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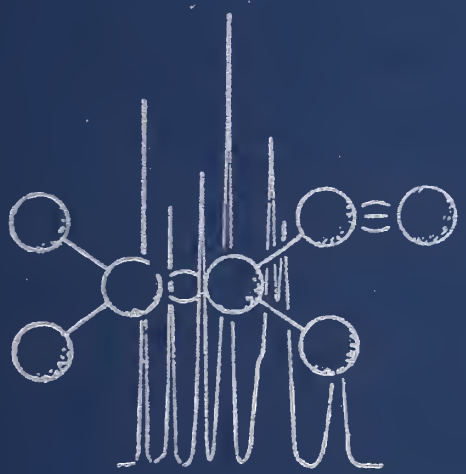

TRACE ORGANIC ANALYSIS: A NEW FRONTIER IN ANALYTICAL CHEMISTRY

TRACE ORGANIC ANALYSIS: A NEW FRONTIER IN ANALYTICAL CHEMISTRY

Proceedings of the 9th
Materials Research Symposium
held at the National Bureau of Standards
Gaithersburg, Maryland
April 10-13, 1978

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FOREWORD

The Center for Analytical Chemistry of the NBS National Measurement Laboratory, a major national center for analytical chemistry research, is concerned with improving the accuracy and precision of analytical measurements throughout the nation and exercises leadership in solving important analytical problems that affect many different segments of the economy as well as the health and safety of our citizens. The NBS Center for Analytical Chemistry is charged with conducting analytical research, preparing and analyzing Standard Reference Materials, and performing service analyses for other Centers within the NBS and the federal establishment. Within the Center for Analytical Chemistry is the Organic Analytical Research Division, which is mandated to monitor and improve all aspects of trace organic analyses. At present, this Division has 25 technical people who are engaged in the development and utilization of trace organic techniques ranging from immunoassay to isotope dilution mass spectrometry.

One important mechanism of the Center for Analytical Chemistry for exercising its leadership is in the coordination of special conferences and symposia and providing a congenial atmosphere in which scientists from throughout the world may exchange their views and ideas in the field of analytical chemistry. Experts are invited to participate in National Measurement Laboratory sponsored conferences, and an attempt is made to advise all scientists of these meetings and to invite them to contribute papers to these symposia.

The first Materials Research Symposium sponsored by the Institute for Materials Research was held October 3–7, 1966, shortly after NBS moved to their new laboratories in Gaithersburg, Maryland. This first symposium, which was coordinated by the Center for Analytical Chemistry, was concerned with trace characterization of materials. Subsequent to that symposium, more specialized conferences sponsored by the National Measurement Laboratory and coordinated by the Center for Analytical Chemistry have been held on such subjects as quantitative electron probe microanalysis, modern trends in activation analysis, and ion selective electrodes. "Analytical Chemistry: Key to Progress on National Problems," held in June 1972 was the American Chemical Society's annual summer symposium sponsored by the ACS Division of Analytical Chemistry and the journal *Analytical Chemistry*, and cosponsored by the Center for Analytical Chemistry. Most recently, the Seventh Materials Research Symposium on Accuracy in Trace Analysis was sponsored by the National Measurement Laboratory.

This volume is the proceedings of the 9th Materials Research Symposium. The theme, "Trace Organic Analysis: A New Frontier in Analytical Chemistry," attracted over 400 scientists, representing a broad spectrum of industrial, governmental, and educational institutions from the United States and from many foreign countries. The format of the symposium consisted of plenary speakers, who discussed specific topics in the areas of sampling, preparation, analysis, and new methodologies, and contributed papers generally expanding on these topics.

The large attendance at this symposium, as well as the lively and interesting sessions, indicate that trace organic analysis is one of the key disciplines needed by materials scientists, environmentalists, biologists, and engineers to understand and ultimately solve the increasingly complex technological problems now facing the nation.

John D. Hoffman, *Director*
National Measurement Laboratory

PREFACE

This volume, the formal report of the Ninth Materials Research Symposium held at the National Bureau of Standards in Gaithersburg, Maryland, April 10–13, 1978, is comprised of the invited and contributed papers given at the symposium. The objective of the symposium was to assess the present state of the art and future direction of trace organic analysis.

Until recently the major emphasis in trace analyses has been in the determination of inorganic substances. However, we are now coming to realize that many of our most pressing problems require a competence in trace organic analysis. These analyses are urgently needed to adequately protect our health and environment, and to ensure the purity and nutritional value of our food. The various factors which contribute to a successful analysis were explored during the symposium. The symposium consisted of three and one-half days of papers. Leading authorities, with broad knowledge of the problems in the areas discussed, were invited to present the keynote lectures. Simultaneous sessions consisting of groups of papers corresponding roughly with the themes of the invited papers were presented in the afternoons.

It is hoped that this volume will provide much of the necessary background information and directions for the future to assist in establishing priority goals and a rationale for solving the difficult problems in trace organic analysis.

Identification of commercial materials and/or equipment by the authors in these proceedings in no way implies recommendation or endorsement by the National Bureau of Standards.

An undertaking of the magnitude of this symposium and of the proceedings, would not have been possible without the cooperation and assistance of a large number of dedicated people. The enthusiastic participation of the symposium Steering Committee, the invited speakers, the session chairmen and those who contributed papers is deeply appreciated. Many members of the staff of the Center for Analytical Chemistry and of the National Bureau of Standards assisted during the symposium in various capacities.

Special thanks are given to Ronald B. Johnson and Robert F. Martin of the National Measurement Laboratory. They provided all of the fiscal management of the symposium and assisted with the other administrative matters associated with an operation of this magnitude. Sara R. Torrence and Joanne Lorden of the Public Information Division, under the direction of R. S. Franzen, deserve a special note of praise for their work in organizing the accommodations, social program, and coordinating the logistical aspects of the symposium. Members of the staff of the Technical Information and Publications Division, under the direction of W. R. Tilley, have given invaluable assistance in the many phases of publishing these proceedings. Special thanks are due to Rebecca J. Morehouse and Miriam K. Oland of the same Division for producing this book using computerized photocomposition techniques.

Within the Center for Analytical Chemistry, special thanks are given to Donna Kline and her colleagues in the Text Editing Facility for assuring the accomplishment of all the details of program planning, correspondence with speakers, and preparation of the book of abstracts. Beverly Clipper and Karen Barkley are owed thanks for their successful efforts in overseeing smooth operation of the symposium and rapid compilation of the proceedings. Thanks also go to Amanda Walker for the editorial assistance she provided in preparing the final bound proceedings.

Stephen N. Chesler
Harry S. Hertz
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ABSTRACT

Researchers in diverse areas must currently perform critical analyses on minute quantities of organic compounds in various matrices. It was the aim of this Symposium to bring together these scientists to discuss their common problems and to explore current and impending technology for organic analyses. Emphasis was placed on the total analysis, from collecting the sample through interpreting the results, rather than upon the measurement only.

The Proceedings consist of a series of invited papers by experts as well as particularly appropriate contributed papers. Topics covered in the Proceedings are as follows: Sampling and Sample Handling for Trace Organic Analysis, State-of-the-Art Analytical Systems, Analytical Techniques on the Horizon, Analysis of Nutrients, Analysis of Organic Pollutants and Their Metabolites in the Ecosystem, Analysis of Drugs in Body Fluid, Analysis of Food Toxicants, and Analysis of Hormones and Neurotransmitters.

Key words: Drug analysis; food toxicants; hormones, neurotransmitters; nutrients; organic pollutants; trace organic analysis.

CONTENTS

Section II. FOOD ANALYSIS

Section III. CLINICAL ANALYSIS

Section IV. ANALYTICAL SYSTEMS FOR TRACE ORGANIC ANALYSIS

Section V. GENERAL ANALYTICAL TECHNIQUES

Section I. ENVIRONMENTAL ANALYSIS

NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

STATISTICAL SAMPLING AND ENVIRONMENTAL TRACE ORGANIC ANALYSIS

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In the field of analytical chemistry, statistical sampling traditionally did not play a prominent role. Samples were usually drawn, or composited, from fairly homogeneous material and the characteristic of interest determined. The results were averaged and claimed to represent the value of the property desired. This procedure has been used in manufacturing and industrial processing for some time, and has shown to be satisfactory for the purpose. For example, in determining percent carbon in steel, only one preliminary grab sample from a melt is taken and analyzed to give the carbon content representing the whole 160 tons.

Once the chemists ventured from manufactured goods with controlled composition to natural products, the variability of the properties among samples began to pose a problem. In determining the sucrose content of a shipload of raw sugar, samples were taken systematically every 300 tons while unloading. The average of the 90 or so samples in a shipload is considered to be "the sucrose content" of the whole shipload by definition. Where the buyer and seller can agree on a specified procedure, the purpose is served.

In environmental measurements, or in analysis of low level contaminants, time and space added dimensions to the statistical sampling problem. Coupled with difficulties in extraction and measurements in trace organic analysis, the problem is indeed formidable. Any knowledge as to sources of variability and pattern of variability, however, would be helpful in dealing with the sampling problems.

It is suggested that the design of a proper statistical sampling scheme depends almost entirely on the purpose for which the results are going to be used. Hence, without an explicit and defined purpose for an undertaking, the design of the sampling scheme cannot be formulated for efficient data collection and for the correct interpretation of results.

Key words: Environmental measurements; modeling; sampling schemes; statistical sampling.

The title of this 9th Materials Research Symposium is "Trace Organic Analysis: A New Frontier in Analytical Chemistry." To me, the words "NEW FRONTIER" can mean only one thing; that is, they are mathematically identical to the statement "We got problems." Problems in one area of research usually generate problems in other areas of research. Problems in trace organic analysis, no doubt, will generate problems in statistics. I am happy to have the opportunity to discuss one particular statistical problem—not from the viewpoint inside the laboratory, but from outside the laboratory—the problems that are there, the problems that remain to be solved, before the taking of a single measurement.

I will be talking about statistical sampling, and touch upon topics in environmental sampling, but not particularly on "compliance sampling." We realize the problem of compliance sampling is an important one, and our group has been pushing for research in this area for some time. However, the problem we have been working on is a different one—in the weights and measures area. There we are dealing with manufactured items produced under quality control: cans, packages, bottles, etc. Here we are dealing with unwanted by-products: hydrocarbon, pesticides, and others. The weighing of a package is a relatively simple operation. The detection of trace organics in ppm or ppb range is involved and time-consuming. The customer who got short-changed in a can of soup suffers a loss immediately, whereas the effects of some of the low level contaminants over the long run are virtually unknown. From the viewpoint of statistics, there may be some general principles which apply to both areas, but the applications and the emphases are

certainly different. I hope that, a couple of years from now, we will have something more concrete to report on Environmental Compliance Sampling.

Statistical sampling problems have been with us for a long time. We are all aware of the use of sampling in opinion polls, in the acceptance of manufactured items, in the determination of heat content of a pile of coal, or in the sucrose content of a shipload of raw sugar. The general procedure is to define first a population of interest and the characteristic of the population to be measured, to construct a frame from which the samples may be identified and drawn, to make observations or measurements to the population characteristics.

In concrete terms, we may use the following example:

Population: Oysters harvested in the Chesapeake Bay area.

Characteristic: Amount of insecticide residue (DDT or DDE) in these oysters.

Frame: All the oyster beds identified and numbered.

Samples: A sampling scheme, a rule, appropriate for the selection of oysters from catches, catches from locations (oyster beds).

Measurement: Gas chromatography.

Inference or Purpose: Safe for human consumption, or complying with some arbitrary health standard.

In fact, practically everything we do involves sampling. For example, the measurement you made yesterday morning is a sample from a series of measurements you could have made yesterday afternoon, today, tomorrow, or sometime next week. Implicit in this statement is that each of these measurements you could have made is as valid as any other measurements. The resulting values may differ somewhat, but there are properties of consistency and constancy among this group of measurements that give you confidence to predict values of future measurements to within certain limits. The population here is the "measurement process" which you have developed, be it gas chromatography or mass spectrometry. You are quite confident that the resulting measurements, as a sample from the measurement process, are valid since the procedure has not changed, the laboratory conditions remain the same and all the instruments have been properly calibrated. Occasionally, though, you may wonder why the last five measurements from the same sample appear to have an upward trend!

Let us call the minute quantity on which you make the analysis a subsample, taken as part of a sample you have received. Assuming the sample has been properly homogenized by you, or by some other laboratory, the measurement made on the subsample may be considered the same as the sample itself.

All samples are collected with great care to avoid contamination and change in properties. Measurement results on these samples, however, will be different since each is a sample from a particular location at a particular time. How to select samples so that these results can be reasonably combined to answer the "question" of interest, the specification of purpose that it is intended to serve, is the central theme of statistical sampling.

In this sense we see that our "measurement process" has been extended to a "sampling and measurement system." The results one obtains from such an extended system are required, similarly, to have properties of consistency and constancy before we can claim that these results are valid and use them for the purpose of prediction. For example, if we aim to determine the hydrocarbon content of intertidal sediments from Katalla River in Alaska at a particular period of time, two laboratories, collecting samples independently during the period, and measuring their samples by accepted methods, should arrive at results reasonably in agreement. For otherwise, one result may be considered as good as the other, or both results could be in error. No rational decision can be made based on conflicting data.

To achieve the desired degree of constancy and consistency sufficient for the purpose intended, one must have control on three facets of the system, namely:

A. The measurements made within each laboratory must be stable and have the desired precision.

B. Measurement results between two laboratories for the same sample must agree to within limits of measurement uncertainty.

C. The sampling scheme employed by each laboratory must have compatible logical structures such that the results given by each laboratory will estimate the same quantity to within some acceptable limits of uncertainty.

We may claim, therefore, that statistical sampling is analogous to statistical design of experiments. A possible difference is that an experiment is usually under the control of the scientist performing the investigation. He knows fairly well the purpose of the experiment, and how to go about getting the answer. This situation may still be true in sampling and measurement systems, but in most instances, the problem involves factors external to the laboratory which may not be under the control of the person performing the measurements. Because of the added complications, the laboratory scientists usually prefer to concentrate on their own specialty: the measurement of samples as given. For example, in the 100 or so papers listed in the program of this symposium, nearly all of the papers deal with either new methods of measuring certain contaminants, or describing laboratory procedures.

Clearly there should be a balance between efforts spent on laboratory work and efforts spent on statistical sampling. If the right measurements are made on samples collected in an ambiguous manner, we may in fact give right medicine based on wrong diagnosis. Definitive and precise measurement methods and techniques are necessary conditions, but by themselves not sufficient in answer to the broader question, "Are you sure that the population characteristic can be inferred from the samples you have analyzed?"

What are the "right" samples to take? Or, what is a "correct" sampling scheme? The answers to these questions are complicated in general, but basically depend on three factors:

- What is the objective of the exercise?
- What are the sources and magnitudes of variabilities one is likely to encounter in each of the several aspects of the undertaking?
- What resources, funding and trained personnel, are available to ensure that the objective is achieved?

These are tough questions, and can only be answered through the joint effort of the subject experts, the analysts, and the statisticians. Most of the time an iterative process is necessary, i.e., estimates are made on the values of the various parameters involved, then a pilot experiment is designed and performed to confirm or revise these values, and then the final design is made based on these revised values of parameters.

What is the Objective of the Exercise?

Certainly we all know why we take the samples and make the measurements. The objective is usually expressible in simple and understandable terms. In the above example, the objective is to determine the trace amount of hydrocarbon contained in intertidal sediment of Katalla River in Alaska, say, in 1977, to serve as a baseline measurement. The question usually asked of the statistician is "How many samples do I need?"

The amount of work involved in translating this simple objective into an operationally meaningful statement is usually underestimated. Some of the questions that have to be answered may be:

Location: Say 144° 35'W, 60° 11'N.

Area: 5 kilometers along the river (or along the shores) between benchmarks, on both the right and left banks.

Intertidal: The 20 meter strips covered between high tide mark and low tide mark.

Time: The third week in August.

Sediments: Top 15 cm layer exclusive of vegetation growth and passing sieve of a certain size. Let us say there is no apparent change of sediment characteristics with respect to visual observation.

Let us assume that the above "defined" the population we have in mind, neglecting the fact that it may have rained heavily the week before, or during the week, or any other unusual events. The characteristic of the population we wish to observe, then, is the hydrocarbon content of this large chunk of mud. How many samples do we need?

A natural counter question at this time would be "How well do you need to know the hydrocarbon content?" This is an extremely important question, since the amount of work and cost involved depends directly on how well we need to know the result. Requirements that are over-demanding would only result in waste of resources; on the other hand, results with large uncertainty may not serve the purpose at all. The "purpose" is usually supplied by external considerations, by geologists, ecologists, by toxicologists, by medical experts, or by regulatory requirements.

Let us say, for purpose of illustration, that we know the total hydrocarbon content at Katalla River is probably less than 5 μg/g, and as long as the amount stays under 10 μg/g, no one is particularly worried. So at that level, we could tolerate a "realistic" uncertainty of nearly 100 percent. Specifically, we can sharpen the requirement to the results of two laboratories. Working independently in sampling and measurements, the averages of the two results could be considered acceptable if the ratio of the results does not exceed 2. Once the total uncertainty of the final result is decided upon, the next step is to gather information on sources of variabilities which contribute to the uncertainty of the final result, and budget our samples in such a way so as to achieve the desired uncertainty in an efficient manner.

Sources and Magnitudes of Variabilities

One source of variability which comes immediately to mind is the uncertainty due to measurements. In trace organic analysis, this is a particular problem since the magnitude of measurement errors can be large in comparison with the value measured. To improve the precision of such measurements is the main purpose of this symposium. After all, the measurement uncertainty is used as a "yardstick" to see if two samples are the same, or are different. A fine, well-calibrated yardstick with graduated scale will allow us to measure a difference in length much easier than a crude one.

A crude yardstick, however, can be used if that is all we have, provided, however, the yardstick stays constant in length. If it shrinks on cold days when used by A, and expands on hot days when used by B, then we have essentially no measurements at all. Hence, whether a measurement is considered precise or imprecise, the basic requirement is that the process must stay stable over time, i.e., the measurement errors must follow a stable distribution, with a constant mean and a constant standard deviation. Only then can we take advantage of the fact that the precision of the average improves as the number of samples measured increases. Since every sample will be measured, and the averages compared, the measurement error in the final result

could be reduced to a suitable percentage of the total, say 20 percent, provided, however, that the effect of the matrix, the percentage of extraction, and the specificity of the procedure are all well established, and the measurement process is in a state of statistical control.

In the above example, the site is downstream from a known oil seep. Hence a gradient along the river is highly probable. We may decide to set up 5 stations 1 kilometer apart along the banks of the river so that this source of variability may be estimated from the results. The other source of variability, that between the right and left banks, can also be obtained. (It is easy to put 10 dots on the map, but I am not sure these locations are logistically acceptable sampling stations.)

The variability between the low tide side of the strip and high tide side of the strip is obviously of interest, hence two substations may be assigned at each station, one at low tide and one at high tide sides of the strip. Perhaps 3 samples can be taken at each substation, a pair of duplicate samples 10 meters apart, and a third one 200 meters away. The total number of samples is

2 banks
5 stations
2 substations at each bank
3 at each substation at each station

or 60 samples.

These 60 samples would allow us to compare differences and estimate variabilities between banks, along stations, between high tide and low tide, and between duplicates. The last one is important at this stage since it allows us to check realistically how well we can perform in the laboratory, assuming that samples 10 meters apart do not change much in their trace hydrocarbon content. Variability estimated between duplicates will be used as our new yardstick to measure other differences.

Sixty samples to take and measure may be considered too many. Perhaps one of the duplicate samples can be deleted if we do not need to check on our measurement capability. Then we are left with 40 samples. If in addition we know the variability between high and low tide areas are small, then we could stagger the 5 stations and do away with the 2 substations per station, leaving us with 20 samples. Whether the 20 samples will yield an average with an uncertainty of 100 percent, of course, will depend on the variabilities between banks, and between stations. The measurement error should not be much of a factor when the 20 results are averaged. When we have some idea on the sources and magnitudes of variabilities, we can take more samples corresponding to the larger sources, so that in averaging, these variabilities can be reduced. A pair of duplicates given the same values obviously supplies very little information.

Suppose now the objective is not to estimate the hydrocarbon content of that part of Katalla River, but to get a "real world" sample to check on eight laboratories doing trace hydrocarbon analysis.

The above example was discussed in detail to illustrate the fact that how many samples and what samples to take depends entirely on (1) the objective and goal of the undertaking, and (2) the various sources of variabilities that are present. In situations where a buyer and a seller are involved, such as a liquified natural gas transaction, the number of samples that have to be analyzed for their heating value depend entirely on the agreement reached between the two parties. In compliance sampling, the regulatory agency could approve or adopt some reasonable guidelines in sampling to ensure pollution or toxicity levels measured are realistic in representing conditions being monitored. Perhaps the most demanding task is that of modeling, to find out the interrelationships between various systems. Here, since we cannot contract with nature or regulate nature's way of doing things, there is no alternative but to learn slowly and painstakingly to accumulate knowledge for the future.

Professor J. S. Hunter of Princeton University [1], in a statement to the Subcommittee on the Environment and Atmosphere, Committee on Science and Technology, commented:

> "Only if the data collection has as its purpose the development of scientific models to elucidate the nature of the environmental problem, and ultimately to lead to control of our environment, will the data be worth having. Good historical records are valuable only if they can be used to construct models and forecasts. Data, if it is of poor quality, becomes itself a pollutant of clear thinking and rational decisions."

In conclusion, let me say that all of us tend to forget that statisticians cannot work in a vacuum. By themselves they can assume $X_1,X_2,...,X_N$ are independently and identically distributed random variables, with their respective variances. They can also come up with a sampling plan which is optimum in some sense. These are mathematical problems that can be attacked and solved. When translated into the real world, it is important to check if all the assumptions are at least reasonably true, to obtain estimates of the values of parameters that have a direct bearing on the design, and to be sure that the results obtained and inferences made from the samples and measurements, combined, do answer the questions raised to a specified degree of certainty.

Reference

[1] Hunter, J. J., Statement to the Subcommittee on the Environment and Atmosphere, Committee on Science and Technology, 9/15/77.

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SAMPLE PREPARATION FOR ENVIRONMENTAL TRACE ORGANIC ANALYSIS

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This paper covers the handling of markedly different sample types, namely gas (ambient air, stack gas), water, and solid wastes. Emphasis will focus upon the sample preparation for the measurement of EPA's priority pollutants exclusive of pesticides and some specialty byproducts.

For gas samples, toxic compounds are present in the gaseous and particulate phases. Volatile components are concentrated by passing the gas through solvent, carbon, or porous polymer resins. They are quantitatively dissolved and introduced into the instrument. After removal of particulates by filtration or cyclonic action, the nonvolatile toxics are measured by GC/MS, GC with and without specific detectors, or HPLC.

In the analysis of water, volatile compounds of low solubility are removed by nitrogen sparging, trapped and analyzed as mentioned above. Nonvolatiles such as polynuclear aromatic hydrocarbons, PCB's, or phenols are adsorbed on porous polymer resins or solvent extracted. The resulting organic concentrate is analyzed in the same manner as particulates.

Solid wastes are simpler in that the toxics are already present in a concentrated form. Volatiles are determined by simply heating the sample in the instrument. Nonvolatile organics are dissolved by solvent in Soxhlet or ultrasonic extractors. Following extensive cleanings the pollutants are determined.

It is convenient and desirable to employ internal standards for quantitation purposes. These may consist of 1) a representative organic known to be absent in the sample to be analyzed, or 2) a compound especially labeled with a halogen, deuterium or ^{14}C atom.

Key words: Gas chromatography; gas chromatography/mass spectrometry; preconcentration techniques; sample preparation; ultraviolet spectrometry.

I. Introduction

In recent years, numerous scientific investigations have indicated that certain organics in the environment are carcinogens. In order to analyze for these toxic materials at the ppb level, new analytical methodology had to be developed. This required innovations in the sampling of various media, the selective concentration of particular classes of toxic materials, and finally the analytical instrumentation which would be used for the final measurement. One of the prime objectives in any of the methods which were developed was to selectively concentrate the toxic materials to a level high enough for their detection with the most sensitive analytical instrumentation.

Although sampling methodology may change depending on whether air, water, or sludge is sampled, the various concentration techniques are similar regardless of sample type. It is the preconcentration techniques which are utilized from the time the sample is obtained and its final measurement that will allow for the detection of trace organics at or below the ppb level.

II. Preconcentration Techniques

In the discussion which will follow, an overview of numerous preconcentration techniques will be presented. It should be realized, however, that for a particular application a method not discussed in this paper may be quite applicable in solving the analytical problem. This may be

especially true in the discussion of adsorptive materials used to preconcentrate organics. Although recent literature has contained methods which utilized adsorbents such as chromosorb [1], silica gel [2], florisil [3], and polyurethane foams [4], this paper will concentrate on some of the more commonly used materials, namely Tenax GC, XAD-2, and charcoal.

In review of the various methods of sample preconcentrations, there are really three: namely, sparging, adsorption on polymer resins, and liquid extraction. In the discussion which follows, the applicability of all three methods will be presented.

A. SPARGING

Although the technique of sparging has been known for some time, various approaches, i.e., the use of new entrapment media, have led to the improvement in the detection limits of organics. A common method is to purge a water sample with an inert gas such as nitrogen and then pass this gas-organic mixture through a thermally stable adsorptive material such as Chromosorb 103 or Tenax GC. Once sufficient time is allowed for the purging of the sample, the trap is then thermally desorbed onto a column suitable for GC or GC/MS analysis. Conclusions presented by Bellar and Lichtenberg on such a concentration procedure showed that this method may be useful for the quantitative measurement of a large range of organics which have a boiling point of <150 °C [5]. The detection limit for the Bellar method was approximately 0.5 μg/L for many of the organics where particular emphasis was given to organohalides.

Clayton McAuliffe has developed a method for the analysis of hydrocarbons dissolved in water [6]. The method is based on the GC measurement of the headspace after repeated equilibrations with helium of the aqueous sample containing dissolved hydrocarbons. Hydrocarbons, including alkanes, cycloalkanes, olefinic and aromatic hydrocarbons up to C_{10}, can be determined. In order to calculate the concentration of the hydrocarbons, the gas chromatographic data obtained from successive measurements are plotted and then back extrapolated to the original hydrocarbon concentration. This must be done for each hydrocarbon. In many cases, sensitivity is approximately 0.01 μg/L.

Investigations by Grob et al. have indicated that organic substances present in water samples may be quantitatively removed with activated charcoal being the adsorption medium [7]. The apparatus utilizes a hermetically closed circuit system whereby air is pumped through the water and then circulated through the activated carbon wafer. Included in his report is an investigation of the use of water vapor (i.e., steam) to purge the aqueous sample. Carbon disulfide is used to desorb and the subsequent analysis is performed by GC or GC/MS. In unpolluted water, the method allows for the detection of hundreds of substances up to C_{24} at concentrations as low as 0.1 ppb. Grob's conclusions were that when dealing with water samples which contained major constituents of about 5 ppt, alkanes up to C_{20} could be extracted by simply stripping with air at room temperature. There were some difficulties in obtaining good recoveries for aromatics, oxygenated and nitrogenated compounds. As a result, the recovery data for such substances must be individually checked. His results showed that stripping of the water sample with steam yielded much better efficiencies for polar materials but poor recoveries for alkanes up to C_{16}, as seen in Table 1.

An interesting method for obtaining both the volatile hydrocarbons by N_2 sparging along with the nonvolatiles by coupled-column liquid chromatography was developed by May et al. [8]. An important advantage of their technique is that there is little sample handling and thus less chance for sample contamination. The volatiles are trapped and subsequently thermally desorbed from Tenax GC, while the remaining liquid is then concentrated on a Bondapak C_{18} packed precolumn for coupled-column liquid chromatographic separation and analysis. The reported sensitivity of this method is approximately 1 μg.

Table 1. *Recovery of test substances (%) under different stripping and concentration conditions [7]*

Substance	Air, 2.5 L/min, 25°			
Substance	20 h, 8 ppt	44 h 8 ppt	44 h 20 ppt	Steam distillation 20 h, 8 ppt
C_{10}-alkane	91	86	92	--
C_{12}	102	97	98	16
Acetophenone	85	90	88	62
C_{14}	92	101	96	48
Methylnaphthalene	49	62	92	50
C_{16}	96	102	97	71
Phenol	--	--	5 (?)	67
1-Decanol	21	36	62	102
C_{18}	86	97	101	72
Fluorene	13	21	47	42
C_{20}	43	78	94	68
Skatole	3	4	11	23
Anthracene	21	32	57	82
C_{22}	21	45	84	67
C_{24}	7	16	47	74

B. Adsorption Traps

In the above discussion on a few of the many sparging techniques described in the literature, one notices that, for the most part, the method uses some sort of adsorption medium in order to concentrate the particular organics or class of organics of interest. Two of the most popular polymeric materials used in the sampling of both ambient air, stack gases and wastewaters are Tenax GC and XAD-2. In a recent report by G. A. Junk et al., recovery data for numerous organics is presented [9]. A summary of their data, broken down by compound type, is shown in Table 2. Although the recovery data looks good for the entrapment of various organics, certain specific compounds such as phenol itself has a recovery of only 40%. This is an important consideration since phenol is one of EPA's priority pollutants. Similar results have been observed with the use of Tenax for the entrapment of hydrocarbons in ambient air and water [10]. Some recovery data for the use of Tenax as an adsorption medium is shown in Table 3.

The other popular medium for the entrapment of organics is activated charcoal. Selected recovery data for the use of this medium to entrap hydrocarbons from air is shown in Table 4 [11]. There are advantages and drawbacks to all three sorbtive media, too numerous to go into in this short survey report. It should be mentioned that, depending on the recovery of the compounds of interest, the method of desorption available (Tenax is normally thermally desorbed, XAD-2 may be extracted with either pentane or dichloromethane, and charcoal is normally desorbed with CS_2), the investigators may select a different sorbtive medium. Also, differences in air sampling from ambient to stack gas sampling will dictate the sorbtive trap. For example, the use of Tenax to trap hydrocarbons in the presence of high acid content in the stack gas is unsatisfactory because Tenax decomposes under high acid concentrations. XAD-2, on the other hand, has been shown to be a more useful material under such conditions.

TABLE 2. *Recovery of organics in water from XAD-2 at the 10 to 100 PPB level [9]*

Compound type	No. compounds tested	Average recovery, %
PNA hycrocarbons	8	88
Halogen compounds	10	87
Esters	16	89
Phenols	7	82
Pesticides, herbicides	6	83
Aldehydes, ketones	7	95

TABLE 3. *% recovery of organics from Tenax at the PPB level [10]*

Compound	Air	Water
C_6 hydrocarbon	100	90
C_{11} hydrocarbon	92	70
Diphenyl	96	
Acetone	97	
Cresol	90	89

Compounds adsorbed at 20 °C and desorbed at 250–270 °C

TABLE 4. *Carbon disulfide extraction efficiencies for organic vapors trapped in charcoal tubes [11]*

Compound, ~0.1 μL	Time min	Recovery, %	σ
Acetone	20	89	±3.1
Toluene	20	98	±3.7
Methanol	15	88	
Benzene	15	97	
Methyl isobutyl ketone	15	104	

C. ION EXCHANGE RESINS

One of the problems with all of the sorbtive materials mentioned above is that although they may concentrate a wide range of organics, they are not commonly used in the selective concentration of particular compound classes. This puts a strain on the ability of the final measuring technique to separate out all of the compounds collected on the trap in order to identify them.

A method for the analysis of phenols which was published by Chriswell et al. and which we have also adapted for the trace analysis of phenolics in wastewater utilizes an anion exchange resin to selectively concentrate phenolic and other acidic compounds [12]. A table of the recovery data for a few of the phenolics on EPA's list of priority pollutants is shown in Table 5.

Although the anion exchange resin is important in the selective concentration of acids and phenolics, equally important is the selection of appropriate GC columns for the final analysis. A detailed discussion of the analysis method and the GC separation of phenols will be presented later.

Table 5. *Recovery of phenols from anion exchange resin [12]*

Compound	Concn., PPB	Recovery, %
Phenol	25	95
2,4-Dimethyl phenol	40	95
4-Chloro-3-methyl phenol	40	95
2,4,6-Trichlorophenol	55	95
Pentachlorophenol	85	80

D. Solvent Extraction

The partitioning of toxic compounds into a suitable organic solvent from an aqueous mixture has long been known as a means by which organics may be removed from water. The extract is then either further purified for a particular class of organics or simply reduced in volume and analyzed by GC or GC/MS.

Although benzene has been demonstrated as an efficient solvent for the extraction of PNA hydrocarbons, its use has recently come under strict federal regulations which makes its use impractical in many laboratories. Methylene chloride, however, has been found to be efficient in the removal of various organics from wastewaters. The efficiency of methylene chloride for the extraction of PNA's was tested on a wastewater sample which contained a 1 μg/L spike of ^{14}C-PNA. The data, which is illustrated in Figure 1 shows that virtually 99% of the PNA was removed from the water after three consecutive extractions. In order to obtain additional confidence in the extraction, the radioassay technique was employed on seven additional wastewater samples. An average of 91% of the radioactivity was recovered with three successive extractions. A similar water sample was spiked with a ^{14}C bis-2-ethylhexyl phthalate at 0.3 ppb. The recovery of the phthalate after three consecutive methylene chloride extractions averaged 87% for four wastewater samples.

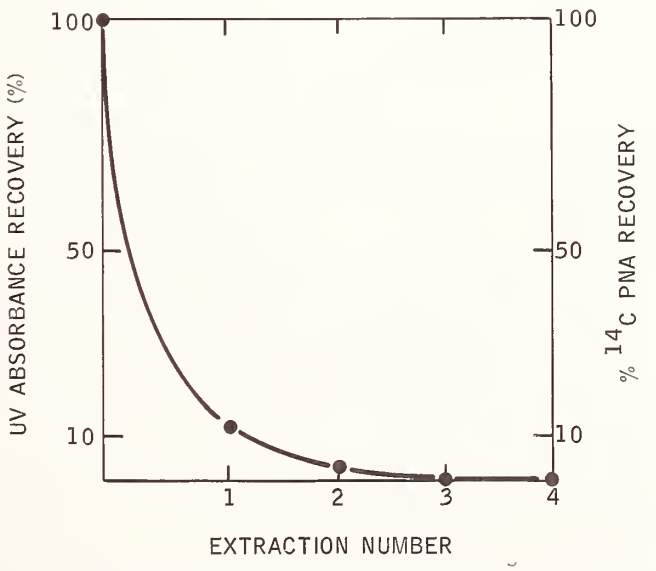

Figure 1. Extraction efficiencies of PNA hydrocarbons from waste water with dichloromethane.

Not only may solvent extraction be used in the removal of organics from wastewater, but more importantly the judicial use of appropriate solvent extraction schemes may help to selectively concentrate organics of interest, while removing interfering compounds. One such sample is the use of DMSO/H_3PO_4 for the separation of PNA's from hydrocarbon matrices [13]. Since our experience has shown that major interfering species may prevent the accurate determination of PNA's, the FDA "wax procedure" [14] was adapted for use in selectively concentrating PNA's from wastewater extracts. Although slight losses are encountered in the tandem back wash procedure, the overall recovery is excellent especially when considering that most of the non-PNA organics have been removed. It has been our experience to date that whenever this extraction procedure alone has been used, sample cleanup is quite adequate. However, for dirty samples or for those which contain polar aromatics, a deactivated Al_2O_3 column may be necessary [15].

E. Concentration by GC Peak Trapping

The use of GC peak trapping has been an important link to the concentration of PNA materials for their final analysis by UV. Based on many years of experience, we have found the trapping device illustrated in Figure 2 to be both convenient and efficient. The normal protocol involves trapping of peaks that are only seconds apart or even partially resolved. Examination of the material in the trap by UV or MS has consistently shown variations in the spectra which demonstrate that the trap is accurately tracking changes in the GC effluent.

There are really two important features of this method, namely to separate and concentrate organic materials. First, although in a following discussion the applicability of the method to PNA analysis will be illustrated, the same technique may be easily adapted to the analysis of other organics. Secondly, since the gas chromatogram often contains overlapping peaks or peaks of questionable retention times, the trapping of the peak and its measurement by UV offers an accurate yet inexpensive means by which the unknown may be quantitatively identified.

An example of the trap efficiency is its ability to resolve overlapping GC peaks such as 2-methyl phenanthrene, 1-methyl phenanthrene and an alkylfluorene as found in the petroleum sample illustrated in Figure 3 [16]. The UV spectra of peaks 5 and 6 of Figure 4 are seen to

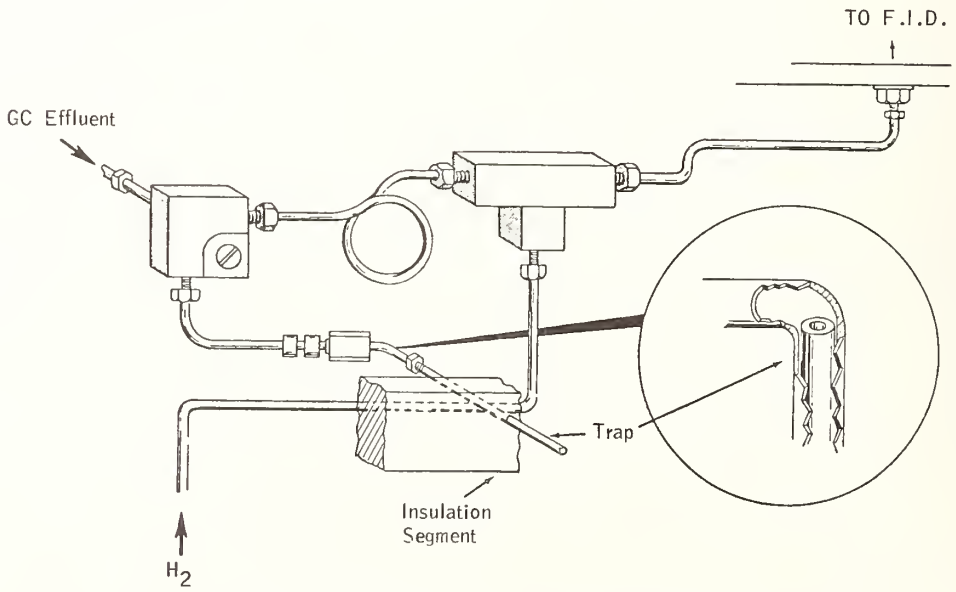

Figure 2. Gas chromatograph splitter and trapping assembly in manifold of PE-900.

closely agree with their respective pure compounds, 2-methyl phenanthrene and 1-methyl phenanthrene. Although a reference spectrum for peak 7 was not available, other data suggests that this peak may reliably be identified as an alkylfluorene. It should be noted that although the GC trace may leave a question as to the compound identification by retention time alone, the examination of the individually trapped peaks by UV spectroscopy shows little smearing of successive traps, thus allowing for easy identification. In addition, since 1-methyl phenanthrene and

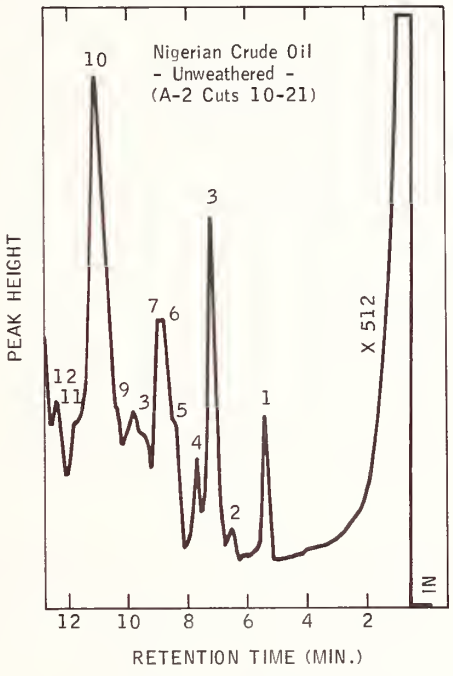

Figure 3. Gas chromatogram of Nigerian crude oil.

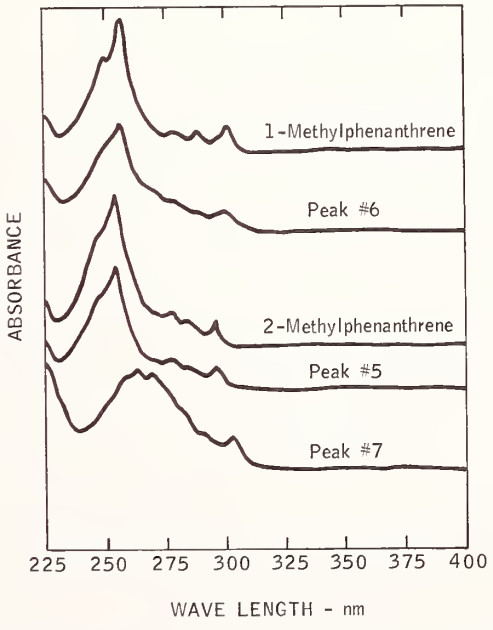

Figure 4. UV spectra of selected GC peaks of Nigerian crude oil.

2-methyl phenanthrene are isomers, the quantification by MS would be very difficult, while UV easily resolves the two compounds.

Of the 42 individual PNA hydrocarbons that have been quantitatively measured by the GC/UV method, only 8 of them occur as mixtures in GC peaks where there is overlap. One such mixture includes benzo(a)anthracene, chrysene, and triphenylene as depicted by a typical UV absorbance spectrum illustrated in Figure 5. Therefore, although the GC peaks overlap, the use of peak trapping coupled with, in this case, UV spectroscopy, offer an important analytical method for the analysis of organics.

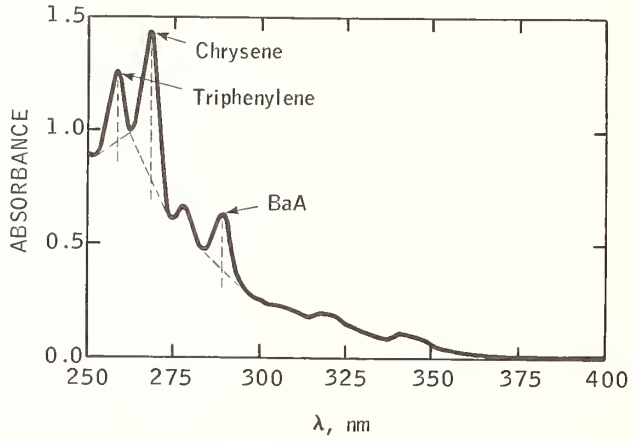

Figure 5. UV spectrum of GC fraction containing benzo(a)anthracene, chrysene and triphenylene.

III. Methods of Analysis

A. Analysis of Phenolics by Ion Exchange Chromatography

Of the 129 compounds on EPA's current list of priority pollutants there are 11 phenolics. A search of recent literature reveals that many attempts have been made to concentrate and quantify individual phenols in wastewater. Preconcentration techniques which involve the absorption of phenols on carbon [17] on XAD-2 [9] have had two serious problems. Low recoveries from charcoal and XAD-2 were obtained. For example, phenol recovery on XAD-2 was 40% [9]. Another distinct disadvantage in the use of these adsorbents was that they were not selective for phenolics but would absorb other organics as well. This made it very difficult to use GC alone in the final analysis due to interferences.

In a recent paper by Chriswell et al., a method which involves the use of an anion exchange resin (Amberlite A-26) has shown good recoveries for phenolics from wastewater at the ppb level. The recoveries along with a brief discussion on the use of exchange resins was presented earlier.

The analysis scheme which they developed is summarized briefly in Figure 6. Samples collected in 1 quart glass containers were preserved with 1 gram of $CuSO_4$ and adjusted to a pH of 4 with H_3PO_4. We have used *o*-isopropyl phenol as an internal standard. The washing of the resin bed with basic methanol will rinse any remaining neutral organics which may subsequently interfere with the final GC measurement. Two GC columns have been successfully used to measure the phenolics. A capillary column coated with 90% didecyl phthalate and 10% H_3PO_4 [18] was used to separate the low boiling phenols. This column has a distinct advantage over other columns in that it is able to resolve all of the dimethyl phenol isomers. Unfortunately, the column temperature cannot be programmed beyond 110 °C due to column degradation. This does not

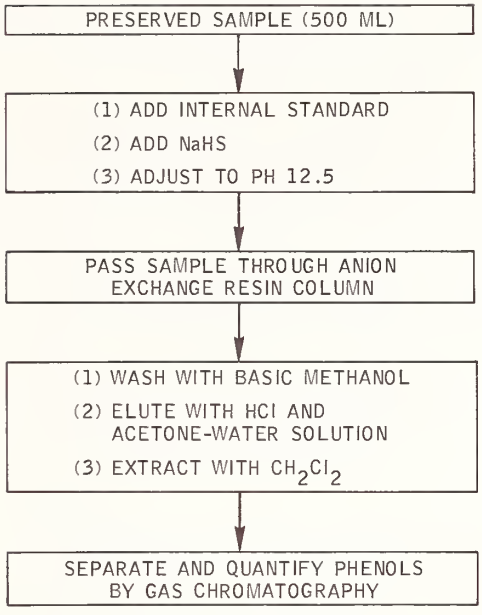

Figure 6. Analysis scheme for phenols in water.

allow for the easy analysis of the high boiling phenols such as pentachlorophenol and 4-nitrophenol. For the analysis of these phenols we have used a 3 ft. glass packed column (coated with SP-1240DA) instead of the more commonly used Tenax column. The Tenax columns were found to cause severe tailing of the higher boiling phenols with nonreproducible results due to tailing and adsorption of low concentration nitrophenols on the Tenax.

Although this method to selectively concentrate phenolics by an anion exchange resin is sometimes tedious and time-consuming, the final concentrate is free from interfering organics commonly present in wastewater streams.

B. Analysis of PNA's by GC/UV Peak Trapping

For many years, industry has been interested in the analysis of wastewater samples for polynuclear aromatic hydrocarbons (PNA). In addition, a review of the current EPA list of 129 priority pollutants shows 13 are PNA's. In an effort to analyze for these and other PNA materials we have routinely used our previously described GC/UV method [19,20]. Although we regularly measure 18 individual PNA's ranging from the 3-ring aromatic, anthracene, to coronene, a 7-ring compound, the method is not limited to this particular group of compounds. Because of the implicit nature of the measurement technique, it is possible to quantitatively identify virtually any parent and many substituted compounds which have reasonably intense UV absorption spectra.

An example of an outline of the analysis method is illustrated in Figure 7. In the methodology carbon-14 labeled benzo(a)anthracene and benzo(a)pyrene are used as internal standards to monitor the recoveries of the PNA materials as they are subjected to the various cleaning, purification and concentration procedures. The initial volume for wastewater samples is approximately 3 liters with the final volume of purified extract being 10 μL. This method has been used to analyze the low concentration ppb levels of PNA compounds.

The extraction methods which were used in the PNA analysis were discussed in a previous section. It should be mentioned, however, that by the use of carbon-14 labeled PNA's it is possible to monitor the recovery of PNA's after each step in the extraction and purification scheme. In addition, an important feature of the GC trapping technique is that the individual ^{14}C B(a)A and ^{14}C

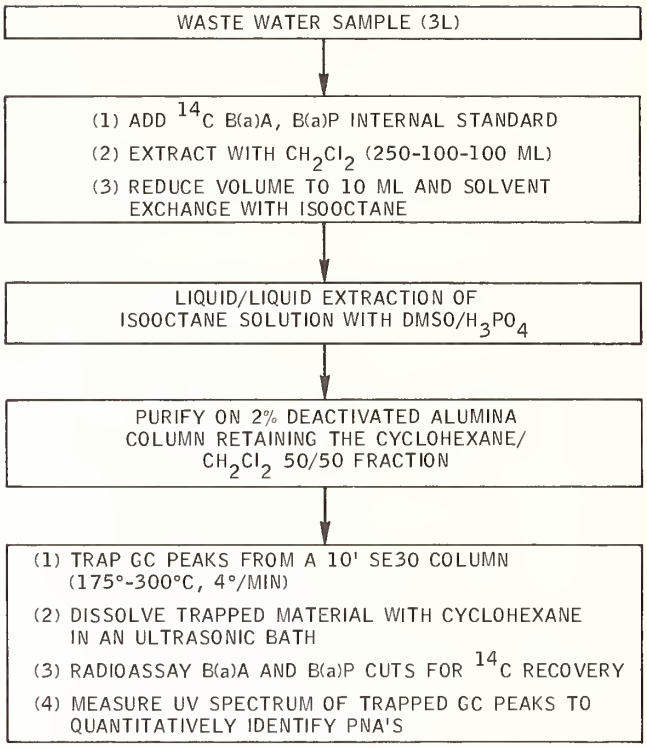

Figure 7. GC/UV procedure for PNA analysis of waste water.

B(a)P may now be separated and assayed for their respective recoveries. These results are then factored into the calculations which are used to quantify the individual PNA hydrocarbons present in the original water sample.

As previously mentioned, the UV spectra of the trapped GC peaks are measured to quantify the individual PNA's present and also, as in the case of B(a)A, chrysene, and triphenylene, UV is used to distinguish between them since they have overlapping GC peaks. Although only 21 PNA hydrocarbons are routinely reported now, calibration data are available for 42 compounds with twice that number being measured if desired.

Quality control over the GC/UV procedure is maintained by assaying a blend of phenanthrene, pyrene, benzo(a)anthracene, benzo(a)pyrene and benzo(g,h,i)perylene at frequent intervals. Although the procedure covers only the GC/UV and radioassay and not the solvent extractions, data from 19 analyses over a 9-month period shows that there is a standard deviation of one sigma (0.20 μg) or a 10% relative error. The recovery of the five compounds was found to be 93%.

The precision for the entire method, which includes the variations in sampling, was found to be within 21 relative percent (one sigma) for concentration range 0 to 30 μg/L and 18 relative percent (one sigma) for 30 to 200 μg/L. As a measure of accuracy, a Delaware River water sample, which contained no detectable PNA's was spiked with μg amounts of 18 PNA's. The results presented in Table 6 illustrate that good recoveries were obtained.

Table 6. *Recovery of PNA added to Delaware River water*

	μg/L	
	Added	Found
Fluoranthene	0.91	1.10
Pyrene	2.34	2.74
Benzo(a)anthracene	0.69	0.74
Chrysene	0.76	0.83
Triphenylene	0.24	0.26
Methylbenz(a)anthracene	0.52	0.50
Dm/Et benz(a)anthracene	0.14	0.11
Benzo(g,h,i)fluoranthene	0.18	0.16
Benzo(b)fluoranthene	0.09	0.12
Benzo(j)fluoranthene	0.07	0.07
Benzo(k)fluoranthene	0.13	0.15
Perylene	0.11	0.12
Benzo(a)pyrene	0.50	0.54
Benzo(e)pyrene	0.27	0.33
Methylbenzo(a)pyrene	0.30	0.50
Methylbenzo(e)pyrene	0.21	0.28
Benzo(g,h,i)perylene	0.11	0.07
Coronene	0.00	0.01

IV. Conclusions

In conclusion, this overview was written to point out some of the similarities in the preconcentration of water and air samples prior to their instrumental analysis. Although there are numerous ways to preconcentrate organics, we have tried to highlight those techniques most commonly in use today. It is without doubt that new developments in the areas of selective concentration of organics, such as in the use of ion exchange resins, will lead to the measurement of toxic materials without the presence of interfering compounds.

Although we have presented a general overview of various preconcentration techniques, we have tried to also specifically detail how they may best be used as in the case of phenol and PNA anlaysis. In addition, the use of GC peak trapping, although specifically applied to PNA analysis, may very well be an important technique for the quantification of other organic compounds. This may take precedence over GC/MS, for example, if specific components cannot be resolved or if an inexpensive technique is desired.

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ANALYSIS OF WATER FOR CHLORINATED HYDROCARBON PESTICIDES AND PCB'S BY MEMBRANE FILTERS

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Pesticide residues in water have been analyzed through the use of cellulose triacetate membrane filters. As a chemisorption separation method DDT analogs, mirex, aldrin, and PCB mixtures have been separated from water by absorption to these filters. Recovery of these compounds has been achieved through elution with diethyl ether. Filters were cleaned before use with diethyl ether.

Adsorption of *p,p'*-DDT, *p,p'*-DDE and Aroclor mixtures 1242 and 1254 were found to be 98–99% complete. Maximum loading studied at this time has been 385 ng/cm^2 for DDT analogs and 1650 ng/cm^2 for Aroclor-1254.

Recovery from membrane filters of these compounds ranged from 69 to 113%. *p,p'*-DDT analogs (*p,p'*-DDT, *p,p'*-TDE, and *p,p'*-DDE) at charge levels of 60, 300, and 1500 ng were recovered from 68 to 89%. Mirax at 300 and 7500 ng charge was recovered at 97–113%. Aroclor mixture 1242 at charge levels of 400, 2000, and 10,000 ng and Aroclor mixture 1254 at similar levels were recovered at 89–112% and 83–84%, respectively.

The recovery of DDT analogs and mirex were found to be similar from water whose pH was 2 and 7 but at pH 12 it dropped to almost zero.

The importance of this new chemisorption separation method is seen by its convenience of operation. Separations can be done in the field and filters stored easily. A high degree of concentration can be achieved.

Key words: Cellulose triacetate filters; gas chromatography; pesticide residues; sampling.

I. Introduction

A number of methods for the separation of pesticides and other organic compounds from water for their analysis have been discovered in recent years to supplant separatory funnel separations. These have been summarized by the author in a recent publication [1]. These include activated carbon, urethane foam plugs, coated Chromosorb W, and most recently XAD macroreticular resins [2,3]. All of these methods are adsorption methods where neutral organic compounds are preferentially adsorbed to solid surfaces in place of remaining in water solution.

The use of cellulose triacetate membrane filters can now be added to this list of adsorbers. This material has been found to separate DDT compounds and PCB mixtures from water with a high degree of efficiency. Because these initial results were so encouraging the study was expanded to include more pesticide compounds and other types of membrane filters. It would be of great value as a means of analysis for pesticides and environmental contaminants in water samples the world over. Additional work was undertaken to determine the total capacity or loading of an Aroclor mixture on this filter medium.

II. Experimental

The experiments in this study have been performed primarily with membrane filters made of cellulose triacetate material (Gelman Corp.) having a pore size of 0.45 μm. Henceforth "filters" refers to those made from this material unless otherwise stated. Initially they were cleaned before use by passing ethyl through them in the normal filtering setup. Later cleaning was done by Soxhlet extraction.

Compounds tested for their absorption to the filters were prepared from analytical standards supplied by the Health Effects Research Laboratory, EPA. Initially, the recovery solutions were prepared from distilled water that was freshly redistilled in a Corning all-glass still. Subsequent supplies of water were obtained from the Milli-Q apparatus (Millipore Corp.) that were purified by ion exchange and organic cartridges as well as by a particle filter of the same dimensions as those used for the work here. Each solution consisted of a mixture of pesticides or a single Aroclor mixture. The water solution was shaken for 8 minutes and allowed to stand for up to 16 hours or more before the solutions were extracted by the filter. Amounts of 30 mL, 150 mL and 750 mL (0.00003, 0.00015, 0.00075 mm^3, resp.) were used to implant desired quantities on the filter at 3 levels of charging. Triplicate samples at each level were filtered with water aspiration. Compounds were desorbed and eluted from the filter by 2 applications of 20 mL ethyl ether each. The solvent was exchanged to isooctane and analyzed by gas-liquid chromatography (GLC).

The water eluting from the filter (the filtrate) was extracted with petroleum ether (hexane/toluene for the parathion, gamma-BHC, heptachlor mixture) and analyzed by GLC.

Three samples of the stock solution applied to the filter were also extracted and analyzed by GLC to determine separator recoveries from water.

Gas-liquid chromatography was accomplished on a 1.5% SP-2250/1.95% SP-2401 column (Supelco, Inc.) heated to 190–210 °C in a Microtek Model 220 gas chromatograph. The detection was done by Ni^{63} electron capture and the signal linearized before recording. Peak areas were computed with a CRS-100 electronic integrator (Infotronics, Inc.).

In testing for the effectiveness of various types of filters for adsorbing Aroclor-1254 150 mL of a water solution containing 1500 ng of the Aroclor was passed through each type of filter in triplicate. Filter types studied were cellulose triacetate, mixed cellulose esters, acrylonitrile/polyvinyl chloride on nylon, and vinyl (all from Gelman Corp.), glass fibre/epoxy reinforced (from Whatman Corp.), and polycarbonate (from Nucleopore Corp.).

The loading capacity of a filter to Aroclor-1242 was determined by successively adding charged water portions to a single filter. Each fraction was 1.3 L (0.0013 m^2) in volume and contained 13000 ng of the Aroclor. The experiment was conducted in 6 batches of 6 fractions each. During the processing of each batch triplicate samples were taken for direct analysis of the Aroclor content. Because of losses occurring due to adsorption in the makeup of the solutions the 36 applied fractions were reduced in size to 28.16 fractions each containing 13000 ng.

The loading of the filter was determined by analyzing the filtrate solutions of each applied fraction in the manner described above. These concentrations expressed as percent recoveries were analyzed by the Statistical Analysis System NLIN regression program (Marquardt method) to fit the mathematical model for the logistic equation represented by

$$y = \frac{c}{1+e^{-(A+BX)}}$$

It was assumed in the loading that the Aroclor not found in the filtrate was adsorbed to the filter.

Gas chromatographic peaks were analyzed for their chlorine content by gas chromatography-mass spectrometry. A Finnigan Model 3200 GC-MS with a Model 6000 data acquisition system accomplished this study.

The pH effect on the adsorbing character of the filter was determined by preparing 400 mL solutions charged with the appropriate amount of pesticide or PCB. The pH was then adjusted with sulfuric acid or potassium hydroxide, analytical grade.

All solvents used in the experiments were pesticide grade (Burdick and Jackson or J. T. Baker).

III. Results

The phenomenon of organic compounds adsorbing to a membrane filter during the course of rapid filtration is becoming well known. In our study of the analysis of water for DDT analogs and polychlorinated biphenyls (PCB's) [1,4], a gallon of water could be filtered in about 4 minutes. During this filtration essentially all of these compounds were adsorbed to the filter.

The precise nature or strength of the adsorption has yet to be studied. Attempting to desorb them, however, by a simple shaking with hexane gave recoveries ranging from one to 45% for DDT analogs (*p,p'*-DDE and *p,p'*-DDT) and from 3 to 62% for Aroclor mixtures (1242 and 1254) [1]. When these same filters were extracted a second time but with acetonitrile the total recoveries were improved: they ranged 31–89% for the DDT analogs and 41–140% for the Aroclors.

Subsequently the desorption was done by passing ethyl ether through the filters [4]. The data for this work is found in Table 1. The *p,p'*-DDT analogs, DDE, TDE and DDT were recovered reasonably well and with small variance. Even better recoveries were obtained with mirex and Aroclors-1242 and 1254. Desorption by elution with ethyl ether was the chosen method for all the work presented in this paper.

TABLE 1. *Recovery of DDT analogs, mires and polychlorinated biphenyls from membrane filters. Application to filter via water solutions. Desorption via ethyl ether extraction. Triplicate runs.*

Compound	Recovery from water solution	Applied quantity	Recovery from filter, corr.	Mean (S.D.)
	%	ng	%	
p,p'-DDE	107	30	67.7	
		150	66.6	67± 0.9
		750	68.4	
p,p'-TDE	-	60	70.4	
		300	72.7	74± 4.3
		1500	78.8	
p,p'-DDT	110	60	80.5	
		300	65.8	69± 9.8
		1500	61.9	
Mirex	66	300	113	105±11.2
		7500	97	
Aroclor-1242	79	400	111.8	
		2000	92.1	98±12.3
		10000	89.3	
Aroclor-1254	79	600	83.2	
		3000	83.6	83± 0.3
		15000	83.0	

A. Survey of Pesticide Compounds Affected by the Cellulose Triacetate Membrane Filter

In order to ascertain which compounds were adsorbed by the cellulose triacetate membrane filter a large number of pesticide compounds were subjected to the adsorption-desorption system just described. For each compound the key calculation desired in terms of usefulness for anaytical purposes was the desorbed to filtrate ratio (D/F)...the recovered amount from desorption of the filter divided by the amount extracted from the water that had been passed through the filter. Ratios of at least 10 to 1 would be needed if this system would be effective in an analytical

scheme. If all of the material were accounted for in the two extractions, this would amount to at least a 90% preference to adsorbance on the filter.

The compounds that I studied were grouped in increasing D/F ratio. There was a surprisingly good correlation between this ratio and the structure of the compound (Table 2). Organophosphorus compounds had D/F ratios ranging from 0.4 to 1.1 which is very poor from the standpoint of including them into a membrane analysis scheme for separation. Aliphatic bicyclo compounds containing an oxygen atom in the structure did not perform much better as their D/F ratios were from 1.1 to 1.7. Favorable but only partly useful ratios were obtained from aliphatic bicyclo chlorinated hydrocarbons, and they were 3.8 to 10.2. Finally the compounds containing an aromatic moiety performed very well with D/F ratios ranging from 19 to 39. Let's examine these in detail, discussing the best adsorbers first.

The aromatic chlorinated compounds tested were the DDT analogs, *p,p'*-DDE, *p,p'*-TDE, *o,p'*-DDT and *p,p'*-DDT. The D/F ratios determined for them came out with flying colors ranging from 19 to 47. Included in this group was the mixture of Aroclor-1254 which also had a very good ratio, 39. These ratios represent a recovery from the filter of 95 to 98%.

Studying mirex and kepone gave additional interesting results. The D/F ratio for mirex was 125 while that for kepone was only 3.1. Mirex has a box structure of 10 aliphatic, saturated carbon atoms with 12 chlorine atoms replacing each possible hydrogen atom. Kepone on the other hand has the same structure as mirex except that one oxygen has replaced two chlorine atoms. Lindane, or gamma-BHC, has six chlorine atoms on a cyclohexane structure and was found to give a D/F ratio of 0.4.

TABLE 2. *Membrane filter adsorption of pesticide and PCB compounds. Overall desorbed/filtrate efficiency at all levels of application*

Compound	Range of application ng	Desorbed/filtrate ratio
Organophosphorus compounds:		
Diazinon, oxygen analog	6000–150000	0.4
Diazinon	500– 12500	0.4
Parathion	1200– 30000	1.1
Oxygenated alphatic bicyclo compounds:		
Heptachlor epoxide	30– 450	1.1
Endrin	60– 1500	1.2
Dieldrin	60– 1500	1.7
Aliphatic bicyclo compounds:		
Toxaphene	1200– 30000	3.8
Heptachlor	120 3000	5.1
	30– 450	5.6
Aldrin	30– 450	6.5
	30– 450	16.5
α-Chlordane	25– 625	9.1
γ-Chlordane	25– 625	10.2
Aliphatic cyclic compounds:		
Lindane (γ-BHC)	90– 2250	0.4
Kepone	180– 4500	3.1
Mirex	60– 1500	125.
Aromatic compounds:		
p,p'-DDT	150– 3750	19.
p,p'-DDE	60– 1500	22.
o,p'-DDT	150– 3750	30.
Aroclor-1254	1500– 37500	39.
p,p'-TDE (*p,p'*-DDT)	150– 3750	47.

A number of pesticides studied have a similar basic structure containing an aliphatic bicyclo grouping. Middle results in the D/F determination were obtained from them. Alpha- and gamma-chlordane gave reasonably good results at about 10 while aldrin and heptachlor were lower at 5 to 6. A value of 3.8 was obtained for toxaphene.

Heptachlor epoxide, endrin and dieldrin gave D/F ratios between 1.1 and 1.7. Heptachlor becomes heptachlor epoxide by replacement of a double bond with an oxygen. Aldrin becomes dieldrin or endrin by the same replacement.

Finally the two organophosphorus compounds studied, diazinon and parathion and the oxygen analog of diazinon all had very low D/F ratios of the order of 0.4 to 1.1.

Huang and Liao [5] studied the adsorption of DDT, heptachlor and dieldrin on three clay minerals. They found the adsorption phenomenon to be very similar to that found in this work. Their explanation for the differential adsorption, therefore, could be used here.

There are strong forces of interaction through hydrogen bonding. Weaker forces include dipole-dipole attractions and van der Waals interaction. DDT and heptachlor each have several hydrogen atoms available for hydrogen bonding (Fig. 1). DDT and PCB's as well can lie pretty flat and have several points of bonding. Heptachlor and those compounds similar to it have a number of hydrogens all on the same end of the molecule. These compounds, however, cannot lie as close to the membrane structure as the relatively planar DDT molecule can. The presence of an oxygen at the end of the dieldrin molecule causes this molecule to have a strong interaction with the membrane surface. In addition the structure is bulkier and the molecule may stick out pretty straight from the membrane structure. Since the attractive forces of the epoxide ring in dieldrin are van der Waals forces, they are weaker than those found in the other molecules.

Further adsorption of the more planar DDT molecule can also be explained by its capability to slip into interlamellar spaces in the membrane.

The attractive forces of halogens must be stronger than those of oxygens. The mirex and kepone structures are almost identical yet mirex is far more preferentially attracted to the membrane structure (Fig. 2).

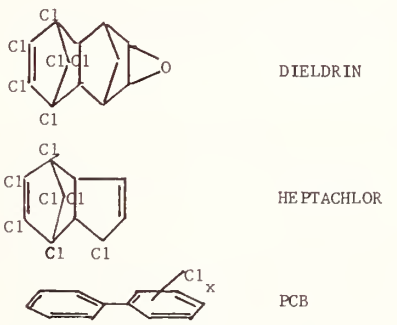

Figure 1. The structures of dieldrin, heptachlor, and polychlorinated biphenyls illustrative of general classes of compounds absorbed to filters.

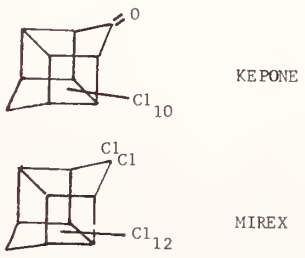

Figure 2. The structures of kepone and mirex.

Two other adsorbent systems have been lately studied for their role in pesticide residue analysis. Porous polyurethane foam plugs have been studied by the group consisting of Gesser, Uthe et al. [6–8]. They reported recoveries of all chlorinated hydrocarbon pesticides discussed here to lie between 92 and 100%. The recovery of Aroclor-1260 was 94%. Lewis et al. examined the recovery of these compounds from vapor state concentrations in air. Gamma-BHC and aldrin gave low recoveries in the 35–58% range but parathion was recovered at higher values of from 66 to 85%, and diazinon at from 70 to 91%. Aroclor-1242 was recovered at 70–80% and Aroclor-1254 at 85%. DDE and DDT were recovered in very good amounts at 83–115% while mirex topped the list at 98–105%.

The macroreticular resin, XAD-2 was found by Coburn et al. [2] as a good adsorbent media for many common chlorinated pesticides. From deionized, distilled water all these compounds including the chlordanes were recovered at greater than 89%. From natural waters spiked samples of them were recovered at lower values ranging from 73–100%. In this example mirex dropped considerably from 97% to only a 52% recovery. XAD-4 has also been reported to be useful for these analyses [3].

B. Survey of Membrane Types Adsorbing Arochlor-1254

A survey was also done in testing the adsorption on various types of membrane filters. Aroclor-1254 was used in all cases for these tests. Mixed cellulose ester membrane filters having a pore size of 0.45 μm gave the highest D/F ratio, 200 (Table 3). Cellulose triacetate filters with the same pore size gave a value of 39. Effectively these two ratios are not much different since the quotient is much greater than one. The triacetate filter of a 5.0 μm pore size also gave a similar value of 59. This indicates that the pore size in this range is not a limiting factor in the adsorption. Acrylonitrile/PVC polymer on nylon filters (pore size of 0.8 μm), vinyl filters (pore size of 5.0 μm), glass fiber/epoxy reinforced, and polycarbonate filters (pore size of 0.6 μm) all produced some adsorption. Their D/F ratios were, respectively 6.1, 5.7, and 5.3. These values would give a maximum recovery of from 84 to 86%. In some cases this amount of adsorption would be large enough to make the filter medium useful for analytical purposes. On the other hand further research may produce selective effects of one material over another which would give increased value for it.

Table 3. *Comparison of desorbed/filtrate ratios for various types of membrane filters*

Filter composition	Pore size μm	Desorbed/ filtrate
Mix cellulose esters	0.45	200
Cellulose triacetate	5.0	59
	0.45	39
Acrylonitrile/PVC on nylon	0.80	6.1
Vinyl	5.0	5.7
Glass/epoxy reinforced	--	5.7
Polycarbonate	0.6	5.3

C. Loading Capacity of a Single Filter with Aroclor-1242

Aroclor-1242 was applied to a single cellulose triacetate membrane filter in an experiment to determine how much of the material would be adsorbed to the filter. It was applied as a water solution of a constant concentration in small fractions. The filtrate for each fraction was analyzed for the PCB.

The total load was found to be 126 μg. Since each filter had an effective area of 9.08 cm^2 ($9.08 \times 10^{-4} m^2$) this amounted to a loading of 14 $\mu g/cm^2$ (or 140 mg/m^2).

The effective filtering portion of the filter was found to have a dry weight of 38 mg. The load on this weight amounted to 3300 $\mu g/g$ or 3300 ppm.

Data was complex to handle. One area of complexity was that the polychlorinated biphenyl mixture in the solution applied to the filter was not entirely recovered from the stock solution. Some had adsorbed to the jug in which it was contained. In addition the recovery was not constant. The recovery values are shown in a plot in Figure 3. Each trial consisted of triplicate determinations. The values follow a second order curve determined by least squares regression. This data was used to correct the fraction "size" so that each fraction in the data presented here represents 1300 ng.

It was found, moreover, that the various compounds that make up the Aroclor-1242 mixture were adsorbed differentially. The gas chromatographic trace of this mixture is given in Figure 4. For the purposes of this paper four of these peaks were selected to minimize the complexity of the discussion. These were chosen to be large peaks, well separated and of a single compound of a given number of chlorine atoms. Those chosen as representative were numbered 1, 4, 7 and 14. Mass spectrometric analysis of the peaks determined that they consisted of a single number of chlorine atoms which were respectively 2, 3, 4 and 5 (Table 4). Caution is expressed in that even though the analysis revealed only a single number of chlorine atoms in the peak that it could in fact contain more than one positional isomer.

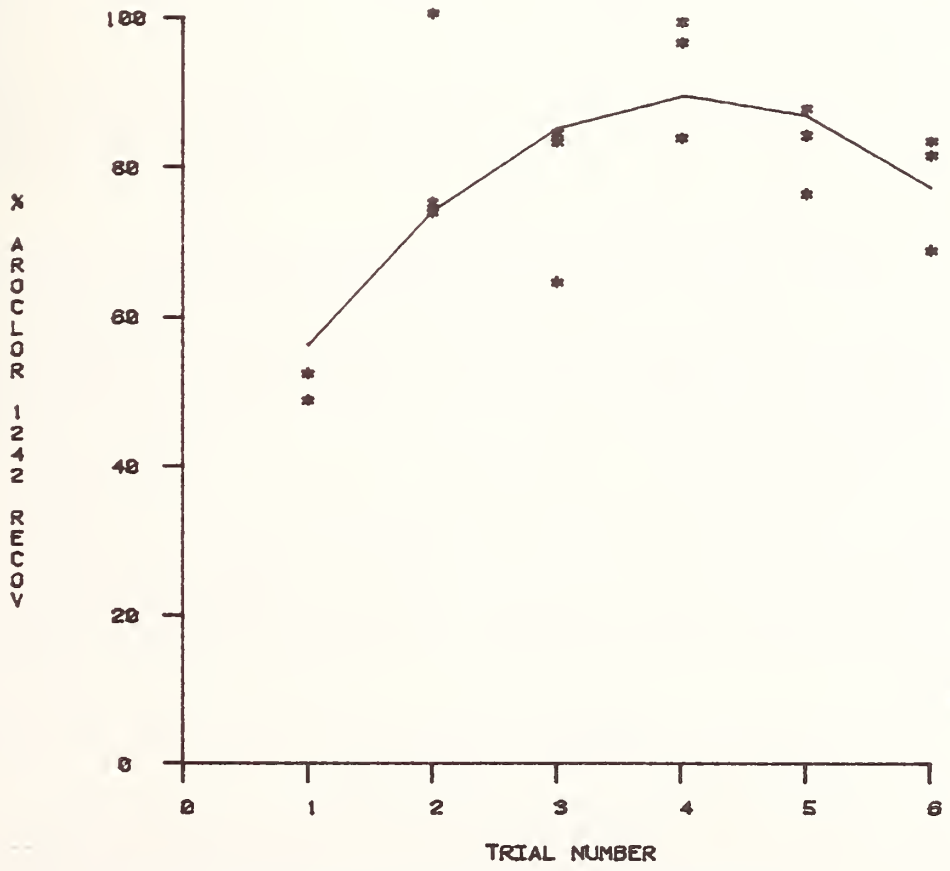

Figure 3. The recovery of Aroclor 1242 from water in sequential trials.

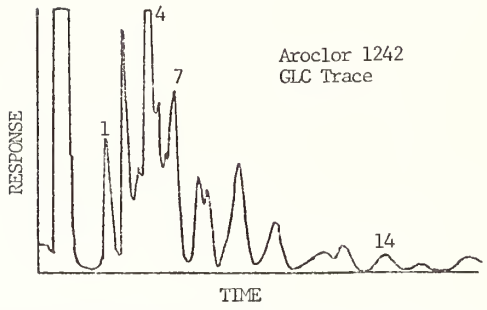

Figure 4. Gas chromatographic analysis spectrum of Aroclor 1242 on a 1.5% SP 2250/1.95% SP 2401 column with electron capture detection. Peaks discussed in text are numbered.

TABLE 4. *Chlorine content of GLC peaks of Aroclor-1242 GLC-MS analysis*

GLC peak no.	No. chlorine atoms per biphenyl
1	2
4	3
7	4
14	5

After 18.2 fractions of PCB solution had been added to the filter three fractions of Milli-Q deionized water not containing PCB were added to determine if there were any desorption with water alone. Following this more PCB solution was passed through the filter to a total of 28.16 fractions, each containing 13000 ng of Aroclor-1242.

Analysis of the filtrate fractions for peak 1 gave data expressed in Figure 5. By fraction 2 only a small portion was found in the filtrate amounting to 86% adsorption. By fraction 6 only 4% was adsorbed. At fraction 10 and beyond all of the impressed Aroclor passed through into the filtrate portion. The large drop in filtrate recovery after the 17th fraction resulted in a sizeable portion having been desorbed by the three portions of pure water that were passed through the column at this point. Succeeding fractions of PCB added gave again adsorption for the compound represented by the peak.

The fast and complete rise to full adsorption and the steep drop after water addition for peak 1 indicated that this peak was not very strongly adsorbed. It was a case of easy-come, easy-go. Peaks 4, 7 and 14 with successively greater chlorine content also showed a consistent rise in adsorbtivity (Figs. 6–8). Peak 4 was slower than peak 1 in reaching saturation and less was desorbed by pure water. Peak 7 was similar. Peak 14 was so strongly adsorbed, at a level of 93% by the 14th fraction, that water removed only a very small part of it as shown by no drop in the curve. The adsorption of these selected peaks at certain portions of addition is summarized in Table 5.

The conclusion from this loading study is that increased chlorine content of the biphenyl molecule results in increased adsorption to a membrane filter.

Calculations were made to determine the total quantity of each peak that was adsorbed in the first 18 fractions. The area to the right and below the curve represents the amount not adsorbed while that to the left and above represents the amount adsorbed. The curve is the solution of the logistic equation that was found by least squares regression of the individual data

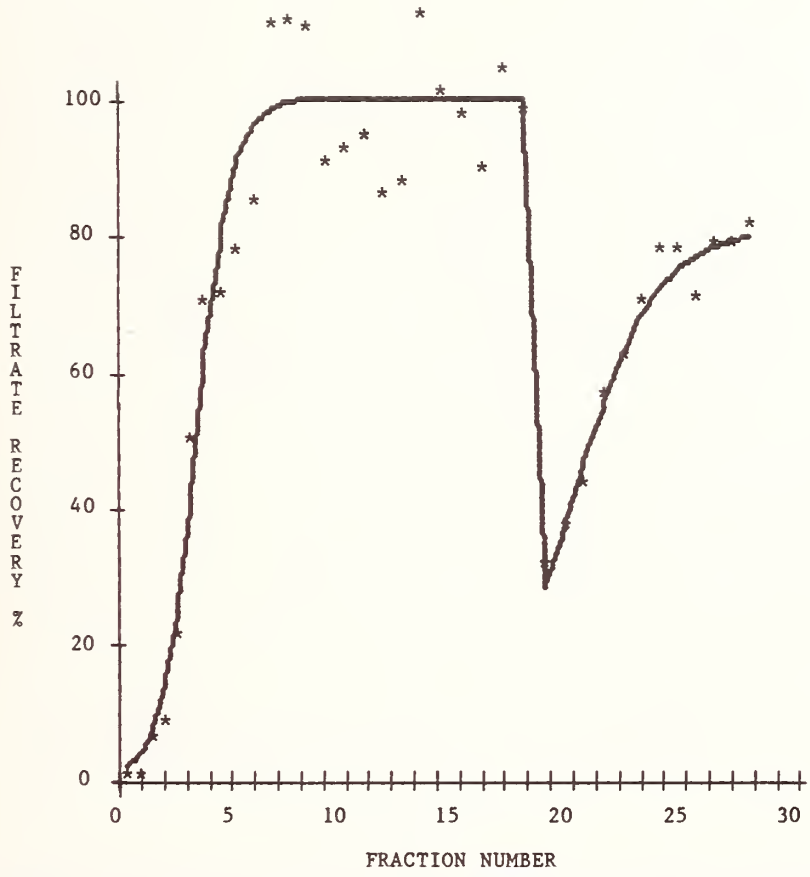

Figure 5. Adsorption of Aroclor 1242, GLC peak 1, on a membrane filter. Values determined are plotted individually (*). Values found by logistic regression are connected (.). Each fraction number represents 13000 ng of Aroclor 1242 applied.

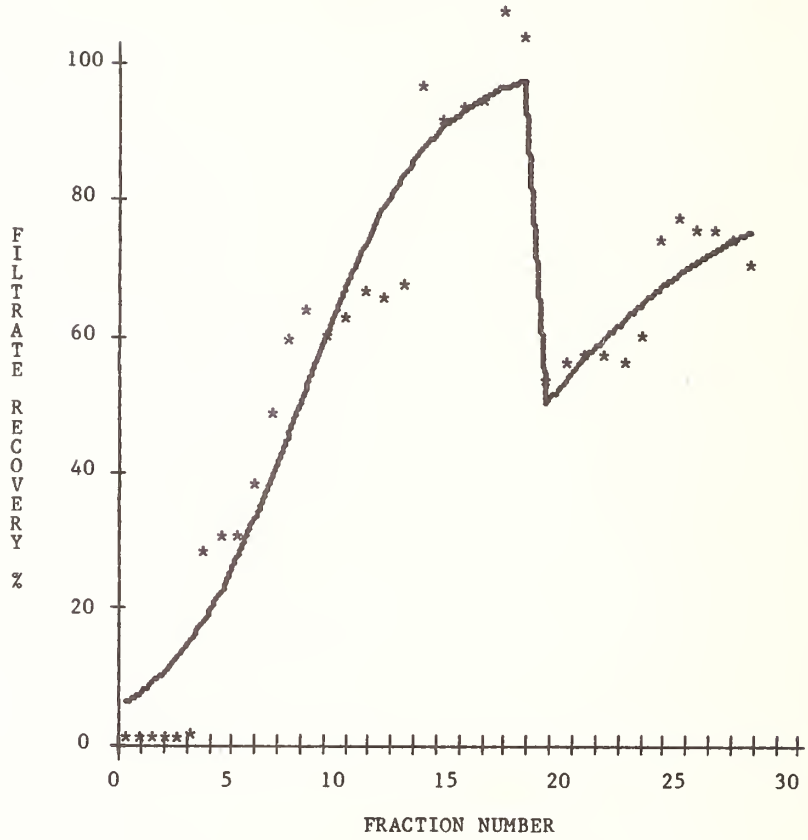

Figure 6. Adsorption of Aroclor 1242, GLC peak 4, on a membrane filter. Values determined are plotted individually (*). Values found by logistic regression are connected (.). Each fraction number represents 13000 ng Aroclor 1242 applied.

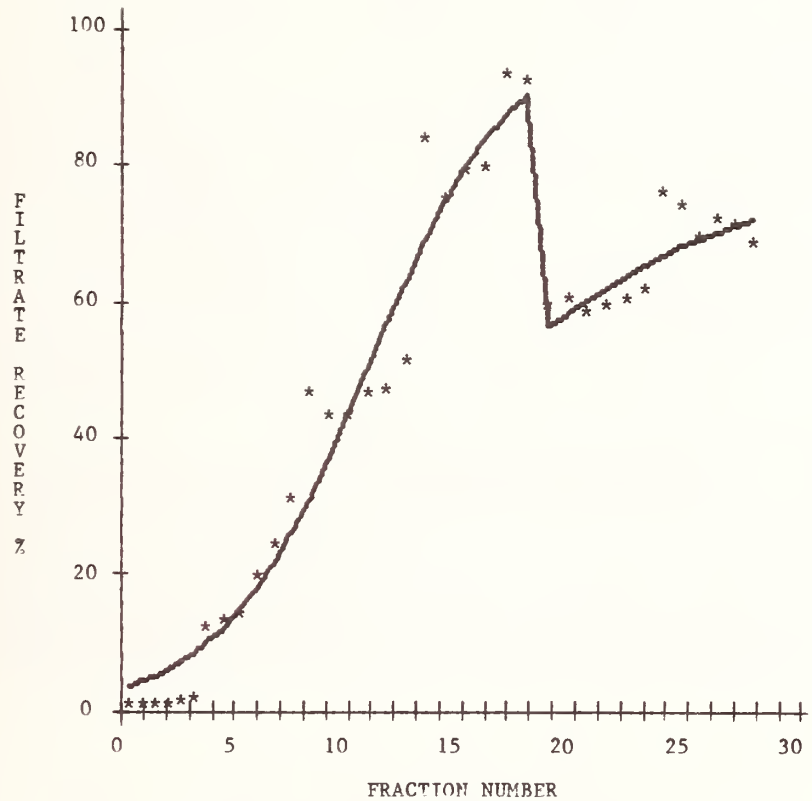

Figure 7. Adsorption of Aroclor 1242, GLC peak 7, on a membrane filter. Values determined are plotted individually (*). Values found by logistic regression are connected (.). Each fraction represents 13000 ng Aroclor 1242 applied.

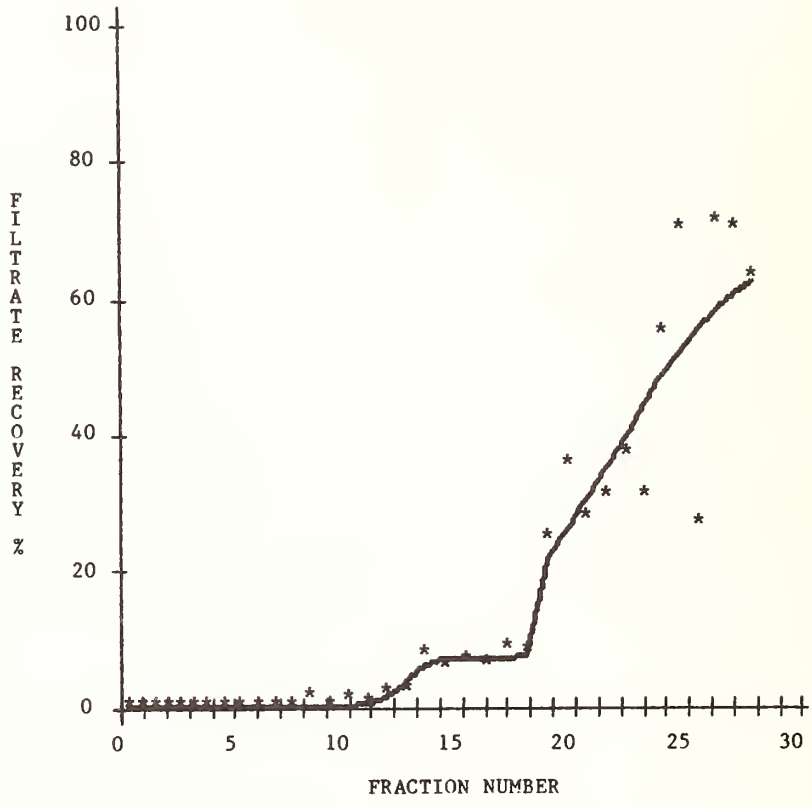

Figure 8. Adsorption of Aroclor 1242, GLC peak 14, on a membrane filter. Values determined are plotted individually (*). Values found by logistic regression are connected (.). Each fraction number represents 13000 ng Aroclor 1242 applied.

TABLE 5. *Adsorption of Aroclor-1242 peaks*

GLC peak	Aroclor-1242 adsorbed, % fraction no.			
	2	6	10	14
1	86	4	0	0
4	90	67	33	10
7	94	82	57	25
14	100	100	100	93

points. This equation when integrated and solved between the limits of 0 and 18 fractions gave the area under the curve. Since 100% recovery represented 13000 ng, the total amount of peak 1 was the product of 13000 ng and 18 fractions times the percentage of the peak in the total mixture. When the integrated data was subtracted from the total applied, the adsorbed amount was determined. This data is presented in Table 6.

The amount of each peak that was desorbed by pure water was also calculated and presented in Table 6. Totals of these two amounts for all 15 peaks were 126 μg adsorbed in 18 fractions and 17 μg desorbed by 2250 mL of pure water.

TABLE 6. *Aroclor-1242 loading on a single filter*

Peak no.	PCB adsorbed, ng 18 fractions	PCB desorbed, ng 3 fractions
1	2110	670
4	19600	3220
7	18200	2340
14	2320	31
Total (15)	126000	17000

The conclusions that can be drawn from this loading data are somewhat limited since only one concentration was applied. It would be interesting to see if this system would obey the Freundlich relationship. To do this would require application of PCB solution at different concentrations. Temperature effects could also be studied.

The study of Uthe et al. [8] showed that there was a common adsorption efficiency between all peaks of the PCB studied in their adsorption to urethane plugs. In their study they did get a higher efficiency when more material was added to the foam plug. On the other hand, Lewis et al. [9] in studying the adsorption of PCB peaks in air to urethane plugs found that earlier peaks eluting from the GLC column were adsorbed less efficiently.

D. THE EFFECT OF PH ON THE ADSORBITIVE QUALITIES OF MEMBRANE FILTERS

Some brief work was done to determine what the effect of pH had on the adsorption qualities of the membrane filter. The first experiment with spring water from the nearby Benner Springs (a source for a State fish hatchery) showed good recovery from adsorption to the filters for the DDT analogs and mirex at pH of 2 and 7. However, at pH 12 the amount recovered from a filter dropped to almost zero (Table 7). A later study using Milli-Q deionized water showed good recoveries at pH 7, 10 and 11 and only a slight dropoff at pH 12 (Table 8). A third study with spring water again (Table 9) showed a good recovery at pH 10 but severe dropoffs at pH 11 and 12.

TABLE 7. *Effect of pH on membrane filter recoveries from Benner Springs water*

Compound	Applied quant. ng	Recovery, %, at pH 2	pH 7	pH 12
Aldrin	7.5	20	32	6
p,p'-DDT	60	80	87	6
Mirex	30	57	66	0

TABLE 8. *Effect of pH on membrane filter recoveries from Milli-Q water*

Compound	Applied quant. ng	Recovery, %, at pH 7	pH 10	pH 11	pH 12
Aldrin	113	90	76	67	25
p,p'-DDT	900	69	94	94	71
Mirex	450	92	72	71	66

TABLE 9. *Effect of pH on membrane filter recoveries from Benner Springs water-II*

Compound	Applied quant. ng	Recovery, %, at pH 10	pH 11	pH 12
Aldrin	113	88	29	20
p,p'-DDT	900	96	31	9
Mirex	450	52	25	15

The pure water result probably indicates the proper effect of pH on the adsorption properties of the filter. Severe dropping off at pH 11 and 12 with the spring water was probably due to adsorption of the compounds to a flocculent precipitate formed at those pH's consisting probably of metal hydroxides.

IV. Conclusion

Cellulose triacetate membrane filters have been shown to be effective filtration agents for certain pesticides and industrial contaminants dissolved in water. Their effectiveness appears to be a function of the aromatic quality, the flatness of the molecule, the absence of oxygen or other similar atoms, and the presence of chlorine. Membrane filters made from other materials were adsorbers of PCB's but were less effective. The loading capacity of the triacetate filter was related directly to the chlorine content of the PCB tested.

These filters can be convenient agents for the separation and analysis of environmental contaminants. Separations from water can be done with portable apparati in the field, transported easily, and stored for later analysis.

V. Acknowledgments

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APPLICATION OF LIQUID AND GAS CHROMATOGRAPHIC TECHNIQUES TO A STUDY OF THE PERSISTENCE OF PETROLEUM IN MARINE SEDIMENTS

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A technique employing high-pressure liquid chromatography (HPLC) and glass capillary chromatography has been used to monitor the long-term persistence of petroleum in marine sediments. Petroleum in sediments was studied using Prudhoe Bay crude (PBC) as the host oil, as part of a combined chemistry/biology study of the impact of oil on marine ecosystems.

Total monoaromatic-diaromatic hydrocarbon concentrations in extracts of oil polluted sediments were analyzed by HPLC on series coupled columns containing Durapak oxypropionitrile on Porasil C. Chromatographic separation of the extracts produced a saturated fraction and a monoaromatic-diaromatic fraction which was approximately 80% resolved as determined by the separation of model compounds. The quantitative analysis of the summation of the monoaromatic-diaromatic components was performed with a calibration curve prepared from a PBC aromatics fraction isolated by an ASTM method. Infrared analysis of carbon tetrachloride extracts of the same sediments provided total oil concentrations as complementary information to the HPLC analysis. More detailed compositional information on individual saturate and aromatic hydrocarbons in oil contaminated sediments was provided by glass capillary chromatography.

The above methods were applied to a study of the persistence of PBC in two types of sediments that were exposed to conditions which simulated possible environmental pollution situations. In a laboratory experiment, a fine-textured sediment containing PBC oil was monitored periodically over a period exceeding 1 year after deposition in a continuous-flow seawater bioassay apparatus. Similarly, the fate of PBC in a field experiment involving a coarse beach sediment located in an intertidal zone was studied. The persistence of PBC in both sediment systems is discussed in terms of total oil concentration, concentrations of hydrocarbons and hydrocarbon classes, and some of the physical, chemical, and biological parameters which affect the degree of persistence.

Key words: Gas chromatography—mass spectrometry; glass capillary chromatography; high pressure liquid chromatography; hydrocarbon persistence; infrared spectroscopy; petroleum hydrocarbons; sediment analysis.

I. Introduction

Much research effort in past years has been directed toward developing analytical methods for determining the source of oil spills in the marine environment. These methods included the techniques of gas chromatography [1,2] and infrared spectroscopy [3]. These, as well as other techniques, were reviewed by Wilson et al. [4]. A study has been conducted to provide hydrocarbon baseline information in anticipation of possible oil spills in a specific area [5]; however, scant information has been provided on the fate and effects of petroleum in the environment employing such methods. Blumer and Sass [6], described the application of gas chromatography to a study of the persistence and degradation of spilled fuel oil, and most recently, methods have been described for the application of glass capillary chromatography and HPLC to monitor petroleum-type hydrocarbons in marine sediments [7–9]. In most cases, little quantitative information is provided.

Our research efforts have been directed toward development of analytical methods to determine the rates and mechanisms of degradation of petroleum hydrocarbons in marine sediment systems, to determine which compound types are persistent in these systems, and to correlate this

information, where possible, with observed effects on the marine biota occupying these systems. Previous Battelle research efforts utilized gas chromatography and infrared spectroscopy (IR) for the study of petroleum pollution in sediments and as suspensions in seawater [10–12]. Total oil concentrations, as measured by infrared spectroscopy, were used to monitor petroleum concentrations in continuous flow bioassay systems in marine organism toxicity studies [13–14]. We used the IR method for monitoring the persistence of petroleum in laboratory and field sediment systems [10], and, in addition, developed a new method employing high-pressure liquid chromatography (HPLC) to monitor total monoaromatic-diaromatic hydrocarbons in marine sediments. This paper describes these experimental techniques and their application to a study of the persistence of Prudhoe Bay crude oil (PBC) in two types of sediments which simulated possible environmental pollution situations.

II. Experimental

A. Materials

PBC oil was supplied by NOAA OCSEAP Office, Juneau, Alaska. All extractions and chromatographies were conducted employing "distilled in glass" solvents (Burdick and Jackson). Samples were stored in vials with Teflon-lined caps prior to analysis. Sediment and system blanks were run periodically to assure a contamination-free analytical system and to account for natural hydrocarbon concentrations.

B. Sample Preservation

Coarse and fine-textured sediment samples obtained from the field and laboratory experiments were stored in glass jars (Teflon-lined caps) or as polyvinyl chloride cores at –20 °C until analyses could be conducted.

C. Sediment Extraction

For IR analysis, 20-gram samples of sediment were added to 100-mL amber glass pharmaceutical dispensing bottles containing 20 grams of anhydrous sodium sulfate (Na_2SO_4). Fifty milliliters of carbon tetrachloride (CCl_4) were added and the samples were shaken for 6 hours, at which time, the CCl_4 was removed and the sediment-Na_2SO_4 mixture was recharged with 50 mL of CCl_4 and shaken a second time over a 16-hour time period. These extracts were appropriately diluted and analyzed by IR. Extraction of uncontaminated sediment was used to correct samples for oil-free background absorbance.

Hexane extracts of sediments were prepared in a similar manner for HPLC and GC analysis using only one 24-hour extraction of the sediments. Based on total oil concentrations from CCl_4 extracts from subsamples of the same sediment, aliquots of the hexane extracts were appropriately concentrated prior to column chromatography and subsequent GC analysis.

D. Total Oil Analysis by IR

Concentrations of total oil in sediments were determined on CCl_4 extracts of sediments with a Beckmann Acculab 6 spectrophotometer by comparison of the amount of absorbance obtained at 2927 cm^{-1} to a calibration curve [15]. Triplicate analysis of known concentrations of oil indicated that the double extraction with CCl_4 removed over 97% of the oil from the sediments.

E. Analysis of Hexane Extracts by HPLC

Hexane extracts of sediments were analyzed by HPLC employing three series-coupled 1/8″×2′ Durapak oxypropionitrile on Porasil C columns (Waters Associates) using hexane as the

mobile phase at a flow rate of 1.0 mL/min. Samples were monitored with a Waters differential refractometer and a Schoeffel UV detector set at 221 nm. The ability of the column system to separate hydrocarbons into saturate, monoaromatic, diaromatic, and polyaromatic fractions was demonstrated by the separation of the model compounds: hexadecane, benzene, naphthalene, and phenanthrene. Samples of hexane extract from sediment contaminated with oil when analyzed under the same conditions produced an overlapping monoaromatic-diaromatic fraction (Fig. 1). The quantitative analysis of the combined monoaromatic-diaromatic fraction was performed with a calibration curve prepared from a PBC aromatics fraction isolated using an ASTM method [16].

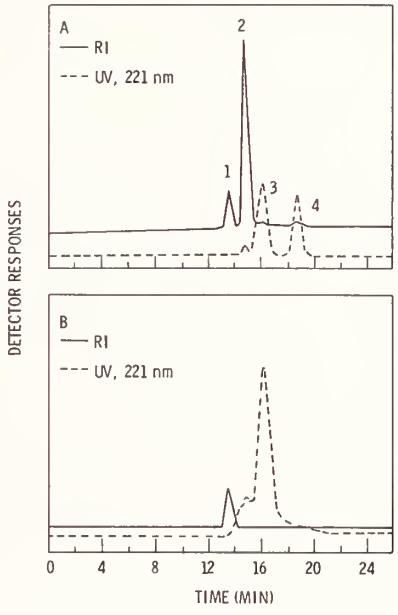

Figure 1. Liquid chromatographic separation of A: hexadecane (1), benzene (2), naphthalene (3) and phenanthrene (4) on Durapak oxypropionitrile on Porasil C columns and B: an aromatic fraction isolated from PBC oil by an ASTM Method.

F. Capillary Gas Chromatography

PBC oil and hexane extracts of sediment samples were analyzed on a Varian Model 2800 gas chromatograph modified to operate with glass capillary columns. Chromatographic separations were conducted on 30 meter SE-30 glass capillary columns (J & W Scientific) programmed from an initial temperature of 70 °C to 250 °C at a rate of 4°/min. Typical operating conditions provided a helium linear velocity of approximately 30 cm/s through the column. A split ratio of 10 to 1 was employed. Nitrogen makeup flow to the flame ionization detector was approximately 80 mL/min. Such conditions produced columns with N_{eff} approximately 60,000 when operating isothermally at 130 °C.

G. Gas Chromatography—Mass Spectrometry (GC/MS)

A Hewlett-Packard 5980A quadrupole mass spectrometer operating in the argon chemical ionization mode and employing single-ion monitoring was used to determine retention times of specific compounds and approximate retention time ranges for specific compound types. Chromatographic separations were performed on a 6′×1/4″ glass column containing 3% SP-2100/0.3% SP-1000 on Chromosorb W 100/120 mesh AW-DMCS. The column was programmed from 80 °C to 250 °C at 8°/min.

H. Analysis of Components of PBC Oil

The separation and analysis of saturate and aromatic fractions of PBC oil was adapted from a chromatographic technique described by Warner [17] with the following modifications: Fifteen grams of silica gel (Grace Davison Chemical Co., 100–200 mesh) was slurried in hexane and packed into a 12 mm ID×250 mm glass column. Saturate (40 mL, hexane) and aromatic (86 mL of 20% CH_2Cl_2 in hexane) fractions were collected in 40 mL conical tubes. Samples were concentrated under a stream of nitrogen without the aid of external heat, transferred to 5 mL solu-vials and concentrated to 1 mL. One milliliter of internal standard (2, 6, 10-trimethyldodecane for saturate fraction and hexamethylbenzene for aromatic fraction) was added to each sample, concentrated to 1 mL or less and analyzed by GC as previously described.

I. Hydrocarbon Recovery Studies

One milliliter of two concentrations of oil in hexane was added in separate experiments to triplicate 20 gram samples of a coarse beach sediment and a fine-textured sediment. Such amendments produced concentrations of hydrocarbons in sediments which approximated the hydrocarbon concentration range for which quantitation in the laboratory and field sediment experiments was conducted. Each of the samples was extracted with hexane and prepared for GC analysis by the methods previously described. Concentrations of individual hydrocarbons in the hexane extracts were quantitated by GC and compared to the concentrations known to be present in the amount of oil amended to the sediment.

Average recoveries of saturate hydrocarbons from fine-textured sediment at the high and low oil concentration levels were 85% and 96%, respectively. For the coarse beach sediment, saturate hydrocarbon recoveries were 86% and 82%. For the fine textured sediment, average recoveries of aromatic hydrocarbons from naphthalene to the dimethylphenanthrenes at the high and low concentration levels were 76% and 92%, respectively. Similarly, for the coarse beach sediment, the recoveries were 88% and 83%. Data reported in Tables 1 and 2 were corrected for recovery.

TABLE 1. *Comparison of component types found in oil from 1-year-old laboratory sediment samples compared to composition in original crude oil*

Component type	A mg component per gram oil in laboratory system	B mg component per gram oil in PBC oil	A/B
C_{12}	0.35	3.95	0.09
C_{17}	0.21	3.39	0.06
Pristane	1.37	2.15	0.64
C_{18}	0.20	3.00	0.07
Phytane	0.97	1.34	0.72
C_{26}	0.18	1.80	0.10
Total saturates	5.86	47.75	0.12
Naphthalene	0.54	1.55	0.35
2-Methylnaphthalene	0.84	2.49	0.34
1-Methylnaphthalene	1.48	2.14	0.69
Dimethylnaphthalenes	5.16	7.97	0.65
Trimethylnaphthalenes	2.40	4.81	0.50
Phenanthrene	0.39	0.58	0.67
Methylphenanthrenes	1.12	1.67	0.67
Dimethylphenanthrenes	1.30	1.67	0.78

Table 2. *Composition of component types found in oil from 7-month-old field sediment samples compared to composition in original crude oil*

Component type	A mg component per gram oil in field system	B mg component per gram oil in PBC oil	A/B
C_{12}	2.03	3.95	0.51
C_{17}	0.72	3.39	0.21
Pristane	3.39	2.15	1.58
C_{18}	0.82	3.00	0.27
Phytane	2.54	1.34	1.90
C_{26}	3.07	1.80	1.71
Total saturates	37.14	47.75	0.78
Naphthalene	0.44	1.55	0.28
2-Methylnaphthalene	1.37	2.49	0.55
1-Methylnaphthalene	0.56	2.14	0.26
Dimethylnaphthalenes	5.59	7.97	0.70
Trimethylnaphthalenes	11.74	4.81	2.44
Phenanthrene	0.52	0.58	0.90
Methylphenanthrenes	4.69	1.67	2.81
Dimethylphenanthrenes	4.37	1.67	2.62

III. Results and Discussion

The HPLC technique was developed to provide a rapid means for providing information on the concentrations of total monoaromatic-diaromatic hydrocarbons in petroleum contaminated sediment. As in the IR technique, solvent extracts of sediments are analyzed directly. Such information is not provided as part of total oil concentration as measured by IR since this technique relies on the measurement of saturate hydrocarbon absorbance at 2927 cm^{-1}. Therefore, measurement of total oil alone is inadequate in the measure of changes that are occurring in the relative composition of compounds and compound types present in petroleum contaminated sediments, particularly, the aromatic components. The HPLC technique provides needed complementary information on classes of compounds which are currently receiving attention from researchers regarding their toxicological effects on marine organism populations [18]. In using the HPLC method, however, it is important to calibrate for quantitative purposes using an authentic sample of the oil under study since the detector response per unit weight of aromatic material can vary significantly from one crude oil to another.

The two techniques (HPLC, IR) were used in the analysis of samples derived from two types of PBC contaminated sediments exposed to conditions which might simulate possible environmental pollution situations. In a laboratory experiment that simulates, for example, pollution that might be encountered in sediment near offshore drilling activities, a fine textured sediment (mud) contaminated with PBC oil, positioned at the bottom of an aquarium tank, and receiving a continuous flow of seawater over its surface was routinely sampled for a period exceeding 1 year for persistence of petroleum hydrocarbons. A control tank was also sampled. Similarly, in a field experiment, the fate of PBC layered on the surface of a coarse beach sediment located in an intertidal zone was studied to simulate a spill situation where oil impacts a beach. The results of these analyses are shown in Figures 2 and 3. For the laboratory experiment (Fig. 2), data obtained for total oil concentration indicate that the rate of depuration of oil from fine, undisturbed sediments is very slow, on the order of several years. These results must be viewed

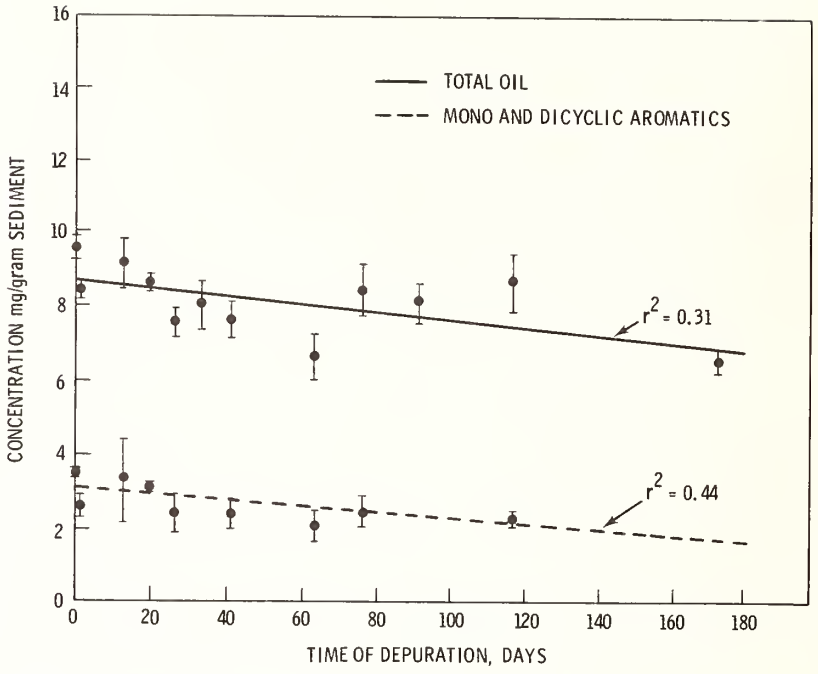

Figure 2. Persistence of total oil and monoaromatic-diaromatic hydrocarbons in laboratory sediment system as measured by IR and HPLC respectively.

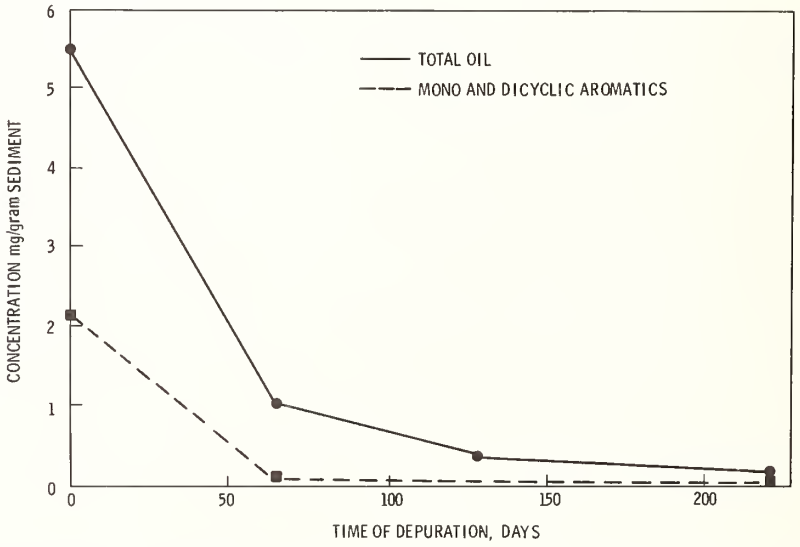

Figure 3. Persistence of total oil and monaromatic-diaromatic hydrocarbons in field sediment system as measured by IR and HPLC respectively.

with caution, however, until a proper field study can be conducted. The data for the aromatics analysis also suggest slow depuration of these components, but at a rate somewhat faster than total oil. Correlation of the data with the linear regression was better for aromatics ($r^2=0.44$) than for total oil ($r^2=0.31$). In both sets, the scatter of data around the linear regression indicates that the oil is not as homogeneous in the sediment as might be desired.

These data are in marked contrast to results obtained in the field experiment (Fig. 3). Because of the sampling restrictions of the original experimental design, the results are based on single analyses of sediment samples taken at four time intervals. Seven months after initiation of the experiment only 2.8% of the total oil as measured by IR and 5.9% of the monoaromatic-diaromatic components, as measured by HPLC, remained. The more rapid loss of oil from the beach sediment can be attributed to greater activity derived from many physical, chemical, and biological mechanisms. Major contributors to these mechanisms include wave and tidal action, surface volatility, photochemical and biodegradative processes, interstitial water solubility and adsorption.

More detailed compositional information on individual saturate and aromatic hydrocarbons and hydrocarbon types in these oil contaminated sediment systems was provided by GC. To determine the persistence of individual hydrocarbons in each system, comparisons were made between the compound type distributions in each sediment system and the original crude oil. The results are shown in Tables 1 and 2. Table 1 refers to changes in distributions of hydrocarbons in the laboratory sediment study. In column A of this table, is shown the relative contribution of major hydrocarbon and hydrocarbon types relative to the total oil concentration of a sediment sample taken 1 year after initiation of the laboratory study. This contribution to total oil is then compared with the concentration of the hydrocarbon or hydrocarbon type in PBC (column B). The ratio of A/B represents the changes in the distributions of individual hydrocarbons and hydrocarbon types in the oil-sediment laboratory system due to the influence of the various physical, chemical, and biological processes previously described. For all hydrocarbons and hydrocarbon types studied in the laboratory system, the ratio of A/B was less than one, indicating the preferential loss of these components relative to other more persistent components in the oil. There was a substantial difference in relative persistence among the hydrocarbons with the aliphatic straight hydrocarbons being lost most rapidly (lowest A/B ratio). Pristane and phytane were present at concentrations approximately 10-fold higher than other saturate hydrocarbons. Aromatic hydrocarbons were more persistent than the saturate straight chain hydrocarbons with increasing persistence following the order of increasing molecular weight. The relative losses of individual hydrocarbons and hydrocarbon types in the field study followed similar trends except that enrichments in pristane, phytane, trimethylnaphthalenes, methylphenanthrenes and dimethylphenanthrenes were observed relative to the original oil composition (Table 2). Differences observed in distribution of individual hydrocarbons and hydrocarbon types between the two sediment systems is a result of the complex combination of physical, chemical, and biological processes. A major physical factor contributing to these distributions is the differences in the energy of the systems. Wave and tidal energy plays a major role in losses of oil in the field system as was shown in the analyses conducted by IR and HPLC. For the same system, similar results have been reported for individual hydrocarbons and hydrocarbon types [10]. The shorter residence time of the majority of the oil in the field system effects the rate of change that occurs in the compositional distribution of individual hydrocarbons and hydrocarbon types remaining in the system due to the concentration dependency of many of the processes that effect the retention of the oil. In the laboratory system where the energy of the system is considerably lower, the biodegradation process plays an important role on the retention time of oil. This is reflected in the results of the laboratory experiment listed in Table 1 where A/B ratios for pristane and phytane are substantially closer to 1 relative to the ratios for saturate straight chain hydrocarbons. Similar results were reported by Blumer and Sass [6] who used C_{17}/pristane and C_{18}/phytane ratios as evidence of biodegradation of oil in the environment. Both systems showed a preferential persistence of aromatic hydrocarbons suggesting their greater immunity to decomposition or removal from the two sediment systems by the processes previously described.

IV. Acknowledgments

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Trade names are supplied throughout this paper to assist the reader in replicating the experiment but do not imply endorsement by Battelle Memorial Institute.

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

THE USE OF A FLUORESCENCE DETECTOR IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ROUTINE ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS IN ENVIRONMENTAL POLLUTION AND OCCUPATIONAL HEALTH STUDIES

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A routine method for trace analysis of nine major polycyclic aromatic hydrocarbons (PAH) by high performance liquid chromatography (HPLC) with fluorescence detection is described. The fluorimetric detection involves a deuterium light source and excitation wavelengths below 300 nm. Careful selection of excitation and emission wavelengths gives a high degree of selectivity and specificity in fluorescence detection and permits complete analysis of individual PAH in a multi-component mixture. The extremely high sensitivity in fluorescence detection has reduced minimum detectable concentration of the PAH close to subpicogram levels, e.g., benzo(a)anthracene 0.19 pg, benzo(k)fluoranthene 0.11 pg, benzo(a)pyrene 0.34 pg. The sensitivity is found to be strongly influenced by the amount of water present in the PAH solution to be analyzed. The HPLC—fluorescence system allows the use of dilute solutions, thus eliminating the usual clean-up procedures associated with trace analysis. The application of the method for the analysis of PAH in environmental, process and occupational health samples is discussed.

Key words: Environmental pollution; fluorescence; high performance liquid chromatography; occupational health; picogram; polycyclic aromatic hydrocarbons; selectivity; sensitivity; specificity; trace analysis.

I. Introduction

Polycyclic aromatic hydrocarbons (PAH) are widespread contaminants of the environment, occurring primarily as a result of combustion and pyrolysis of organic materials. A significant number of these PAH are either known or suspected carcinogens [1]. There is now considerable interest from environmental and health protection agencies in the development of reliable, sensitive and rapid techniques for the analysis of PAH in environmental and occupational health samples. Many analytical techniques involving high performance liquid chromatography (HPLC) have been described in the literature [2–7] for the characterization of complex mixtures of PAH in environmental samples. None of these published methods has been developed into a satisfactory routine procedure for the quantitative analysis of PAH in such samples.

There are several problems involved in the analysis of PAH. A major difficulty is their isolation from other interfering organic substances. Clean-up procedures for separation of the PAH from interfering components are usually practiced. Such steps may be associated with adventitious contamination or loss of PAH. Elimination of clean-up steps in trace analysis is therefore desirable. In addition, a high sensitivity of detection is required for the analysis of the low concentrations of PAH normally encountered in the environment.

This paper describes a new routine method for PAH determination. The procedure reported is a combination of HPLC in the reverse phase mode with a highly efficient microparticulate column and a fluorescence detector with a deuterium lamp source. The extremely high sensitivity in fluorescence emission has reduced the minimum detectable concentration of most PAH close to subpicogram levels. The procedure is both rapid and simple, and the successful application of this

new technique for routine monitoring of PAH in environmental, process and occupational health samples is discussed.

II. Experimental

A. Reagents

Nine PAH standards were selected for this study (see Fig. 1 for PAH structures). The PAH standard samples were obtained from the following sources and were used without purification: fluoranthene, B(a)A, chrysene, B(k)F, B(a)P, B(e)P from the stock of the laboratory of Air Pollution Control Directorate, Department of the Environment, Ottawa, and perylene, dibenz(ah)A and benz(ghi)perylene from Aldrich Chemical Company, Inc., N.J. Spectrograde cyclohexane and acetonitrile were obtained from Burdick and Jackson Lab., Inc. Distilled, deionised water shown to be free of fluorescent impurities was used.

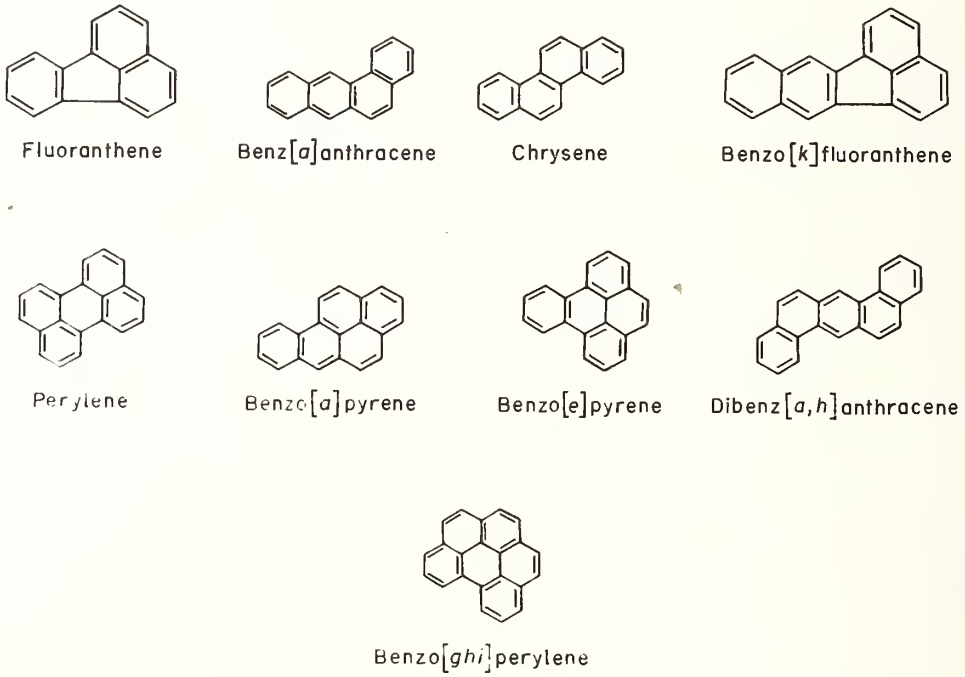

Figure 1. Structures of polycyclic aromatic hydrocarbons.

B. Apparatus

A spectra-physics Model 3500B Liquid Chromatograph equipped with a thermostatted oven and a variable wavelength fluorescence detector, Model FS 970 Spectrofluoro Monitor (Schoeffel Instrument Corporation, Westwood, N.J.) was used. The FS 970 Spectrofluoro Monitor had a low volume flow cell (5 μL) and was equipped with a deuterium light source for generating excitation energy below 300 nm and a set of six interchangeable emission cut-off filters, 370, 389, 418, 470, 550 and 580 nm. For UV detection, a variable wavelength detector Model SF 770 Spectroflow Monitor (Schoeffel Instrument Corporation) was used.

C. HPLC Operating Conditions

HPLC in reverse phase mode was performed isocratically with either 82% or 84% CH_3CN in water. The flow rate was 0.3 mL/min, the column pressure was between 300 and 500 psi, and the temperature was 25 and 30 °C maintained by a thermostatted oven. The injections were made with a sample loop operated by a rotary valve using a 10 μL injection volume. The wavelengths used in UV detection were 254 and 280 nm, and the following three sets of excitation and emission wavelengths conditions were used in fluorescence detection:

	λex 280 nm		λex 250 nm	λex 240 nm
a)		b)		and c)
	λem > 389 nm		λem > 370 nm	λem > 470 nm

D. Sample Preparation

The following samples were investigated: three environmental, two process and one occupational health (personal monitoring) samples. The type of sample and the method of sample preparation were as follows:

Sample A

This was a particulate matter sample from a coke oven stack emission. The sample containing 14.1 mg particulate matter was extracted overnight (~16 hr) with spectrograde cyclohexane in a Soxhlet extractor (~63.1% cyclohexane soluble). 1 mL from the total 100 mL extract was carefully evaporated to dryness under a slow stream of dry nitrogen and the residue dissolved in 75% CH_3CN. After appropriate dilution (1:440), the solution (10 μL injection) was analyzed.

Sample B

This was a gaseous phase sample from a coke oven stack emission. It was collected on a Tenax Adsorbent tube placed after the particulate filter in the stack emission sampling train. The adsorbent was extracted with pentane for 24 hours. Final volume of extract used was 50 mL. 1 mL from the 50 mL extract after dilution (1:20) was analyzed.

Samples (A) and (B) were provided by the Air Resources Branch, Ministry of the Environment, Ontario. Sample (A) was collected according to EPA method [8] and Sample (B) was collected according to the method developed by Battelle Labs [9] and accepted by EPA [10].

Samples C and D

Sample (C)—coal and Sample (D)—coke represent two process samples from a coke oven plant.

Coal (11.17 g) and coke (10:44 g) samples were Soxhlet extracted with cyclohexane (100 mL). The dilution required for 1 mL of 100 mL extract was 1:5 for the coal sample. The coke extract required concentration from 100 mL to 25 mL prior to analysis.

Sample E

This was a sample of recycled waste water from the quenching operation in a coke oven plant. The suspended particulate matter present in the sample was removed from a 1 litre volume by centrifugation, and the aqueous effluent was analyzed by direct injection.

Sample F

This occupational health (personal monitoring) sample was collected on 0.8 μm porosity silver membrane filters according to NIOSH method [11]. The sample provided by the Health

Protection Branch, Ministry of Labour, Ontario, was from a coke oven plant. The sample was Soxhlet extracted with 50 mL cyclohexane and a dilution of 1:1.5 was made prior to analysis.

E. Identification and Quantitation of Peaks

Peaks observed in the sample chromatographic profiles were identified by a combination of four methods: retention times in comparison with standards, coinjection with standards, differential fluorescence technique, i.e., analyzing samples under three optimal fluorescence conditions (λex 280/λem >389, λex 250/λem >370, and λex 240/λem >470), and peak height ratios at two different wavelengths (λex 280/λem >389 and λex 250/λem >370). Peak height was used for quantitation of PAH in the samples.

III. Results and Discussion

The HPLC chromatographic profiles of the PAH obtained with UV detection at 254 nm and 280 nm shown respectively in Figures 2(A) and 2(B) show that the microparticulate column gives good separation of fluoranthene, B(a)P, dibenz(ah)A and benz(ghi)perylene, but fails to resolve either chrysene from B(a)A or B(k)F, B(e)P and perylene from each other in the multicomponent mixture. It is also observed that the PAH of interest exhibit UV absorption at both 254 and 280 nm wavelength, the main difference being in their relative intensities. The UV method of detection at the above wavelengths is therefore non-selective. PAH determination on multicomponent systems is not feasible by such a detection technique unless the PAH are separated from each other.

A fluorescence detection system can often overcome the problem of resolution in HPLC by the fact that two wavelengths, excitation and emission are available for measurement and by careful selection of excitation and emission wavelengths, a high degree of specificity and selectivity in fluorescence detection can be obtained. This selection permits the determination of PAH in a multicomponent system.

Three chromatograms shown in Figure 3 illustrate this. Following the general rule that the optimum excitation wavelength for fluorescence equals maximum absorption wavelength, the PAH were excited at three absorption maxima, namely 280, 250 and 240 nm. Studies with individual PAH and with a synthetic mixture have shown that all PAH with the exception of chrysene and perylene show strong fluorescence when excited at λex 280 nm. A chromatogram of the synthetic mixture at λex 280 nm with emission wavelength set at λem >389 nm is shown in Figure 3(A).

The unresolved single peak from chrysene and B(a)A as observed with UV detection (Fig. 2) is now due to B(a)A free of interference from chrysene. Similarily, the unresolved peak for B(k)F, B(e)P and perylene in the UV detection now separates into two well resolved peaks attributed to B(k)F and B(e)P respectively free from perylene interference.

Chrysene displays poor excitation and therefore little fluorescence at λex 280 nm. The chromatogram shown in Figure 3(A) was obtained with 57 pg B(a)A and 42 pg chrysene (B(a)A/chrysene ratio of 1.35) with a 10 μL injection. At this ratio chrysene does not interfere with the B(a)A determination. It was observed that B(a)A can be selectively measured in the presence of chrysene at λex 280 and λem >389 nm up to a B(a)A/chrysene ratio of 0.025 (5 pg B(a)A/200 pg chrysene) in the mixture. Below this ratio, the contribution from chrysene becomes noticeable.

The selectivity of the fluorescence detector can also be used to monitor chrysene in the presence of B(a)A. As seen in Figure 4 work with individual PAH demonstrates that chrysene shows excitation at λex 250 nm but not at λex 380 nm, whereas B(a)A is excited at both wavelengths. Similar results were obtained with the synthetic mixture as shown in Figure 3(B), where the emission cut off filter (λem >370 nm) passed emission energy from all PAH except B(e)P, dibenz(ah)A and benz(ghi)perylene upon excitation at λex 250 nm.

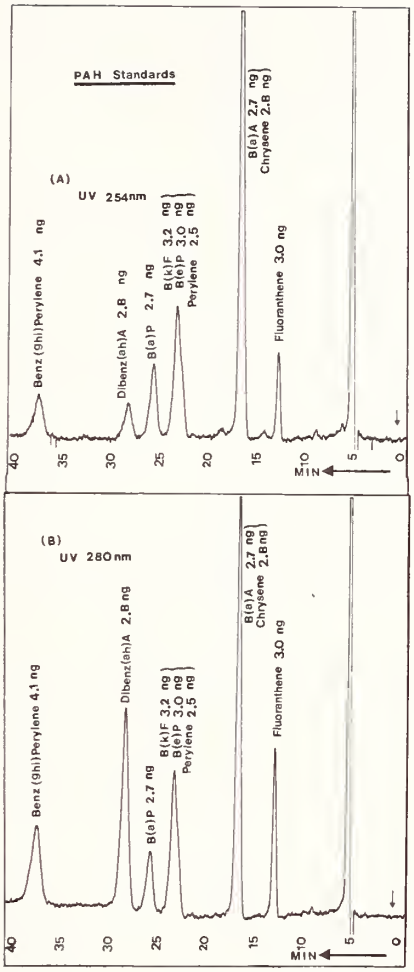

Figure 2. Liquid chromatogram of a mixture of nine polycyclic aromatic hydrocarbons with UV absorption detection. Chromatographic conditions: 2.1 mm i.d.×25 cm Zorbax ODS column, 82:18 (v/v) $CH_3CN:H_2O$, 25 °C, 300–500 psi, flow rate 0.3 mL/min, 10 μL injection of PAH dissolved in 75% CH_3CN. (A) UV 254 nm @ 0.01 AUFS (B) UV 280 nm @ 0.01 AUFS.

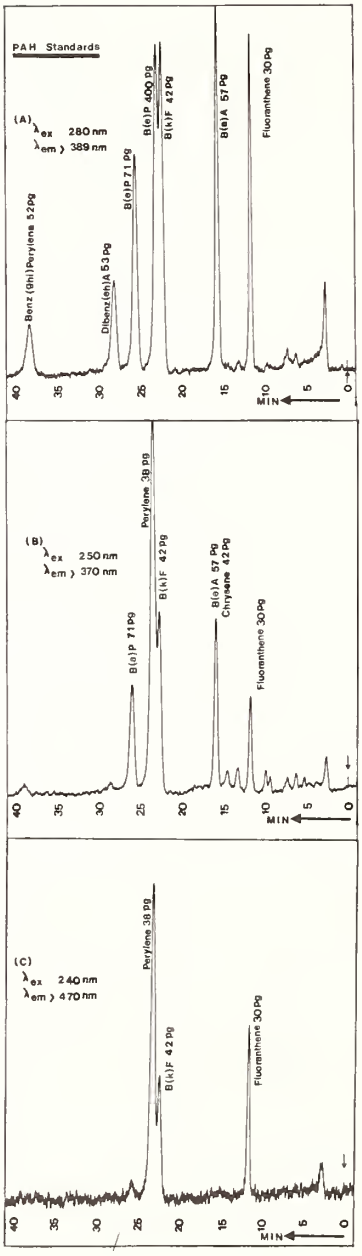

Figure 3. Liquid chromatogram of a mixture of nine polycyclic aromatic hydrocarbons with fluorescence detection. Chromatographic conditions: same as in Figure 2. Fluorescence detection conditions: (A) λex 280 nm, λem >389 nm; emission filter cutoff type KV 389, sensitivity 5.52, range 0.2 μA, time constant 5 sec, (B)λex 250 nm, λem >370 nm; emission filter cutoff type KV 370, sensitivity 5.25, range 0.2 μA, time constant 5 sec, (C) λex 240 nm, λem >470 nm; emission filter cutoff type KV 470, sensitivity 6.10, range 0.2 μA, time constant 5 sec.

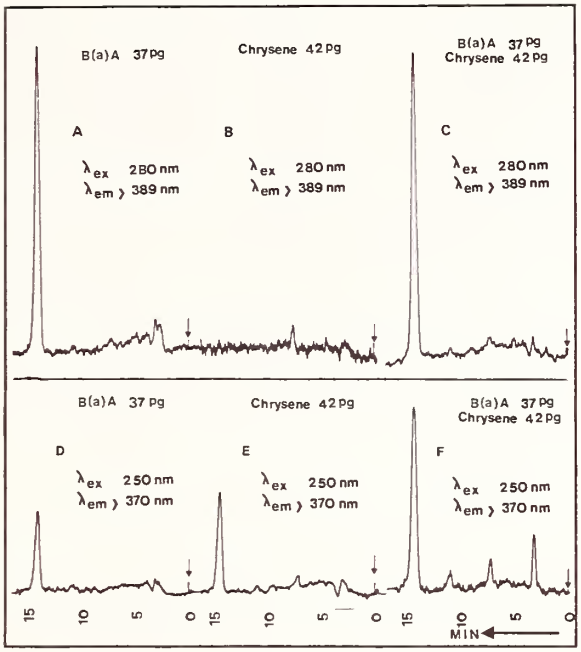

Figure 4. Liquid chromatogram of B(a)A, chrysene and their mixture with fluorescence detection. Chromatographic conditions same as in Figure 2. Fluorescence detection conditions: (A) (B) and (C) same as in Figure 3(A) and (D) (E) and (F) same as in Figure 3(B).

This selectivity in fluorescence detection permits the determination of chrysene in the presence of B(a)A by the use of the following equation.

$$(a) = (b) - \frac{(c)}{(d)} \qquad (1)$$

where

(a) = peak height contribution due to chrysene at λex 250 and λem >370 nm

(b) = peak height of B(a)A and chrysene at λex 250 and λem >370 nm

(c) = peak height of B(a)A at λex 280 and λem >389 nm, and

(d) = a constant (3.91) representing the ratio of peak heights of B(a)A of known concentration at λex 280, λem >389 and λex 250, λem >370 nm (see Figs. 4(A) and 4(D)).

The validity of eq. (1) for measuring chrysene in the presence of B(a)A was demonstrated by a series of experiments with the chrysene/B(a)A ratio varying from 0.36 (24 pg chrysene/66 pg B(a)A) to 4.36 (120 pg chrysene/27.5 pg B(a)A) in the synthetic mixture. The ratio (d) in eq. (1) was determined from several measurements of different but known concentrations of B(a)A and is found to be constant irrespective of changes in HPLC operating conditions (e.g., flow rate, temperature, nature and composition of mobile phase), provided identical chromatographic conditions were used for fluorescence measurements at λex 280, λem >389 and λex 250, λem >370 nm.

Another isomeric pair which is difficult to separate by HPLC is the 5-ring perylene and B(e)P. Throughout this study it was observed that B(e)P had a poor fluorescence response under any fluorescence excitation and emission conditions relative to other PAH. For example, as shown in Figure 3(A), 400 pg of B(e)P gave a signal comparable in intensity to 42 pg B(k)F at λex 280 and λem 389 nm. Depending on the relative concentration of B(e)P and perylene in a mixture, the

poor response of B(e)P permits their selective determination in such a mixture. This is illustrated in Figures 3(A) and 3(B). These chromatograms were obtained with a synthetic mixture containing 38 pg perylene and 400 pg B(e)P. Perylene exhibits weak fluorescence when excited at λex 280 nm and strong fluorescence on excitation at λex 250 nm. Conversely, B(e)P displays fluorescence at λex 280 nm, which is much stronger in intensity than when it is excited at λex 250 nm. It is seen from Figure 3(A) that at the relative concentration of B(e)P (400 pg) and perylene (38 pg), the contribution from perylene to B(e)P is negligible at λex 280 and λem >389 nm. Similarly, at the same relative concentration, there is no contribution from B(e)P to the perylene peak at λex 250 and λem >370 nm (Fig. 3(B)). However, in samples where the concentration of one is large compared to the other, quantitative determination of the individual PAH of the isomeric pair connot be achieved under the above specified fluorescence conditions.

In addition to selectivity, a fluorescence detector also possesses a high degree of specificity to PAH. This specificity is demonstrated in the chromatogram shown in Figure 3(C). The fluorescence conditions used were λex 240 and λem >470 nm, and perylene, B(k)F and fluoranthene are the only PAH observed since the others are completely suppressed. This detector specificity is achieved due to the difference in emission wavelengths among PAH.

A. Detection Limits, Reproducibility and Linearity of Response

When applicable, fluorescence is one of the most sensitive means of detection for HPLC. Sensitivity of fluorescence detection at λex 280 and λem >389 nm was studied with solutions at sub ppb level concentration of a mixture of seven PAH dissolved in water and in 100%, 80%, 50%, 10% and 5% CH_3CN in water. The fluorescence chromatograms shown in Figures 5 and 6(A) indicate clearly that sensitivity is strongly influenced by the amount of water present in the PAH solution to be analysed. As for example, under identical HPLC—fluorescence conditions with the same amount of PAH injected, the highest sensitivity is observed with the PAH solution in 10% CH_3CN (Fig. 6(A)). This observation is rather unexpected and further studies are needed to substantiate this.

Figure 6(B) is a fluorescence chromatogram at λex 280 and λem >389 nm of a mixture of nine PAH dissolved in 10% CH_3CN. The mixture contained 0.18 ppb of fluoranthene, 0.15 ppb B(a)A, 0.48 ppb chrysene, 0.12 ppb B(k)F, 1.20 ppb B(e)P, 0.23 ppb perylene, 0.53 ppb B(a)P, 0.56 ppb dibenz(ah)A and 0.81 ppb benz(ghi)perylene (chrysene and perylene in the mixture do not respond under the fluorescence conditions used). The detection limits (twice the signal to noise ratio) of the PAH are estimated from the chromatogram and are listed in Table 1. Also shown in the table are the detection limits of chrysene and perylene determined individually at λex 250 and λem >370 nm.

These results show that the lower limits of detection for most of the PAH are close to subpicogram levels, which are about three orders of magnitude lower than the minimum detectable concentration obtained by UV detection.

The retention times, peak areas and peak heights of the PAH were found to be highly reproducible. Calibration plots for the PAH are shown in Figure 7. The HPLC/fluorescence response was found to be linear over a wide concentration range from 1 to 100 pg of each PAH mixture [0–500 pg for B(e)P].

B. Applications

Case studies to test the applicability of the described fluorescence detection technique were carried out by HPLC analysis of PAH in environmental samples. Figures 8 and 9 show respectively the chromatograms of the particulate emission and vapour phase emission samples from a coke oven plant. The particulate sample which was run under three optimal fluorescence conditions (λex 280/λem >389, λex 250/λem >370 and λex 240/λem >470) is found to contain four major PAH, namely fluoranthene, B(a)A, B(k)F and B(a)P. The chromatograms (Fig.

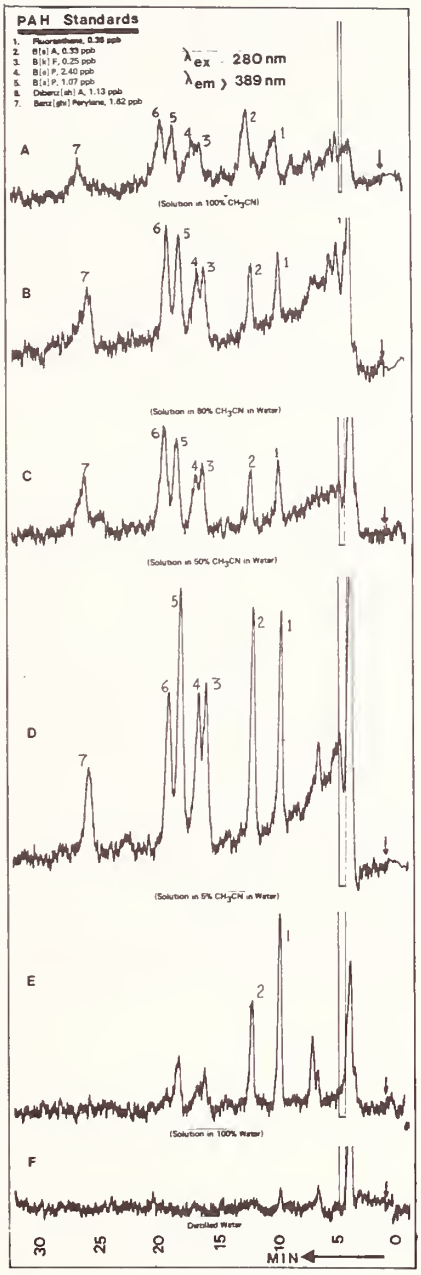

Figure 5. Liquid chromatograms of a mixture of polycyclic aromatic hydrocarbons showing sensitivity of fluorescence detection. Chromatographic conditions: column, flow rate and pressure same as in Figure 2, 84:16 (v/v) CH_3CN:H_2O, 30 °C and 10 μL injection. Fluorescence detection conditions: λex 280 nm, λem >389; emission filter cutoff type KV 389, sensitivity 5.70, range 0.1 μA, time constant 5 sec.

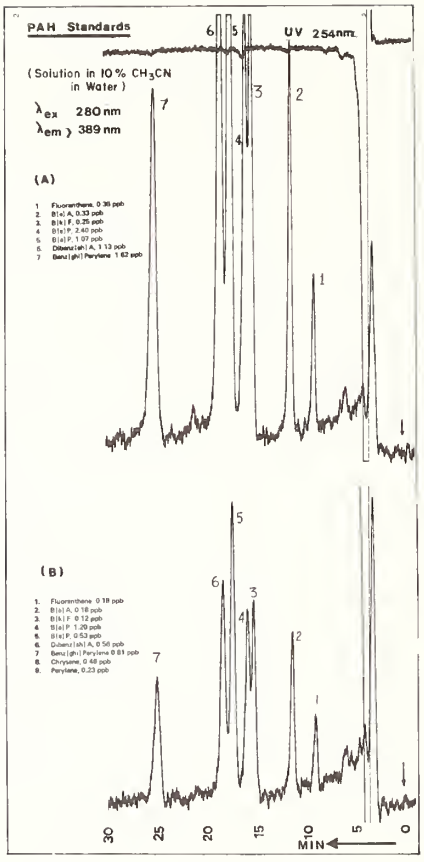

Figure 6. Liquid chromatograms of a mixture of nine polycyclic aromatic hydrocarbons dissolved in 10% CH_3CN showing sensitivity of fluorescence detection. Chromatographic and fluorescence detection conditions same as in Figure 5.

TABLE 1.—*Detection limits of standard PAH*

Compound	Amount (pg)
Fluoranthene (λex 280/λem > 389)	0.45
B(a)A (λex 280/λem > 389)	0.19
P(k)F (λex 280/λem > 389)	0.11
B(e)P (λex 280/λem > 389)	1.20
B(a)P (λex 280/λem > 389)	0.34
Dibenz(ah)A (λex 280/λem > 389)	0.50
Benz(ghi)perylene (λex 280/λem > 389)	1.24
Chrysene (λex 250/λem > 370)	0.75
Perylene (λex 250/λem > 370)	0.19

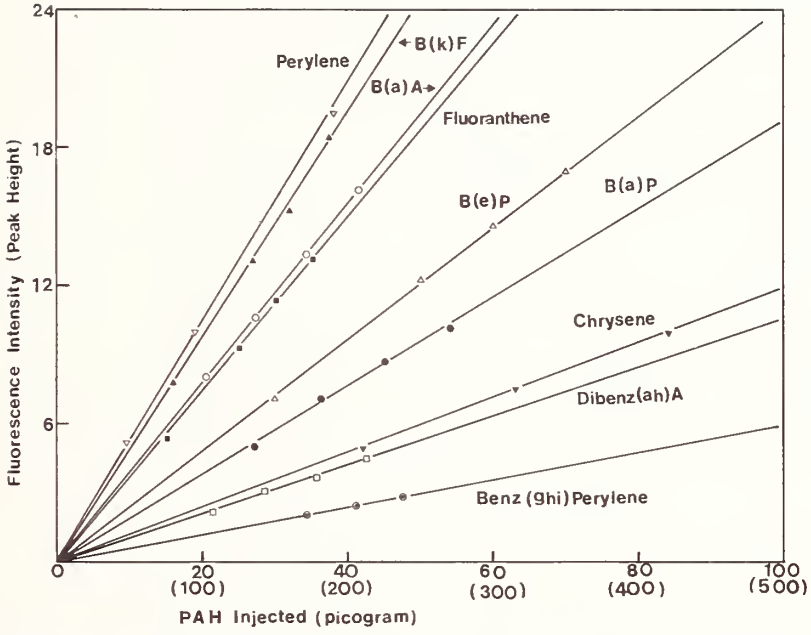

Figure 7. Calibration curves of polycyclic aromatic hydrocarbons showing linearity of HPLC/fluorescence analysis. Chromatographic conditions same as in Figure 2. Fluorescence detection conditions same as in Figure 3(A) except for chrysene and perylene, where conditions are same as in Figure 3(B) and 3(C) respectively. 0–100 pg concentration range for all PAH except B(e)P (0–500 pg).

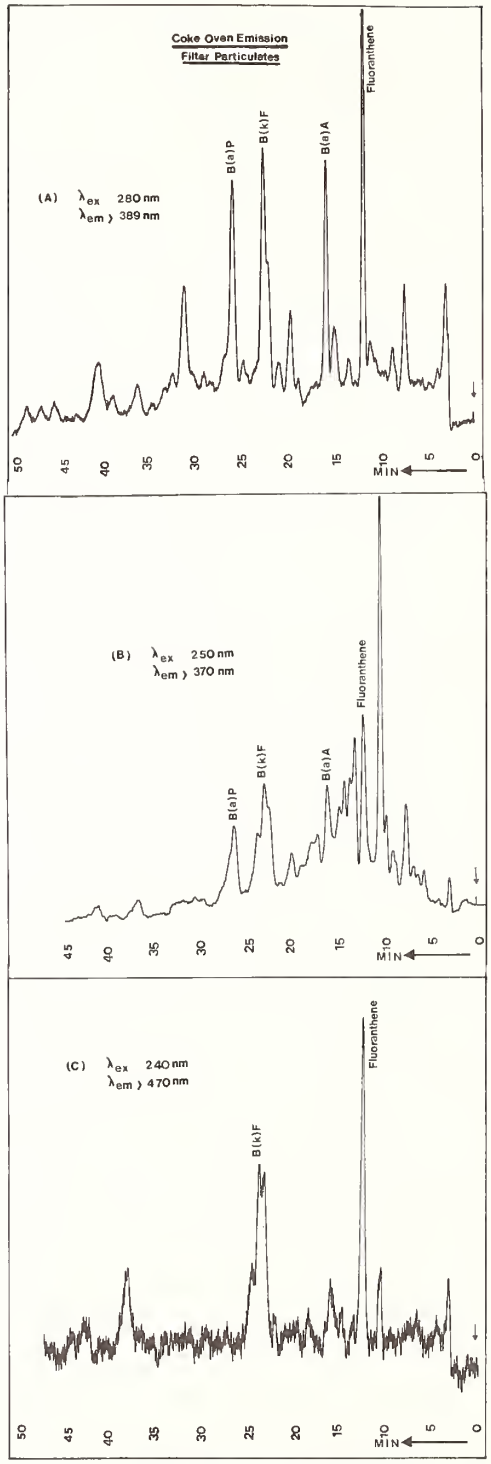

Figure 8. Polycyclic aromatic hydrocarbons in particulate emission from a coke oven plant. Chromatographic conditions same as in Figure 2. Fluorescence detection conditions: (A) and (B) same as in Figures 3(A) and 3(B) respectively, (C) is same as in Figure 3(C) except range 0.1 μA.

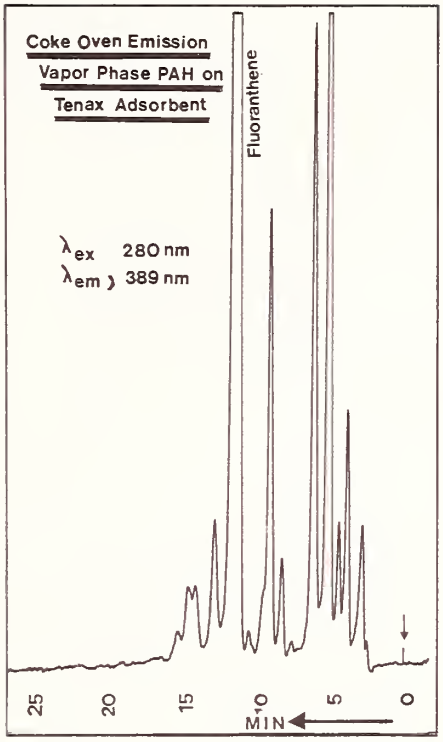

Figure 9. Polycyclic aromatic hydrocarbons in vapour phase emission from a coke oven plant. Chromatographic conditions same as in Figure 2. Fluorescence detection conditions same as in Figure 3(A).

8) show a large number of peaks but other PAH of interest, namely chrysene, perylene, B(e)P, dibenz(ah)A and benz(ghi)perylene are not detected in the sample. The extract from the Tenax adsorbent shows the presence of only the volatile and low molecular weight PAH (Fig. 9). This suggests that the major PAH are contained on the particulate matter rather than in the vapour phase.

Figures 10 and 11 show respectively the chromatograms of the coal and coke from a coke oven plant. The coal sample (Fig. 10) which was run under three optimal fluorescence conditions shows the presence of a large number of well resolved peaks, including fluoranthene, B(a)A, B(k)F and B(a)P. As expected, the sample of coke (Fig. 11) was found to contain no PAH except the ubiquitous fluoranthene.

The analysis of the recycled waste water from the coke oven plant was carried out by directly injecting the dilute effluent. The major PAH detected are fluoranthene, B(a)A, B(k)F and B(a)P (Fig. 12). These four PAH were also identified in a personal monitoring sample from a coke oven plant. This is shown in the chromatogram of Figure 13.

The case studies illustrated above demonstrate the feasibility of the fluorescence detection technique in the HPLC analysis of PAH in environmental, process and occupational health samples. The salient feature of the method is that it allows the use of extremely dilute solutions. Consequently pretreatment by concentration and prefractionation (clean-up) of the sample prior to HPLC is avoided. Such pretreatment is often associated with poor recovery of PAH, and the introduction of interfering impurities, both of which contribute to lack of reliability in the analytical method.

It is concluded that the methodology described offers considerable promise as a simple, reliable and sensitive method of trace PAH analysis.

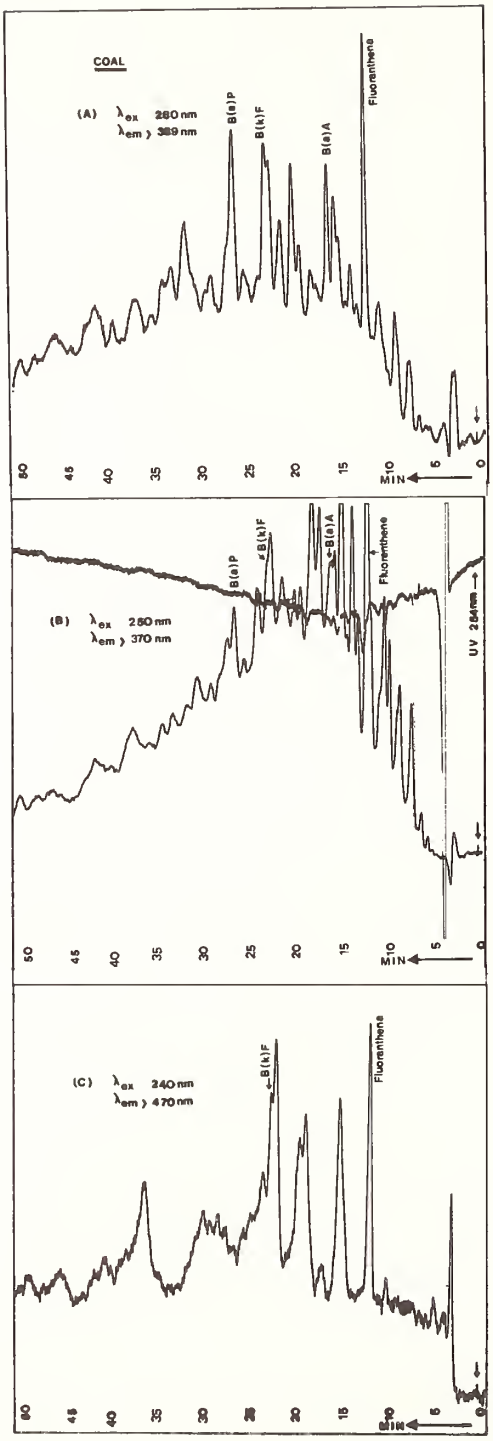

Figure 10. Liquid chromatogram of a coal sample from a coke oven plant. Chromatographic conditions same as in Figure 2. Fluorescence detection conditions: (A) (B) and (C) same as in Figures 3(A), 3(B) and 3(C) respectively.

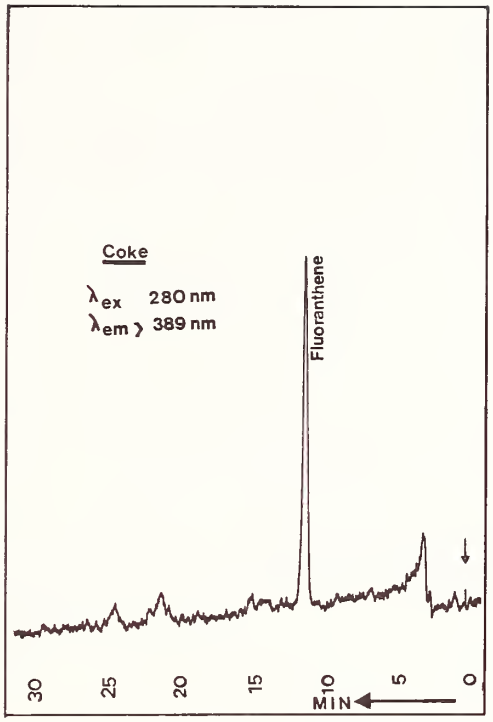

Figure 11. Liquid chromatogram of a sample of coke from a coke oven plant. Chromatographic conditions same as in Figure 2. Fluorescence detection conditions same as in Figure 3(A), except range 0.1 μA.

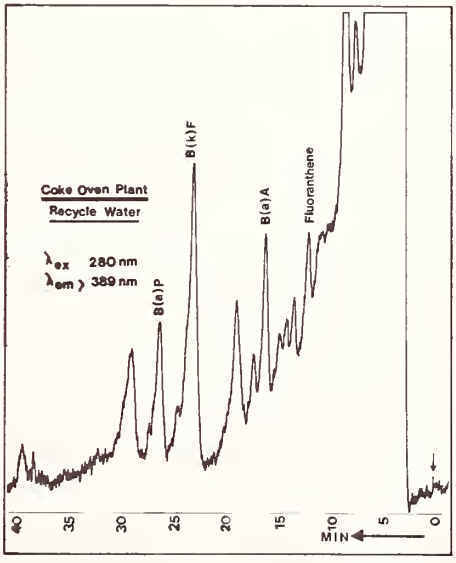

Figure 12. Analysis of coke oven plant dilute recycle waste water for polycyclic aromatic hydrocarbons by direct injection. Chromatographic conditions same as in Figure 2. Fluorescence detection conditions same as in Figure 3(A) except range 0.1 μA.

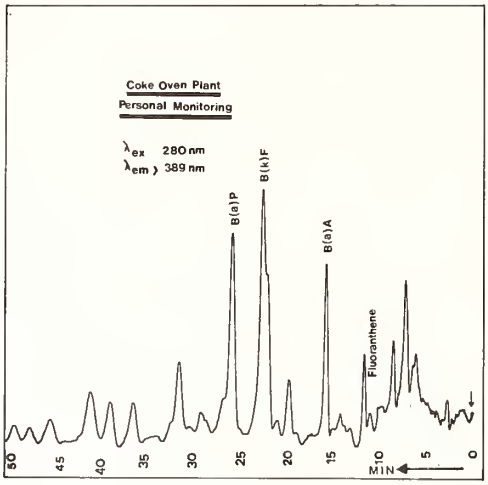

Figure 13. Polycyclic aromatic hydrocarbons in a personal monitoring sample from a coke oven plant. Chromatographic conditions same as in Figure 2. Fluorescence conditions same as in Figure 3(A) except range 0.5 μA.

IV. Acknowledgment

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STUDY OF ORGANOMETAL SPECIATION IN WATER SAMPLES USING LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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A new measurement technique for trace organometal analysis employing liquid chromatography with electrochemical detection is described. Important considerations in the optimization of sensitivity and selectivity of this detection approach are discussed.

A column preconcentration for methyl- and ethylmercury from water samples is outlined.

Key words: Electrochemical detection; liquid chromatography; methylmercury; organometals; water preconcentration.

I. Introduction

Mounting concern over the presence and role of toxic substances in the environment and living systems has created the need for selective and sensitive measurement techniques. Methodology now exists for the analysis of heavy metals (an important class of pollutants) in a variety of matrices, and considerable effort is being devoted to the collection of data on the levels of these elements in biota and water samples. However, some toxic elements can be transformed into organometals by biological and chemical processes. Organometallic species have different properties from their inorganic counterparts. Thus, in order to fully understand the role of these toxic materials, it is necessary to measure the exact chemical form of the element in the sample.

This paper describes some important considerations in developing an electrochemical detection system for measuring organometals separated by high-performance liquid chromatography. Also, a column preconcentration procedure for water samples is described for methyl- and ethylmercury.

II. Measurement System Development

Many organometal species are reducible at potential below −1.0 V vs. SCE [1,2] and therefore should be amenable to electrochemical detection.

There are, however, several special considerations in using liquid chromatography with electrochemical detection for the measurement of reducible analytes. Oxygen must be purged from the solvent and samples, and the pump and detector cell should be enclosed in an oxygen-free atmosphere to prevent reentry through Teflon fittings. The working electrode should have a wide cathodic potential range and low residual current. The familiar dropping mercury electrode (DME), used in polarographic analysis, is unsuitable for high sensitivity liquid chromatography. The DME has high capacitative charging currents (caused by the periodically growing drop), poor

Identification of commercial materials and equipment does not imply recommendation or endorsement by the National Bureau of Standards.

hydrodynamics, and is inconvenient to use. We have developed a solid electrode that retains the cathodic potential range of the DME but has none of the attendant disadvantages. The GAME (gold amalgamated mercury electrode) is prepared by epoxying a 1.0 mm gold wire into a plexiglass electrode holder similar to that developed by Kissinger [3]. The electrode is polished flat with gem polish and then a slightly convex layer of mercury is applied by floating the holder in a pool of mercury. This electrode has a potential limit of –1.2 V and needs refinishing only once a week. Further details on the system construction may be found in other references [1,4].

The selectivity of electrochemical detection can be controlled by the type of waveform applied to the working electrode. Amperometric detection, illustrated in Figure 1, consists of applying a constant potential and monitoring the resulting current. If a potential of E_1 is applied, only reducible species A will generate a faradaic response. Using E_2 will provide a response for both A and B; therefore, it will be incumbent on the chromatography to resolve these two species. This mode of detection has a limit of detection in our system of about 4 ppb or 2×10^{-8} mol/L for $(CH_3)_3Pb^+$ and a linear dynamic range of 10^{-7} to 10^{-3} mol/L.

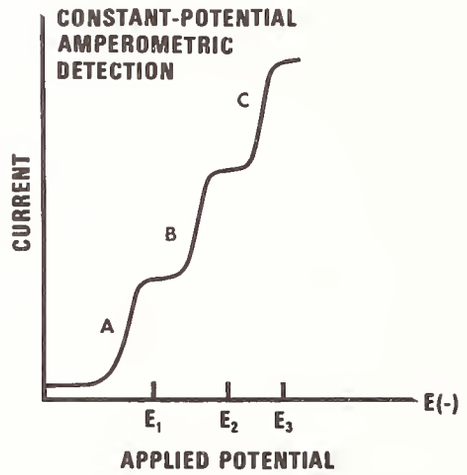

Figure 1. Amperometric detection in liquid chromatography.

The primary advantage of amperometric detection is that several electroactive analytes can be measured simultaneously with good selectivity. Figure 2 illustrates the multicomponent capability of this approach. The detector potential is set at –1.0 V so that even analytes with very different reduction potentials such as CH_3Hg^+ (reduction potential -0.35 V) and $CH_3Ø_3Sb^+$ (reduction potential –0.80 V) can be detected simultaneously. The main disadvantage of amperometric detection is that unknown electroactive components in the sample matrix can also give a response at the detector and may not be chromatographically resolved from the analyte species. A prior separation, for example by thin layer chromatography [5], may be necessary for an interference-free analysis in this mode of detection. Furthermore, this detection approach is very flow sensitive [6] requiring pulse-free pumping and very low faradaic background currents (as the detector noise is proportional to this quantity).

An alternative waveform that has higher selectivity is differential pulse detection shown in Figure 3. A constant base potential is applied but a periodic potential pulse of short duration (about 50 ms) is superimposed every 0.5 to 1.0 second. The current is monitored for about 15 ms before the pulse and near the end of the pulse. The differential of the two currents provides the readout. Species B in the figure will cause a large current difference, but A and C will provide no response. Thus, the differential pulse mode of electrochemical detection provides virtually "species specific" selectivity. The pulse height may be varied from 5 to 100 mV using a Princeton Applied Research model 174A polarograph. The small pulse heights give the greatest selectivity, whereas

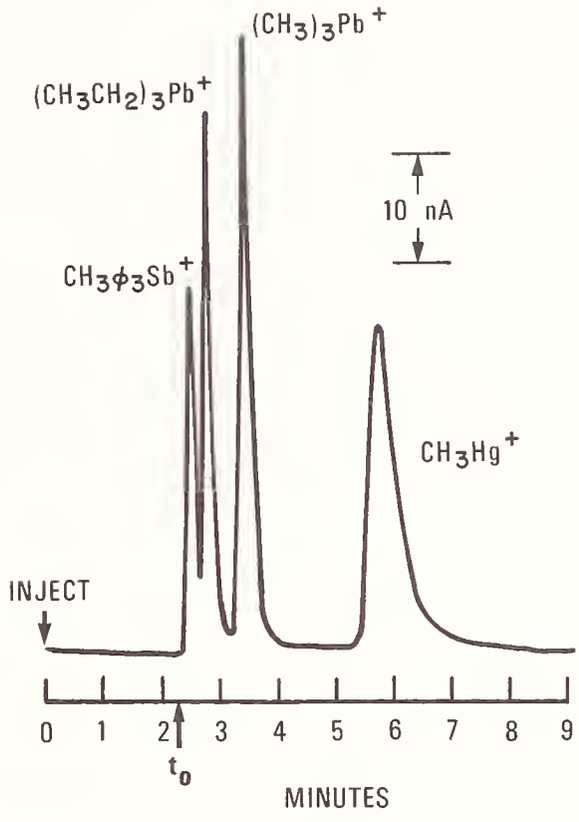

Figure 2. Simultaneous amperometric detection of some organometals. Conditions: detector potential –1.0 V, column Altex μNH_2 4.6×250 mm (10 μm particles), solvent 40% MeOH, 0.06 mol/L NH_4OAc, pH 5.5, flow rate 1.0 mL/min., sample $\sim 5\times10^{-5}$ mol/L.

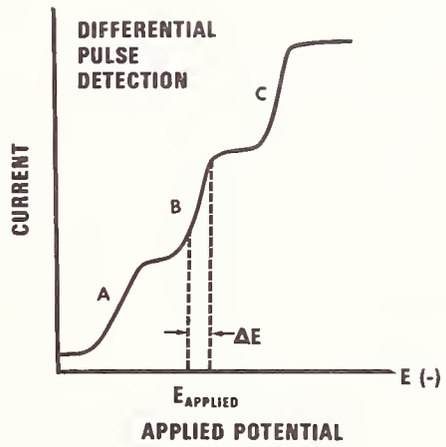

Figure 3. Differential pulse detection in liquid chromatography.

the large pulse heights provide the highest sensitivity (100 mV pulses give about a factor of 3 lower detection limits than 5 mV pulses).

The differential pulse mode of detection is also less sensitive to flow pulsation [6]. It provides a linear dynamic range of 10^{-8} to 10^{-4} mol/L and detection limits of 2 ppb or 1×10^{-8} mol/L for CH_3Hg^+.

The selectivity obtained in the differential pulse mode is illustrated in Figure 4. The sample contains 10^{-5} mol/L CH_3Hg^+, Cu^{++}, Pb^{++}, and Cd^{++}. The divalent ions are easily reducible and would be expected to be found in polluted water samples along with methylmercury. The upper chromatogram shows the result with the amperometric mode of response. The divalent ions could pose an interference especially at low levels of CH_3Hg^+. The lower figure shows the same solution in the differential pulse mode using 50 mV pulses. The first peak is a small response for Cd^{++}

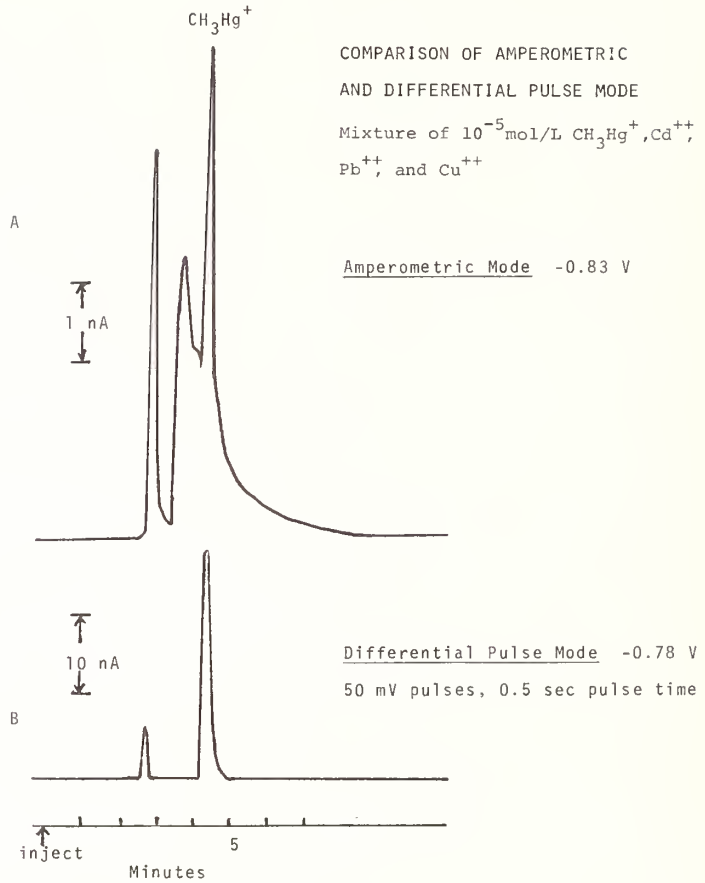

Figure 4. Comparison of the selectivity of amperometric and differential pulse detection. Conditions: column Spherosorb ODS 4.6×250 mm (5 μm particles), solvent 40% MeOH, 0.06 mol/L NH_4OAc, pH 5.5, 0.1% V/V 2-mercaptoethanol, flow rate 1.0 mL/min.

combined with the response for the solvent front. Certainly, using the differential pulse mode, a much more reliable result can be obtained for complex samples without the necessity of extensive "sample clean up" before analysis.

The disadvantage of using differential pulse detection is that the number of simultaneously detectable analytes is very limited. There are two ways to circumvent this "single species" response. For analytes with very similar electrochemical behavior (such as homologs), a compromise detector potential can be chosen. Figure 5 shows the current voltage response for methyl-, ethyl-, and phenylmercury using 5 mV pulses. A compromise detection potential can be chosen at about −0.70 V. A second method to increase the number of detected analytes would be to change the detector potential at the appropriate times during the chromatography to optimize the response for each species. This approach is quite feasible in this mode of operation as little baseline shift is noted when the detector potential is changed. This contrasts the large, slowly decaying background response to potential change observed in the amperometric mode of detection, caused by changing faradaic and capacitative currents.

Thus the choice of waveform depends primarily on the selectivity required for the sample to be analyzed. For very easily reduced (or oxidized) analytes in fairly "clean" matrices, the amperometric mode of detection is preferable. Analytes in complex matrices that are more difficult to reduce (or oxidize) will require the differential pulse mode of detection. In our system there is little difference in the dynamic range or detection limits for the two waveforms.

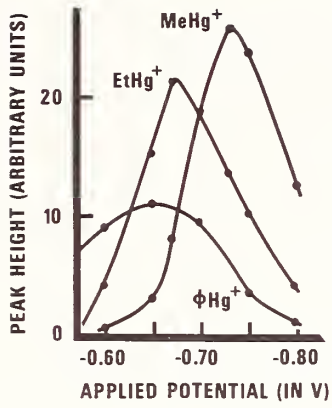

Figure 5. Detection of methyl-, ethyl-, and phenylmercury in the differential pulse mode of detection.

III. Water Sample Preconcentration

Past work in the environmental analysis of water samples for toxic heavy metals has concentrated either on the total element content or the inorganic forms only. Recent attention has shown the importance of organometals (particularly the organomercury species) in natural ecosystems. The organomercury species are often quoted as being two orders of magnitude more toxic than inorganic mercury (II). Thus the measurement of the exact mercury species is important.

Past work has shown levels of total mercury, even in polluted waters, is in the sub-ppb range [7]. Thus, a preconcentration of methyl- and ethylmercury would be necessary to apply this analytical technique for real water samples.

The preconcentration procedure we are developing employs the retention of neutral iodide complexes of the cationic species on a nonpolar adsorbant and subsequent elution with an organic solvent. This enhances the concentration of the analytes almost two orders of magnitude and eliminates many interferences expected in natural water samples.

The cationic organomercury species, CH_3Hg^+ and $CH_2CH_2Hg^+$, would be expected to be bound by sulfide ion or mercaptans in virtually all water samples [8]:

$$CH_3Hg^+ + S^= \rightleftharpoons CH_3HgS^- \quad K_f = 10^{21}$$

These complexing agents interfere with the chromatographic separation developed for the organomercury species shown in Figure 6. The interference of these ligands results from their large formations constants relative to the 2-mercaptoethanol ligand used to form the neutral species most suitable for the reversed-phase separation. Reducible metals (as mentioned earlier) also can pose an interference in the amperometric mode of detection.

The organomercury cations can be freed from their weak acid ligands by acidification to around pH 2 [8] eliminating the interference of sulfide and mercaptans. The preconcentration setup is depicted in Figure 7. A 100 mL sample is collected in a Pyrex bottle containing 100 μL of concentrated nitric acid. The sample is filtered through an 8 μm Millipore filter, rinsing with small portions of 0.1% v/v nitric acid. The washings are combined with the sample and one milliliter of 2 mol/L ammonium citrate (pH 3.0) is added, adjusting the pH to 2.5. Feeding from an opaque glass reservoir, the 100 mL sample is mixed with 1.0 mol/L iodide at a volumetric flow rate of 10 to 1 controlled by the diameter of Teflon tubing connected to a tee joint before the chromatographic pump. The iodide solution contains 0.05% v/v of 2-mercaptoethanol preservative to prevent the formation of I_2 by oxidation of I^- in acidic media by atmospheric

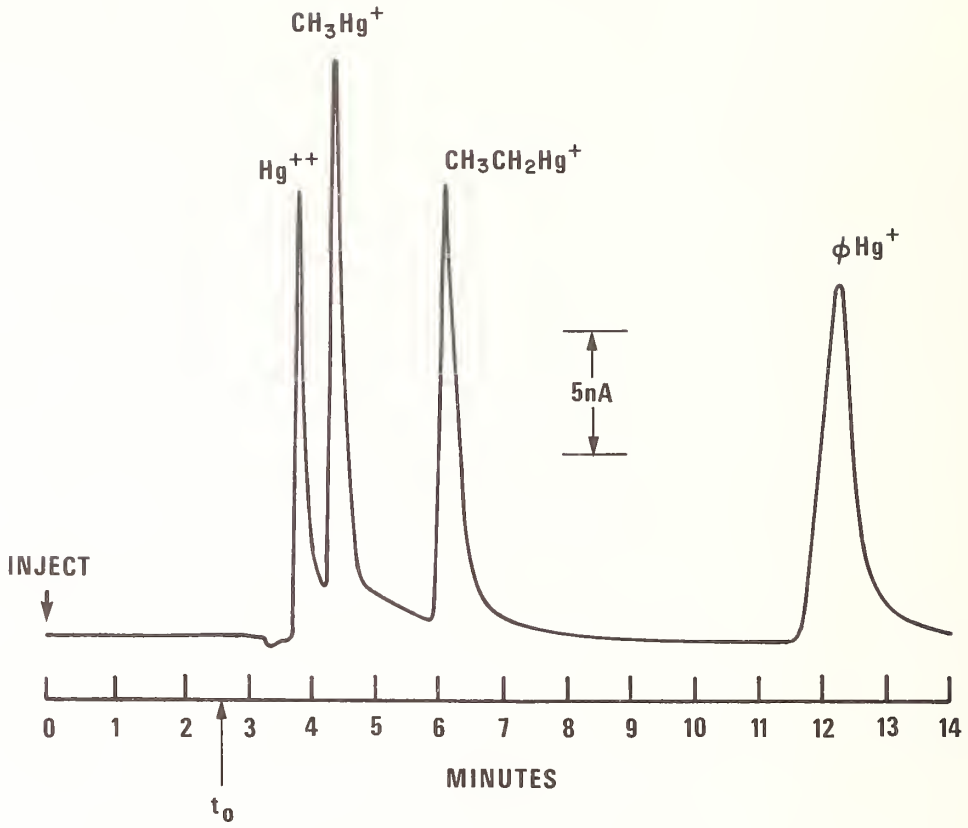

Figure 6. Separation of some organomercury species. Conditions: column Spherosorb ODS 4.6×250 mm (5 μm particles), solvent 40% MeOH, 0.06 mol/L NH_4OAc, pH 5.5, 0.1% V/V 2-mercaptoethanol, flow rate 1.0 mL/min.

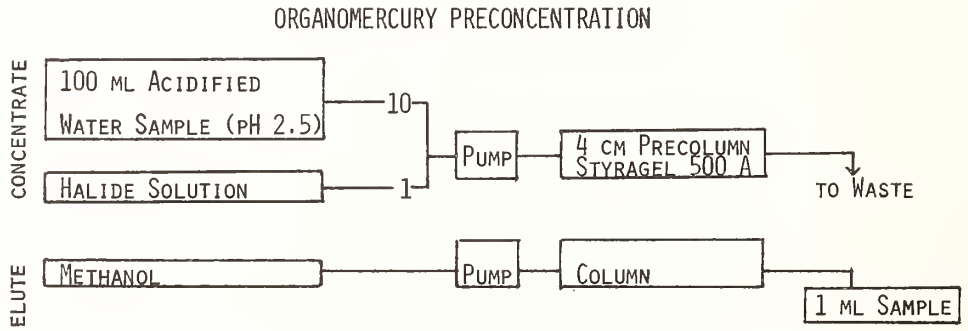

Figure 7. Preconcentration setup.

oxygen (I_2 rapidly oxidizes methylmercury). A simple metering pump (Milton Roy minipump) is used to deliver a flow rate of about 2 mL/min. A short column (40×3.0 mm) is dry packed with Waters Associates Styragel 500 A (<37 μm particles). This polystyrene material is being used as a neutral adsorbent, not as a permeation gel. The adsorbed RHgI species are removed with 100% methanol (the pump must be preflushed with methanol) at a flow rate of 0.1 mL/min. The first 200 μL eluted is discarded and then the subsequent 1.00 mL fraction is collected in a volumetric flask and stored in the dark to prevent photodecomposition.

Preliminary studies indicate the preconcentration factor (volume ratio×fraction analyte recovered) is about 95 for CH_3Hg^+ and 70 for $CH_3CH_2Hg^+$ with a breakthrough volume for aqueous samples of about 150 mL. The effects of the following potential interferences (at the 10^{-5} mol/L level) were evaluated for a 10^{-6} mol/L solution of RHg^+ species: $S^=$ and CN^- (strong complexing agents), and Cu^{++}, Pb^{++}, Cd^{++}, Fe^{+++}, and Hg^{++} (easily reduced metal ions). No interference was noted using the amperometric mode of detection for any of these ions.

Two "real" water samples were also preconcentrated: a sample of the NBS fresh water pond and also Danish I.A.P.O. Standard Sea Water (chlorinity 19.374 parts per thousand). No RHg^+ was found to the 100 parts per trillion level. However a 10^{-7} mol/L CH_3Hg^+ and $CH_3CH_2Hg^+$ spike added to each was recovered. Future work is needed to determine the precise recovery of these two organomercury cations. It may also be possible to extend the procedure with only small modifications to include phenyl- and dimethylmercury as well.

IV. References

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

AN AUTOMATIC SAMPLER, A MASTER ANALYTICAL SCHEME, AND A REGISTRY SYSTEM FOR ORGANICS IN WATER

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A three-phase analysis program for organics in water is described. In the first phase, a prototype automatic sampler was constructed to sample and concentrate organic compounds from dilute systems, including finished drinking water. The sampler collects and seals up to 26 "grab" samples for analysis of purgeable organics and concentrates up to 14 samples on various accumulators, such as XAD resins, for analysis of non-purgeable volatiles and perhaps non-volatiles. Samples are collected according to preset sampling frequencies, sequences, flow rates, and collection periods. The sample water is exposed only to Teflon and glass components. The sampler control circuits are AC or DC powered; valving and switching mechanisms are pneumatically operated using bottled gas. The sampler is refrigerated to 4 °C and is capable of unattended operation for up to 7 days in a remote location.

The wide distribution of organics in water emphasizes the need for development of a master analytical scheme that will serve as a general protocol for analytical surveys in which all volatile organic compounds above a specified level in any type of water are identified and quantified. In the second phase, such a scheme, which incorporates modern GC-MS-computer analytical techniques, is currently being developed by the U.S. Environmental Protection Agency (EPA), and should be available in early 1980. In developing the scheme, emphasis will be on comparison, selection, and optimization of techniques for sampling, extraction, cleanup, and separation of all gas chromatographable organics in water. A computer program for quantification will be based upon internal standards selected for each major functional group and volatility class of organics, and upon predetermined recovery and detector response data obtained using several model compounds from each class. The scheme will be tested and improved using samples of surface water, drinking water, and industrial and municipal effluents.

In the third phase, a comprehensive data collation and retrieval system for organics in water will be developed by the EPA. This system, called the Distribution Register of Organic Pollutants in Water (WaterDROP), will include a means for continual updating and dissemination of new information on organics identified in water. WaterDROP will include various chemical and geographical descriptors for each compound, as well as data on concentration, source, analytical method, etc. The data base will reside in computer storage to allow interactive data retrieval by a remote operator and will, in addition be available in a slightly abbreviated cross-indexed printed version. WaterDROP will be searchable by several parameters to locate compounds of particular interest to analytical chemists, epidemiologists, enforcement groups, and others.

Key words: Accumulators; analysis of organics in water; automatic sampler; master analytical scheme; non-purgeable organics; organic pollutant; purgeable organics; WaterDROP.

I. Introduction

Analysis of organic compounds in water begins with sampling, incorporates a comprehensive analytical scheme, and concludes with the interpretation and registration of the data. The U.S. Environmental Protection Agency has begun a three-part program to satisfy these analytical steps. In the first phase, an automatic sampler, designed to sample and concentrate organics from dilute

systems, was constructed. In the second, a master analytical scheme for all volatile organics in all types of water will be developed. To complete the program, a data registry and retrieval system that will incorporate distribution and occurrence records for all specific organic compounds identified in water will be established. This paper describes the development of the program phases.

II. Automatic Sampler

Chemical analysis starts in the field with the collection of a true sample that is representative of the environmental medium of interest. The sampling step for water analysis is complicated in that different types of samples, or different modes of collection, are optimum for different chemical species. For example, no-headspace grab samples are required for purgeable organics [1], whereas large continuous samples are needed for extraction of non-purgeable volatiles by accumulator columns, such as columns of XAD resin [2].

A. Current Sampling Technology

To collect the gamut of volatile (gas chromatographable) organics in water, at least two types of samples are necessary: a no-headspace grab sample for highly volatile, purgeable compounds such as chloroform that are easily lost from the aqueous phase and a grab, composite, or continuous sample for the non-purgeables. Continuous samples are the most representative of the natural environment. No method is presently available for continuous sampling for purgeables, however; the best samples for these compounds is one carefully composited of a series of grab samples. On the other hand, accumulator columns are now available that allow continuous, simultaneous extraction and concentration of many non-purgeable organics from water. Although analytical techniques are sensitive enough to allow detection and identification of sub-microgram-liter concentrations of many compounds in a grab or composited grab sample of 1 to 5 liters, such samples are often not representative. Accumulator columns can provide a representative sample, simultaneous extraction with a high degree of concentration, and low detection limits.

Currently available automatic samplers [3,4] usually are limited to composite (by flow or volume) sampling into a single container. Sample container volume limitations prohibit sampling over a long period, and a sequence of different samples usually cannot be collected. Some other deficiencies of current samplers include: (a) cooling of the sample for preservation over extended periods of time is difficult, (b) contamination can occur from the pump or delivery tube, (c) extreme care is required in transferring collected samples to the laboratory, (d) the sample must be extracted (no accumulators), and (e) purgeable organics are not quantitatively collected.

Columns of activated carbon [5] and other accumulator columns [6] have been used in the field for many years to extract organics from water, but no automatic sampler has been designed especially for the use of accumulator columns. There is no automatic sampler currently available for the collection of water for the analysis of purgeables.

B. The EPA's Automatic Sampler

Midwest Research Institute (MRI), under EPA contract, recently constructed a prototype automatic sampler to meet the specific need for sampling water for purgeable and non-purgeable organics. This new sampler, which is adaptable to both laboratory and field operations, is designed to collect and preserve all types of water samples, including finished drinking water, for analysis of trace organics.

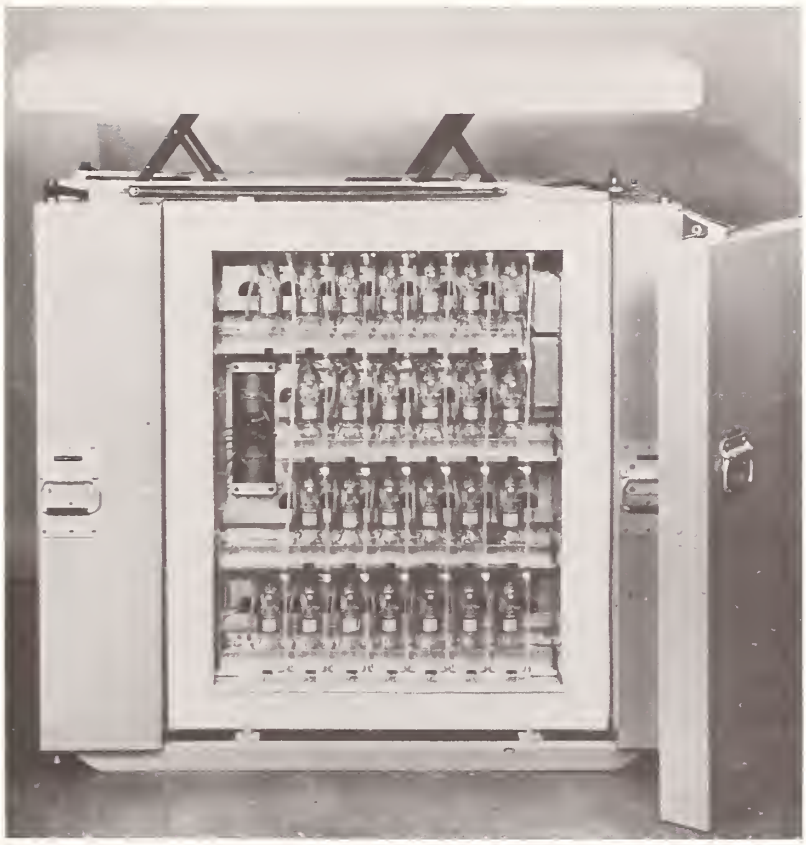


Figure 1. Automatic sampler opened to show the 26 purgeable sample bottles in position.

The sampling system collects 26 purgeable samples (Fig. 1) in special 140-mL sample bottles (Fig. 2) designed to be flushed and filled from the bottom in selected times ranging from 1 to 9 minutes, and then sealed by a rotating Teflon joint. These bottles can be removed from the sampler and their contents transferred to a regular volatile organic analysis (VOA) [1] purging apparatus using gas pressure. Several of these samples can be composited for more representative purgeable analysis or for analysis of non-purgeables by liquid-liquid extraction or other techniques.

The sampler also collects as many as 14 samples on accumulator columns (Figs. 3 and 4) for analysis of non-purgeables. The accumulator column size is variable; the one currently in use holds a 1.8×27-cm column packed with XAD or Tenax resin, activated carbon, or any other accumulator material. The flow rate is adjustable between 50 and 500 mL/min, and each column can be programmed as to volume of water sampled or time span of sample collection. Two or more accumulator columns can be programmed to collect a sample in parallel or in series, and the sequence of columns used can be changed.

The sampler can be operated in a laboratory or a remote site and is capable of unattended operation for up to 7 days. Control circuits are powered by either ac or dc current, the latter using a rechargeable 5 A-h storage battery. Valving and switching mechanisms are pneumatically operated with bottled gas such as propane or compressed air. The sampler is refrigerated to 4 °C, which provides for sample preservation for up to 7 days. The refrigeration system, shown in

Figure 2. A 140-mL purgeable sample bottle for the automatic sampler.

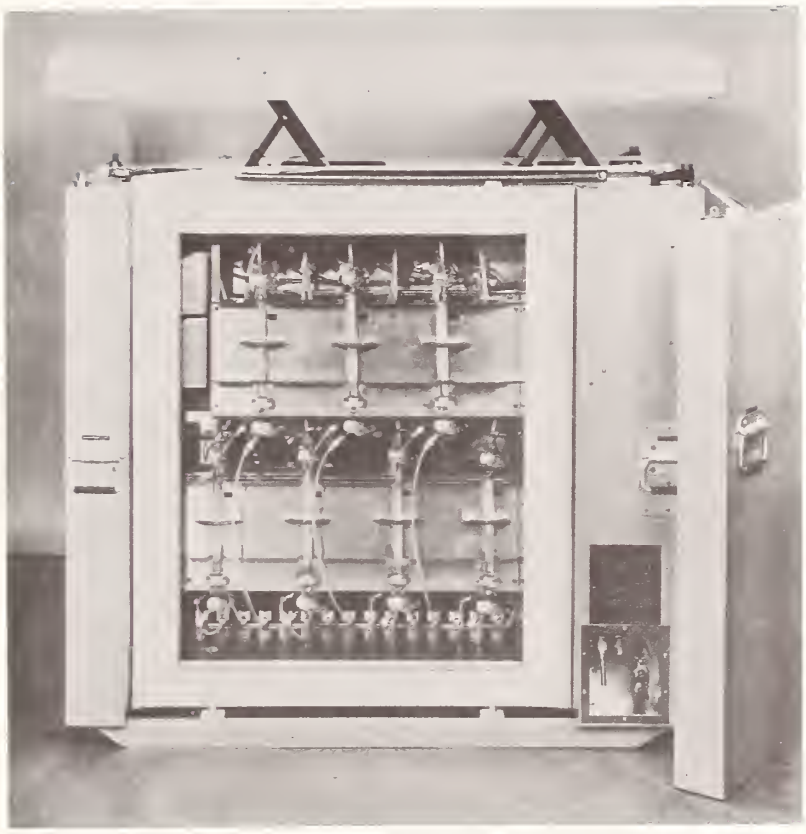


Figure 3. Automatic sampler opened to show 7 of the 14 accumulator columns. Another bank of 7 is located behind the visible bank.

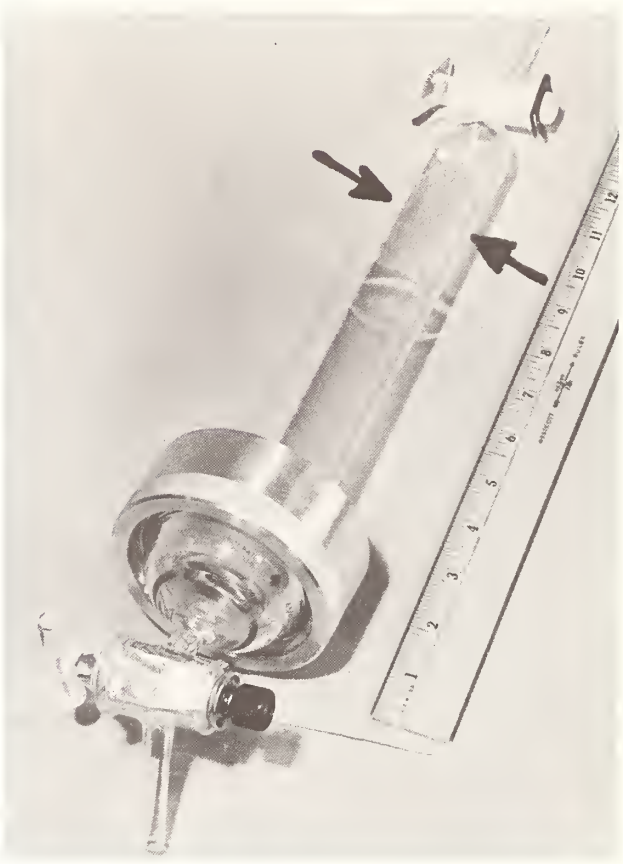


Figure 4. A 1.8×27-cm empty accumulator column for the automatic sampler.

Figure 5, is powered by propane gas (or ac current), being similar to units used in recreational vehicles. To avoid contamination, sample water is exposed only to Teflon and glass components.

The Teflon-bellows sampler pump (Fig. 6), which was especially designed by MRI, is capable of supplying up to 500 mL/min of sample water during the 7-day sampling period. The total sampler unit in its closed and secured configuration is shown in Figure 7.

An endurance test of the battery and the electronic systems representing the worst-case condition indicated that the battery was capable of operating the sampler for 8 days without recharge. The pneumatically operated pump required 55 liters of liquid propane for 7 days of operation at a typical water sample flow rate of 100 mL/min. About 8 liters of liquid propane was required to operate the refrigeration system for 7 days in a 24 °C ambient environment. The sampler temperature did rise to 5 °C on the seventh day, but this is considered acceptable.

The first MRI prototype sampler was received at EPA's Environmental Research Laboratory in Athens, GA, in March 1978 and was immediately subjected to laboratory performance tests. Tests were also begun to evaluate various accumulators that can be used in the sampler. These tests will involve Athens tap water and, later, water at a field site such as a local sewage treatment plant. If the sampler performs as expected, it will be used to sample water from the Great Smoky Mountains National Park, a Biosphere Reserve presently being studied by the EPA's Environmental Monitoring and Support Laboratory, Las Vegas.

This is a first generation automatic sampler. It is obviously overdesigned, especially with regard to the number of individual sample bottles and columns. Thus, the sampler is now too bulky, heavy, and expensive for wide application. The present design should, however, allow a

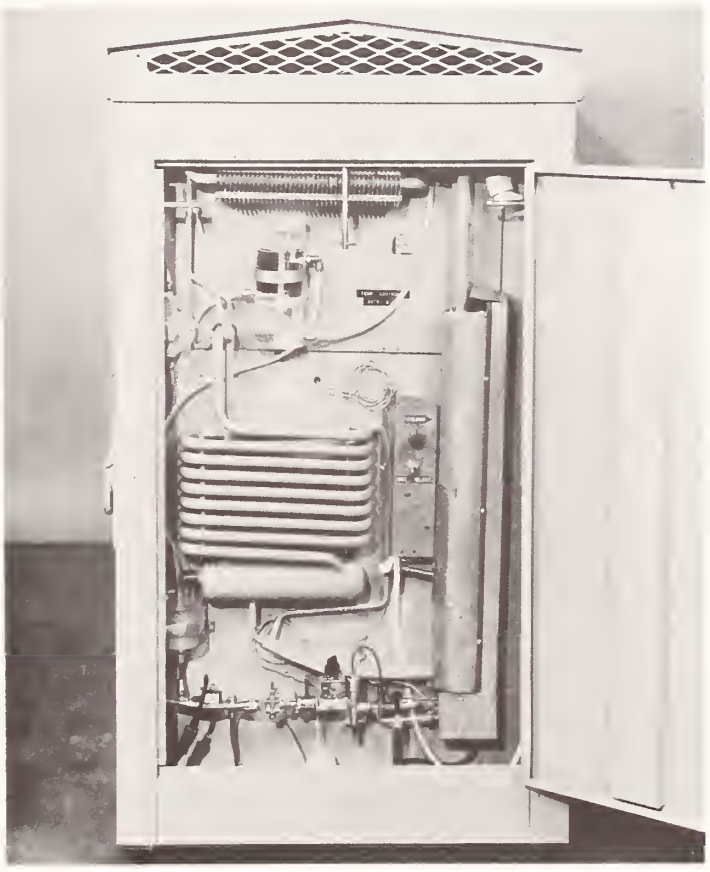


Figure 5. The refrigeration system.

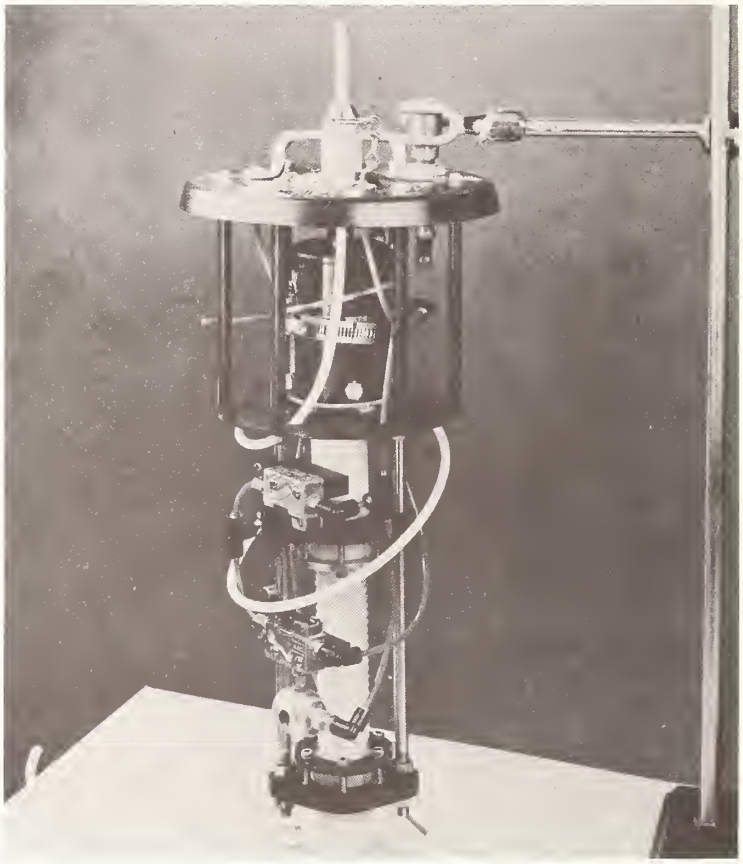


Figure 6. Automatic sampler pump with container removed. Teflon bellows are at the bottom.

Figure 7. Automatic sampler, closed and secured; dimensions are 134×69×130 cm. Pump is in its black container.

thorough evaluation of the maximum capabilities of an automatic sampler. This evaluation will probably lead to the development of a smaller, lighter, and less expensive sampler that should find wide application.

III. Master Analytical Scheme

Although much effort has been expended by several research groups over the last few years on development of methodology for identification and quantification of selected volatile (gas chromatographable) water pollutants, many gaps still exist. For example, extraction and separation conditions have not been optimized, and the recovery of any identified pollutant is not known without repeating extraction and separation procedures with a standard compound. Internal reference standards have not been selected for all groups or classes of compounds. Specific techniques are available for analyzing many volatile and semi-volatile organics in water, but methods are not comprehensive enough to cover the wide range of functional groups of potential environmental importance.

Several protocols have been written for special situations, but there is a need to draw all the common elements together into a general scheme, incorporating the best of the latest advances in sample preparation and quantification that have been generated piecemeal. In mid-1978, the EPA will award a contract to develop an analytical scheme that, when coupled with modern GC-MS-computer identification techniques, will serve as a general protocol for analytical surveys in which all volatile organic compounds above a specified level are searched for and quantified. The scheme

should be a flexible guide for future work in the analysis of volatile organics in water of all types and be specific enough for ordinary situations, but adjustable by experienced personnel to fit unusually complex samples.

A. Use and Applications of the Master Scheme

In applying the master analytical scheme, the user will be directed how to sample any industrial or municipal effluent, surface water, or drinking water in such a way as to obtain sufficient artifact-free sample for qualitative and quantitative analysis of any organic compound that will pass through a gas chromatograph (GC) or can be derivitized to pass through a GC. Lower detection limits will depend upon compounds being analyzed for and the type of water being analyzed and will be specified in the scheme. Generally, expected detection limits are 0.1 μg/L for drinking water, 1.0 μg/L for surface water, and 10.0 μg/L for effluents. Collection may be by grab sample or accumulator column. The user will be told what internal standards to add, and how and when to add them, for any class of organics. Guidance will be provided in handling and preserving the liquid sample or accumulated organics. Extraction, concentration, and clean-up techniques will be applied as specified by the scheme to allow maximum separation and recovery of the compound or class of compounds of interest to the user, or to allow survey analysis of all organics to which the total scheme applies.

The user will then be directed to use specified gas chromatographic columns and conditions for separation and detection of all compounds of interest, after applying prescribed derivatization techniques when necessary. Once compounds are detected, the user will identify them by previously established GC-MS-computer techniques, then quantify all identified compounds with a computer program developed under this contract. The raw data input to the computer program will consist of retention times of sample components and marker compounds (internal standards), intensities of MS signals for sample components and marker compounds, chemical class of sample components (obtained automatically from an on-line mass spectral search system), sample water type, and volatility class. The computer program will include stored MS detector response factors and recovery factors for marker and model compounds for each class of compound within each volatility fraction for each type of water sample. Figure 8 shows how these stored data will be used to calculate, from the raw data, the estimated concentration of each compound of interest within a

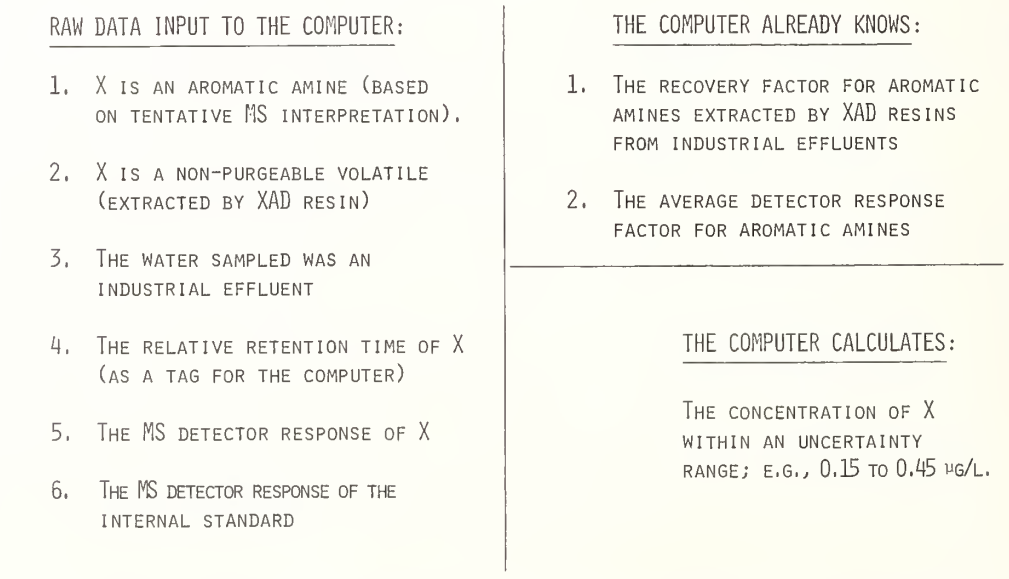


Figure 8. Example of computerized quantitation of compound X using the Master Analytical Scheme.

calculated uncertainty range. The user will apply quality control procedures, as specified by the scheme, during application of the total scheme.

The user will not be bound to use all steps of the scheme, but will be able to select segments appropriate for the analysis of a single selected compound or one class of compound. The scheme will also be flexible enough for the user to adapt it to rapid screening for specific organics with less quantitative accuracy than expected for detailed application, eliminating time consuming techniques designed for unusual functional group classes.

B. Work Necessary for Development of the Scheme

The emphasis of the contractor's experimental work will be on sampling, sample preparation, separation, and quantification. The total scheme will be oriented towards identification and quantification by GC-MS using internal standards. Development of guidelines for the use of marker compounds (internal standards) will be part of the contract; the National Bureau of Standards will assist in the selection of these compounds. Recovery studies will be performed using model systems involving widely varying members of each major functional-group class of organic compound. Extraction techniques will be optimized for each class of compound and each type of water sample, considering the desired level of detection. Accumulator columns will be optimized to collect intermediately volatile compounds. The contractor will be expected to determine optimum GC columns (capillary and packed) and GC conditions for each class or appropriate category of organic compound. State-of-the-art GC-MS-computer systems will be used to detect and identify compounds used in protocol development and to develop techniques for quantification based on internal standards.

Finally, the contractor must prove the protocol by showing its application to a variety of chemical compounds and water types. Spiked and unspiked samples of surface water, drinking water, and industrial and municipal effluents will be specified. (Effluents from energy-related industries will be included.) Model compounds used in recovery studies and compounds for spiking will be based on EPA's priority pollutant list and other chemicals of interest in the water supply program.

The published master scheme is expected to be available in mid-1980.

IV. Distribution Register of Organic Pollutants in Water

The number of organic compounds identified in water is increasing rapidly as scores of laboratories examine effluents, surface water, and drinking water for various reasons. This mass of data needs to be continually updated, classified, and registered to allow development of analytical methodology, to permit accurate health effects studies, and to provide an accurate data base for setting and enforcing pollution regulations.

A. Current Compilations

Shackelford and Keith [7] have published an EPA report listing organic pollutants found in all types of water in North America and Europe. A June 1977 update of this report contained 6944 entries of 1282 different organic compounds. The report contains lists of compounds sorted alphabetically by name, by their location or reference to a published study, and by the type of water in which they were found (29 types are included). It also includes tables that summarize the frequency of occurrence of each compound, the location or reference, and the water type. This list, however, is limited to compounds identified during survey-type analyses. In using these data, one must keep in mind that results of studies that include only analyses for specific compounds are *not* included; polychlorinated biphenyl monitoring studies, for example. Neither does this report include quantitative data.

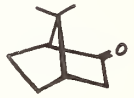
Another valuable compilation of organic compounds found in water is that maintained by the Water Research Centre of England for the Commission of European Communities (CEC) COST-Project 64b [8]. For each compound identified, this listing gives concentrations (if available), type of water sampled, date and location of sample, and reporting laboratory. Compounds are listed by major chemical or use class. Literature searches and regular submissions from laboratories participating in the COST-Project 64b, supplemented by private communications, provide additional entries to this extensive listing. Although the bulk of the compounds listed are those found in western European waters, many data from the U.S. EPA are included. Shackelford and Keith's EPA report [7] includes the CEC data as of October 1975.

B. WATERDROP

A computerized/printed library of organic compounds in water will be the ultimate answer to handling the mass of data described above. In the summer of 1978, the EPA will award a contract for development of a comprehensive collation, registration, and data retrieval system. This system, to be called WaterDROP (Distribution Register of Organic Pollutants in Water) will include a means for continual updating and dissemination of new information on organics in water. It will include various chemical and geographical descriptors for each compound, as well as data on concentration, source, etc. The data base will reside in computer storage to allow interactive data retrieval by a remote operator and will also be available in a slightly abbreviated, cross-indexed, printed version. WaterDROP will be accessible by several modes to search for compounds of particular interest to analytical chemists, epidemiologists, enforcement officials, and others. Figure 9 shows one possible format for WaterDROP data [9].

CAMPHOR

Bornane, 2-oxo
2-Bornanone
2-Camphanone
} Synonyms



Chemical Class: ketone, aliphatic, substituted

C10 H16 O; MW: 152.26: CASRN: 000076222; WLN: L55 A CVTJ A A B

Sampling Date	Sample Site	Conc. (µg/l)	Water Type	Identified By	Lit. Reference	Contact Reference
4-75	Miami, FL (USA)	0.5	Drinking	MS, GC*	1	1
4-75	Cincinnati, OH (USA)	0.1	Drinking	MS, GC*	1	1
4-75	Ottumwa, IA (USA)	0.1	Drinking	MS, GC*	1	1
4-75	Seattle, WA (USA)	0.5	Drinking	MS, GC*	1	1
3-72	Interstate Paper Corp., Riceboro, GA (USA)	90	Waste SIC:2611	MS, GC, IR*	2	1
3-72	Interstate Paper Corp., Riceboro, GA (USA)	20	Waste SIC:2611	MS, GC, IR*	2	1
3-72	Unidentified Kraft Pulp Mill, GA (USA)	45	Waste SIC:2611	MS, GC, IR*	2	1
1-73	Weyerhaeuser Paper Co., Springfield, OR (USA)	400	Waste SIC:2611	MS	3	2
12-73	Weyerhaeuser Paper Co., Everett, WA (USA)	60	Waste SIC:2611	MS	3	2

*Confirmed with a standard

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Contact References:

1. Keith, L. H., Radian Corporation, Austin, TX, 78766, USA. Telephone: (512) 454-4797.
2. Hrutfiord, B. F., College of Forest Resources, Univ. of Washington, Seattle, WA, 98195, USA. Telephone: (206) 543-1714.

Standard Industrial Classification (SIC) Numbers:

2611 Pulp mills.

Figure 9. One possible format for WaterDROP data output.

The data input to WaterDROP will include:

Compound name	Method of identification
Synonyms	Degree of confirmation
CAS Registry Number	Concentration
Wiswesser line notation	Sample site
Empirical formula	Water type
Molecular weight	Sampling date
Chemical class	Literature reference
Structure	Source/laboratory reference
	WaterDROP entry date

The development contract will call for a design phase of 3 months to determine sources of data, methods for evaluation of data, a system for input and continual updating of data, best data output format, necessary computer storage and terminal facilities, the most useful ways to retrieve data from the system, etc. The second contract phase will involve comprehensive data collection from sources throughout the world, evaluation of data to determine quality, collation and storage of the data, development of computer software for data retrieval, and publication of a printed handbook of the data.

It is anticipated that WaterDROP will become part of the Chemical Information System (CIS), a world-wide, publicly accessible chemical information network developed by EPA and the National Institutes of Health. Software for data retrieval will allow the WaterDROP user to query the system for specific compound occurrences according to compound name, geographic area, type of water, molecular structure, CAS Registry Number, and chemical class. If WaterDROP does reside in the CIS, users will be able to interact directly with the other modules of CIS such as Substructure Search, Mass Spectral Search, NIOSH Registry of Toxic Effects of Chemical Substances (RTECS), etc.

The most available product of the WaterDROP contract will be a printed handbook containing all the compound data in the computer files. Manual data retrieval from the handbook will not be as flexible or fast as with the computer system, but suitable indexes will still allow the most useful occurrence data to be located. The handbook will be periodically updated (perhaps every 6 months) with the latest data that has been submitted to the contractor. It is expected that this handbook will be available in the fall of 1979.

V. Conclusions

The automatic sampler, the master analytical scheme, and WaterDROP will all help provide significant advances in our knowledge about trace organics in water. For example, a systematic nationwide survey for specific organics is badly needed in the United States; there is a surprising degree of ignorance regarding the occurrence and distribution of volatile organics, to say nothing of non-volatiles, in water. The analytical advances discussed in this paper should each be applicable to such a survey.

VI. Acknowledgments

Appreciation is expressed to William T. Donaldson, Chief of the Analytical Chemistry Branch (ACB) of the Athens Environmental Research Laboratory, who originated the ideas for the automatic sampler and the master analytical scheme; to Larry Keith, formerly of the ACB staff and now of Radian Corp., Austin, TX, who did much of the original development work on WaterDROP; and to Walter Shackelford of the ACB for his consultation on the master scheme and WaterDROP.

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IDENTIFICATION AND QUANTITATION OF AROMATIC HYDROCARBON METABOLITES IN MARINE BIOTA

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Many marine organisms metabolize polynuclear aromatic hydrocarbons by adding oxygen directly to the aromatic rings. Typical chemical species resulting from such oxygenations are epoxides, dihydrodiols, phenols, and quinones. A variety of conjugates may be formed in subsequent reactions.

Separations of a number of conjugated and non-conjugated metabolites were achieved by high-pressure liquid chromatography (HPLC). The analytical procedures employ an internal standard of metabolites obtained from the urine of rats exposed to radio-labeled naphthalene. By use of the internal standards it was possible to identify metabolites in fish tissues, regardless of whether the compounds were structurally identified, and obtain recovery data on those metabolites that were common to both tissue extracts.

Techniques to purify radiolabeled aromatic hydrocarbons by HPLC and to quantitate hydroxylated and conjugated metabolites of naphthalene are described.

Key words: High-pressure liquid chromatography; marine biota; metabolites; polynuclear aromatic hydrocarbons.

I. Introduction

Marine organisms metabolize polynuclear aromatic hydrocarbons (PAHs) to compounds that may be more toxic than the parent hydrocarbons [1–2]. A number of these metabolic products arise by the direct addition of oxygen to aromatic rings and by subsequent reactions. Typical PAH metabolites resulting from this process are epoxides, dihydrodiols, phenols, quinones, and a variety of conjugated derivatives; several of these metabolites have been shown to be cytotoxic, acting as precursors for mutagenesis or carbinogenesis in mammalian systems [3]. Accordingly, in experiments involving exposures of marine organisms to petroleum or to individual aromatic hydrocarbon constituents, it is important to employ sensitive and reliable analytical procedures for the detection and measurement of biodegradation products.

Analytical techniques developed in our laboratories for studying the formation of metabolites of PAH in marine organisms are presented. Previous methods for extracting total metabolite fractions from homogenates of animal tissues include partitioning between hexane and aqueous formic acid [4], Soxhlet extraction [5], adsorptions on activated charcoal [6,7], and extractions into ether [8,9] or ethyl acetate [10,11]. Separations of individual metabolites from biological samples have employed paper chromatography [5–7,11], adsorption chromatography on columns [7], or thin-layers [4,6,11,12], and adsorption of the parent hydrocarbon into polyethylene [12].

To develop sensitive and reliable quantitative procedures for isolating and identifying metabolites of PAHs in complex biological extracts, we investigated various methods for extracting, concentrating, and quantitating both hydroxylated and conjugated metabolites of naphthalene from tissues and fluids of marine and terrestrial animals. The procedures involve extraction of metabolites with ethyl acetate, concentration in silanized glassware under nitrogen,

and separation by HPLC using ultraviolet (UV) absorbance or radiometric detection. We devised qualitative and quantitative analytical techniques which were based upon both external and internal standards.

In the course of our work, we found that purities of radiolabeled PAH must be checked if credible biological experiments are to be conducted with such compounds. Purifications of commercially obtained PAH that may have decomposed on storage and in laboratory handling were achieved by use of standard analytical HPLC columns.

II. Experimental

A. Materials

Reagents. Methanol and ethyl acetate were Mallinckrodt ChromAR: 95% ethanol was U.S.P., and all other organic solvents were pesticide quality. Standard solutions for qualitative analyses were made up in methanol using α-naphthol (28 ng/μL), β-naphthol-α-D-glucopyranoside (4 ng/μL), α-naphthyl sulfate (8 ng/μL), and α-naphthyl-β-D-glucuronic acid (16 ng/μL) obtained from Sigma Chemical Company. Naphthalene (gold label) and dimethylchlorosilane (DMCS) were obtained from Aldrich Chemical Company. Some of the standard solutions described above contained racemic 1,2-dihydrodihydroxynaphthalene (12 ng/μL) which was synthesized by reduction of 1,2-naphthoquinone with lithium aluminum hydride [13]. Tritium (^{3}H) and carbon-14 (^{14}C) labeled compounds were obtained from commercial sources. Dilutions were made with methanol or water that was filtered through activated charcoal and distilled in an all-glass apparatus. Corn oil used for intraperitoneal injections was U.S.P. quality. All other chemicals were reagent grade.

Apparatus. A high-pressure liquid chromatograph (Spectra-Physics Model 3500) equipped with a 254-nm UV absorbance detector (Spectra-Physics Model 8200) and interfaced to a calculating recorder (Hewlett-Packard Model 3385) was used for high-pressure liquid chromatography. A stainless-steel column, 4 mm i.d.×50 cm was packed in our laboratory with 10-μm LiChrosorb RP-18 (E-M Laboratories) reverse-phase liquid chromatographic column material, using a stirred-slurry column packer (Micromeritics Model 705). Eluted fractions were collected in glass vials by a fraction collector (Fractomette Alpha-200). Radioactivities of the fractions were measured using Aquasol (New England Nuclear) cocktail and a liquid scintillation counter (Packard Tri-Carb Model 3003). Laboratory glassware was treated with either Siliclad (Clay Adams) or with a solution of DMCS in petroleum ether unless otherwise indicated.

Test Animals. Sprague-Dawley rats weighing about 200 g were used. Coho salmon *(Oncorhynchus kisutch),* weighing about 100 g, were raised at this Center. Rainbow trout *(Salmo gairdneri),* weighing about 150 g, were obtained from a commercial fish farm. Both species of fish were maintained in fiberglass tanks without food for 2 weeks prior to exposure.

A. Methods

Extractions. The general extraction procedure includes: (a) homogenization of *ca.* 2 g of tissue or bile with 5 mL of water in a Potter-Elvehjem tissue grinder with a Teflon pestle, (b) transfer of the homogenate to a 50 mL centrifuge tube, (c) rinsing of the residual homogenate into the 50 mL tube with 5 mL of acetone to precipitate proteins, (d) addition of 0.2 g of NaCl crystals to the mixture, and (e) extraction with two 12.5 mL portions of ethyl acetate (rotated for 10 minutes on a test tube rotator). These extracts were concentrated just to dryness at ambient temperature under a stream of nitrogen, and the residues were dissolved in methanol for subsequent analysis.

To determine recoveries, aliquots of the standard solution were extracted, concentrated, diluted to the original volume, and then chromatographed by HPLC with UV detection at 254 nm (see Fig. 1, *upper*). Extraction/concentration efficiencies were calculated for each component by

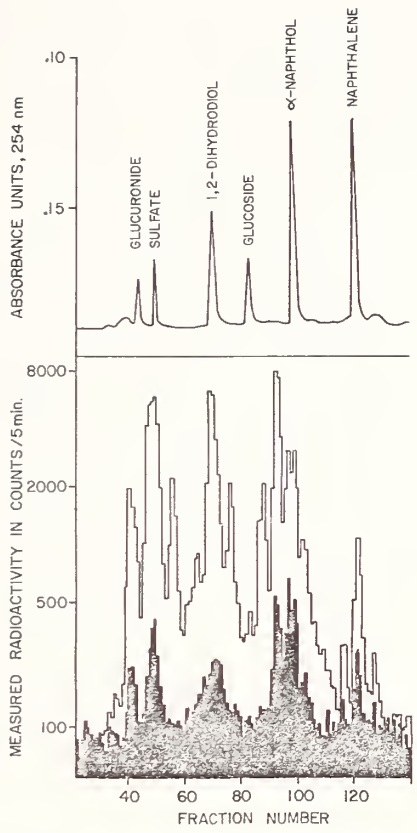

Figure 1. *Upper.* Elution pattern of naphthalene and selected metabolites injected in methanol. Elution: 60 min linear gradient from 5×10^{-4} mol/L KH_2PO_4, pH 5, to 100% methanol. HPLC column: 0.5 m×4 mm 10-μm RP-18 reverse-phase packing. Flow rate: 1 mL/min. UV absorbance measured at 254 nm.

Lower. Measured radioactivity vs. fraction number for combined extracts of rat urine samples after intraperitoneal injections of ^{3}H- and ^{14}C-labeled naphthalene. Shaded area indicates ^{14}C radioactivity. Fractions collected every 30 s. HPLC conditions same as above.

dividing the chromatographic response for each compound in the extract by the corresponding response for the original standard solution.

Chromatography. After 10 minute equilibrations of the column with the buffer (5×10^{-4} mol/L phosphate, pH 5.0), sample aliquots (98±0.6 μL) in methanol were applied to the HPLC column. Flow rates were either 1.0 or 2.0 mL/min, and the column was maintained at 40°±1 °C. The column was eluted by linearly increasing the concentration of methanol with time to 100% over a 30–60 min period. Eluate fractions were collected every 30 s. In some cases 95% ethanol was used instead of methanol.

Purifications. Radioactive compounds were purified by HPLC, collecting the eluate corresponding to the retention time of the compound of interest. Aliquots of this eluate were rechromatographed by HPLC and the radioactivities of the fractions were measured by liquid scintillation counting (LSC). Identities of the compounds of interest were verified by HPLC retention data.

Rat Urine Extracts. Ethyl acetate extracts were prepared from urine collected from rats which had been injected intraperitoneally with radiolabeled (^{14}C or ^{3}H) naphthalene. Urine was collected from animals held in a plastic rodent urine collection cage (Econo-Lab). The urine was saturated with sodium chloride and extracted four times with volumes of ethyl acetate equal to the urine volume. These extracts were combined and evaporated to dryness under a stream of nitrogen

at ambient temperature. The residue was dissolved in methanol to yield a stock solution of about 6×10^5 counts/min/mL for ^3H or 3×10^4 counts/min/mL for ^{14}C. The rat urine extracts were used to obtain recovery data on metabolites isolated from tissue homogenates. Recovery data on metabolites common to both the internal standard and the unknown were obtained, regardless of whether structural identifications had been made of the compounds in the internal standard.

III. Results and Discussion

A. HPLC Sample Preparation

A number of extraction and concentration techniques were evaluated in the present work. Ethyl acetate and methylene chloride were found to be superior to diethyl ether, petroleum ether, and hexane as solvents for the extraction of α-naphthol. However, residual methylene chloride in the concentrates led to variable HPLC retention times for α-naphthol, so ethyl acetate was the preferred solvent. Saturation of the homogenate with added sodium chloride increased the extraction efficiencies of standard solutions of naphthyl glucuronide, sulfate, and glucoside into ethyl acetate by factors of 1.5, 6.0, and 1.0, respectively.

Methods for concentrating the organic extracts with untreated glassware gave erratic recoveries for α-naphthol. Silanizing the glassware with DMCS improved recoveries of α-naphthol more than did treatments with Siliclad or washing with dilute hydrochloric acid; thus DMCS-silanized glassware was used henceforth. Using nitrogen instead of air drying improved the recovery of α-naphthol.

By employing the extraction technique described, concentrating fractions under a stream of nitrogen and employing glassware treated with DMCS, acceptable recoveries (see Table 1) were obtained for all compounds in the standard solutions, except naphthalene. More exhaustive extraction or a lower pH improved the recoveries of naphthyl glucuronide, which generally was obtained in *ca.* 25% yield from homogenates.

TABLE 1. *Extraction efficiencies for metabolites of naphthalene*

Compound	Standards $X \pm \sigma_{N=8}$
Naphthyl glucuronide	0.25 ±0.08
Naphthyl sulfate	.86 ± .07
Naphthalene-1,2-dihydrodiol	.75 ± .09
Naphthyl glucoside	.92 ± .08
α-Naphthol	.64 ± .20
Naphthalene	Erratic

B. Purifications

Several of the radiolabeled hydrocarbons purchased for metabolic studies contained polar impurities having HPLC retention times similar to those of hydrocarbon metabolites. Comparison of elution profiles depicted in Figures 1 and 2 shows that the polar radioactive impurities in the ^3H-l-methylnaphthalene (Fig. 2) eluting in the earlier fractions could be easily mistaken for metabolites. Removal of these impurities in starting materials was achieved by the HPLC purification techniques described. Purifications similar to those given in Figure 2 were obtained with ^3H-styrene oxide, ^{14}C- and ^3H-naphthalene, and ^3H-phenanthrene in methanol; and ^3H-glutathione in water. Commonly employed analytical HPLC columns were used, obviating the need for more expensive, large-capacity preparative columns. Moreover, small amounts of a single aromatic hydrocarbon can be isolated in the presence of large proportions of other components.

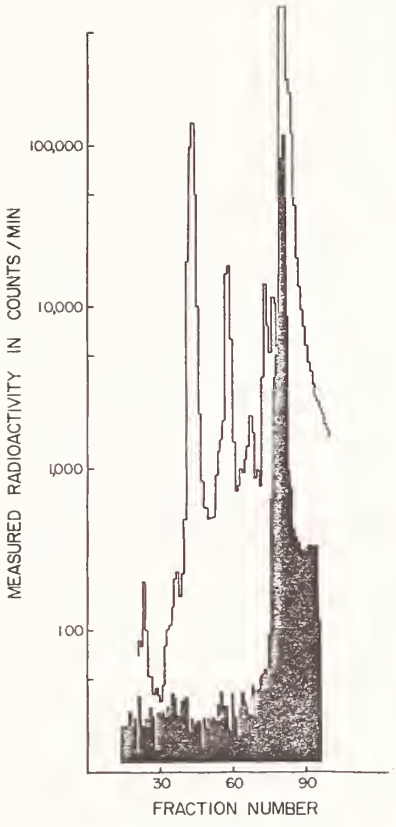

Figure 2. Radioactivity vs. fraction number for HPLC separations of commercially obtained and purified ^{3}H-1-methylnaphthalene. Shaded area represents purified compound. Fractions collected every 30 s by 30 min linear gradient from 5×10^{-4} mol/L KH_2PO_4, pH 5, to 95% ethanol. HPLC column and flow same as in Figure 1.

C. Analysis of Metabolites by HPLC

Ethyl acetate extracts of urine from rats injected with ^{3}H- or ^{14}C-labeled naphthalene revealed a number of radioactive metabolites when analyzed by HPLC (Fig. 1, *lower*). Several of these metabolites had HPLC retention times that corresponded with those of non-radioactive naphthalene metabolites (Fig. 1, *upper*) typically formed in mammalian systems. The non-radioactive metabolites of naphthalene were detected by HPLC using UV absorption monitoring.

In employing the cochromatographic technique with rat urine, the structural identities of individual metabolic products in the unknown extract need not be established. This approach is useful for investigating hydrocarbon metabolites from a test organism that has similar retentions as those obtained from rat urine. The general similarity of metabolic pathways for aromatic hydrocarbons among mammals [14] suggests that marine vertebrates perform essentially the same types of conversions as mammals.

Because a number of metabolites are produced even for a relatively simple and symmetrical PAH molecule, such as naphthalene, it is important to employ an analytical system that has high resolving power, such as HPLC. The use of HPLC, as we have described, minimizes the occurrence of "overlapping" fractions. Clearly, if test animals are exposed to ^{14}C-labeled naphthalene, the internal standard should be prepared from rats exposed to ^{3}H-labeled naphthalene. This approach is preferable to the use of a ^{14}C-labeled internal standard because low

levels of ^{14}C-labeled metabolites may be measured by LCS without interference from ^{3}H-labeled metabolites.

D. METABOLITE QUANTITATIONS

Naphthalene metabolites from test organisms were quantitated as follows: (a) an aliquot of previously assayed urine extract from rats given ^{3}H-labeled naphthalene was added to test-animal homogenates containing metabolites of ^{14}C-labeled naphthalene, (b) each homogenate was then extracted by the ethyl acetate procedure, chromatographed, and the fractions counted by LCS, and (c) the amount of each ^{14}C-labeled component in the sample, M_c, was calculated as

$$M_c = \frac{A_c}{[A_h/A_{u,h}]\ [V/V_u]S_c e} = \frac{A_c}{E\,[V/V_u]S_c e}\ , \qquad (1)$$

where S_c is the specific radioactivity for the ^{14}C-naphthalene used, A_u is the measured radioactivity for the component from the rat urine extract, A is the measured radioactivity for the component from the sample extract, e is the LSC counting efficiency for ^{14}C, E is the extraction/concentration efficiency for the component, V is the volume of the chromatographed extract, V_u is the volume of rat urine extract added to the homogenate, and subscripts c or h designate ^{14}C or ^{3}H radioactivity, respectively. The interference of ^{14}C LSC energies in ^{3}H energies is readily corrected by subtraction prior to these calculations when the ^{3}H activity far exceeds the ^{14}C activity.

This internal standardization with previously assayed ^{3}H-labeled rat urine extract is especially advantageous when HPLC results for metabolites from various ^{14}C-dosed organisms are being compared. The ^{14}C-metabolite data for these organisms may be interrelated via the ^{3}H-metabolite patterns (see Fig. 3) as follows: (a) add the same amount of ^{3}H-labeled extract (e.g., 100 μL) to each tissue homogenate before extraction, and (b) after HPLC calculate radioactivity ratios between corresponding metabolites for both the ^{14}C eluates and the ^{3}H eluates from two samples being compared (e.g., exposed vs. control animals). Then, the radioactivity ratio for the ^{14}C eluates from exposed and control samples divided by the ratio for their corresponding ^{3}H eluates gives a relative concentration parameter, R, which is independent of extraction efficiencies. That is,

$$\frac{\left[\dfrac{A_{1,c} \pm \sigma A_{1,c}}{E_1 \pm \sigma E_1}\right]}{\left[\dfrac{A_{2,c} \pm \sigma A_{2,c}}{E_2 \pm \sigma E_2}\right]} \div \frac{\left[\dfrac{A_{1,h} \pm \sigma A_{1,h}}{E_1 \pm \sigma E_1}\right]}{\left[\dfrac{A_{2,h} \pm \sigma A_{2,h}}{E_2 \pm \sigma E_2}\right]}$$

$$= \left[\frac{A_{1,c} \pm \sigma A_{1,c}}{A_{2,c} \pm \sigma A_{2,c}}\right] \left[\frac{A_{2,h} \pm \sigma A_{2,h}}{A_{1,h} \pm \sigma A_{1,h}}\right] = R, \qquad (2)$$

where A, σ, and E indicate measured radioactivity, uncertainty, and extraction/concentration efficiency, respectively, for a particular eluted component, subscripts c or h indicate ^{14}C or ^{3}H radioactivity, and the subscripts 1 and 2 indicate members of a set of two samples (e.g., exposed vs. control).

$E_c \pm \sigma E_C = E_h \pm \sigma E_h$, since the extraction efficiency is not expected to be different for a ^{3}H- and ^{14}C-labeled eluted compound when extracted together in the same solutions. As a result, the relative concentration parameter for the respective ^{14}C eluates from the two samples is corrected for variations in extraction/concentration efficiencies because

$$\frac{E_1 \pm \sigma E_1}{E_2 \pm \sigma E_2} = \frac{A_{1,h} \pm \sigma A_{1,h}}{A_{2,h} \pm \sigma A_{2,h}} \qquad (3)$$

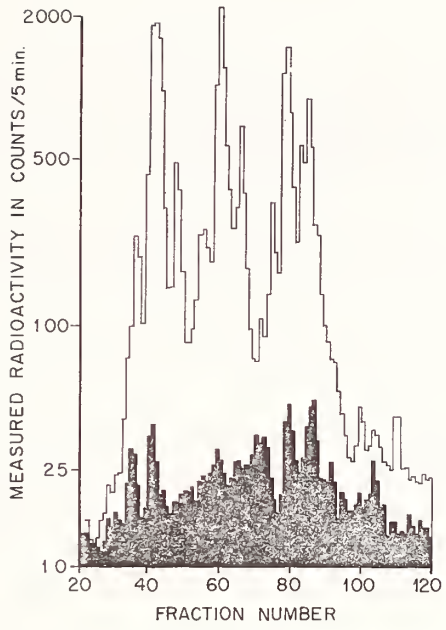

Figure 3. Radioactivity vs. fraction number for bile extract from rainbow trout injected intraperitoneally with ^{14}C-labeled naphthalene. Urine extract from a rat injected intraperitoneally with ^{3}H-labeled naphthalene was added to trout bile as an internal standard. Shaded area indicates ^{14}C. HPLC conditions same as in Figure 1.

Prior radioassay of the individual metabolites in the internal standard is not required. Relating samples through the use of biogenic internal standards improves statistical confidence in comparisons of metabolite concentrations among different sets of samples. Moreover, the biogenic internal standard contains a number of metabolic components, revealed through HPLC analysis, that closely resemble metabolic components in samples from other species (including HPLC eluates of unknown identity). The procedures described should have wide use in the analysis of a variety of biological samples where structural identifications are difficult to obtain because of the lack of availability of pure standard compounds.

IV. Summary

Rat urine extracts containing ^{3}H-naphthalene metabolites were cochromatographed on HPLC columns with extracts from marine organisms exposed to ^{14}C-labeled naphthalene. Thus, metabolites from marine organisms were correlated with those of both known and unknown structure that have been found in rat urine. Moreover, the use of rat urine extracts as internal standards made it possible to obtain recovery data on a variety of metabolites isolated from homogenates obtained from fish tissues.

Impure radiolabeled compounds were refined by HPLC on normal analytical columns to isolate radioactive hydrocarbons in high purity (>99%) for use in metabolic studies.

V. Acknowledgment

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THE TRACE-LEVEL DETERMINATION OF ORGANICS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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High-pressure liquid chromatography holds great promise as a tool for the rapid, trace-level determination of multi-residue samples of organics in water. Because of the great versatility available in selecting mobile phase/stationary phase combinations, many organic compounds can be collected and determined by HPLC without the concommitant problems of extraction, derivatization, clean-up, etc., that plague other methods. In addition, HPLC can be used as a tool to fractionate multi-residue samples of organics into smaller fractions, for further study.

This paper will discuss several applications of HPLC to the analysis of organics in a number of matrices.

One application to be discussed is the analysis of pentachlorophenol in waste effluents. This HPLC method is rapid, requiring no derivatization of the PCP, specific for PCP and can determine PCP in the low ppb range.

Another application is the multi-residue determination of chlorphenoxy acid herbicides in drinking water. Contrary to the gas-chromatographic method, which requires several hours per sample, the HPLC requires only minutes, and determines the herbicides in the low ppb range.

A third application that will be discussed is the isolation of a single pesticide metabolite froma soil extract containing a wide-array of organic components.

Key words: Extraction; herbicides; high pressure liquid chromatography; organics in water; pesticides.

The recent advent of a host of environmental legislation has posed some very challenging and unique problems for analytical chemistry. The requirements of each law vary, but each challenges the analytical chemist to devise new methodology, or improve current methodology, which: 1) allows analysis of trace level components, 2) in complex media, 3) for either individual components, or multiple residues of organics of both similar and dissimilar chemical classes.

This paper will discuss two aspects of the trace organic analysis of water (and waste-water) samples by high pressure liquid chromatography: the extraction process, and the analysis. Liquid-liquid partitioning of a water sample with an organic solvent (or organic solvents) to extract the organic components is a longstanding method. There are several drawbacks to this procedure however. The extraction is usually less efficacious than desired, requiring large volumes of sample to extract the minimum amount of material necessary for detection. When one desires to determine multiple-residues in a water sample, more than one organic solvent is often required if the polarities of the organic components are quite different. The large degree of sample handling often results in cross-contamination of the sample, sample loss, as well as the potential for worker-exposure to toxic components and solvents.

An increasingly common technique used in place of liquid extractions is the technique called *trace enrichment.* Trace enrichment (as shown in Figs. 1–3) involves passing a water sample through a column which is packed with a non-polar packing material. The packing is a silica particle with a non-polar organic moiety bonded to it, creating a non-polar bonded liquid phase. Trace level organics present in the water sample partition to the non-polar packing from the polar water matrix; the efficiency of removal from the water depends upon the relative affinities for the two phases. In this way, the trace organic components of a water sample are both collected and

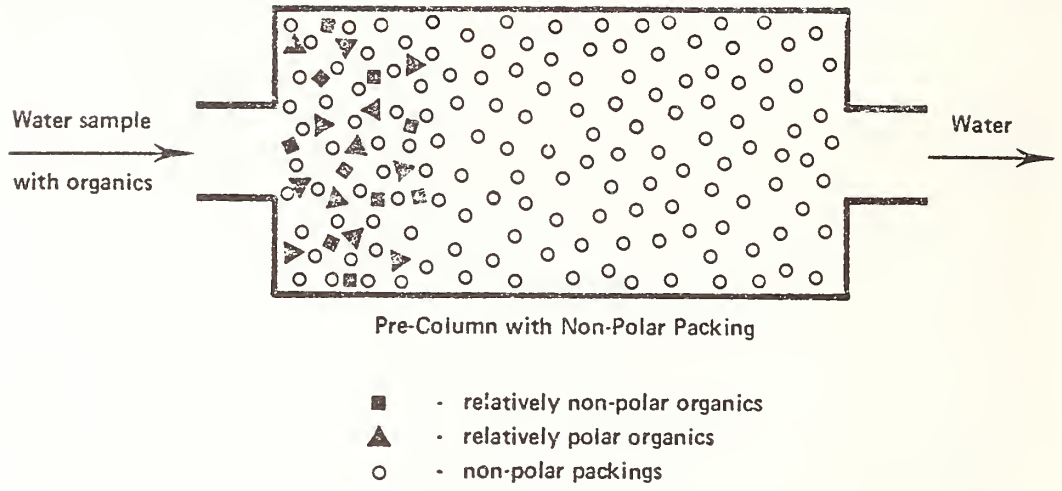

Figure 1. The trace-enrichment process, collection and concentration of organics.

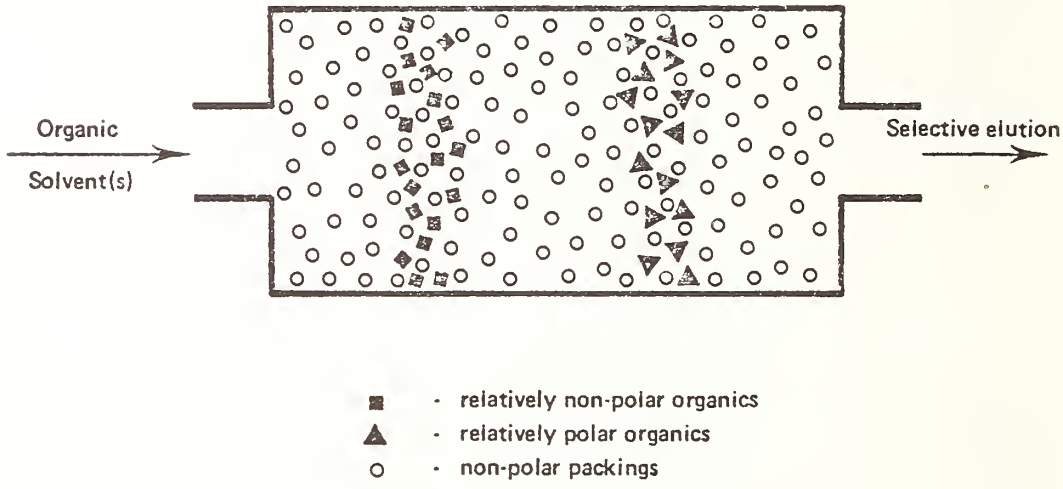

Figure 2. The trace-enrichment process, collection and concentration of organics.

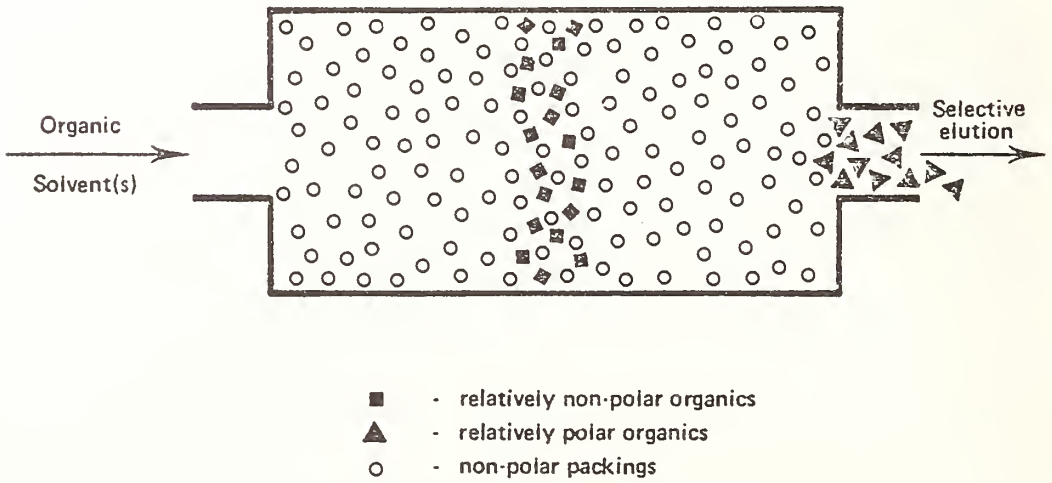

Figure 3. The trace-enrichment process, collection and concentration of organics.

concentrated in one step, on the column. Organic components originally present in the water sample at undetectable levels can be concentrated on the column to a detectable level.

The analysis of the chlorphenoxy acid herbicides particularly lends itself to trace enrichment/HPLC analysis. Monitoring of these herbicides and methoxychlor in drinking water is required by a Federal statute—the Safe Drinking Water Act. Since these herbicides are applied to agricultural lands as different chemical forms (esters, free acids, amine salts), efficient extraction with one solvent is difficult. The currently used GC method requires hydrolysis, esterification and clean-up steps to convert all forms of the chlorphenoxy acids to a form amenable to GC analysis (the methyl ester). The authors experimented with an LC system which would be compatible with the presence, in water, of the different chemical forms of the herbicides. Figure 4 depicts an attempt to separate 2,4-D acid, Silvex acid and 2,4-D dimethylamine salt on a reverse phase column; no separation was achieved using acetonitrile/water mobile phase. However, when the water in the mobile phase is acidified to pH 3.6 (1% HAc), there is retention and separation of the two acids (Fig. 5), while the dimethylamine salt hydrolyses to the corresponding free acid. Apparently, the acid suppresses the ionization of the chlorphenoxy acids, leading to a partitioning

Figure 4. Pesticide residues in drinking water, resolution effect with unbuffered solvent.

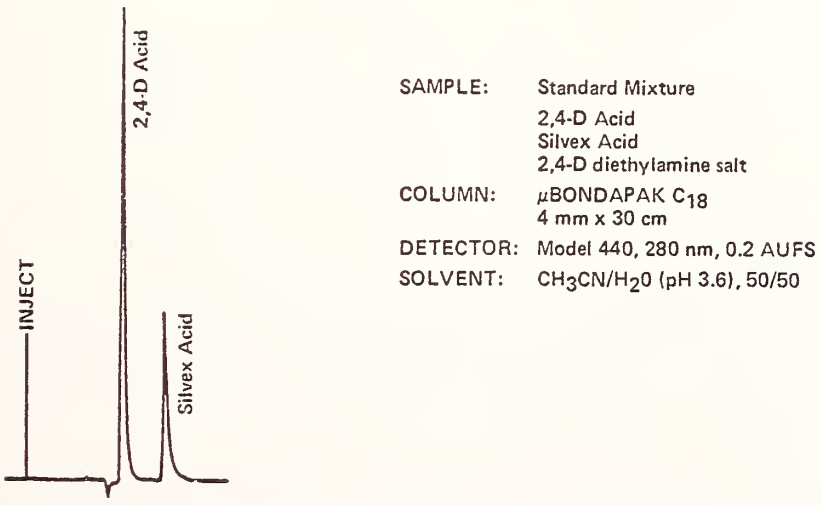

Figure 5. Pesticides in drinking water, resolution effect with buffered solvent.

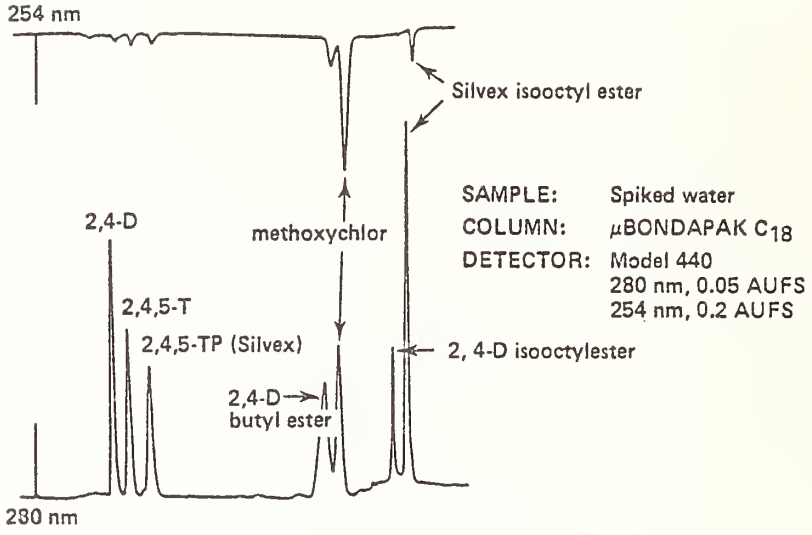

Figure 6. Chlorphenoxy acid herbicides in water, rapid multiresidue analysis.

to the non-polar packing. Utilizing this information, analytical conditions were optimized for the separation of acids, esters and methoxychlor in a relatively short time (approx. 10 min) as shown in Figure 6.

The real importance of this method lies not solely in the final analysis of the herbicides, but also in the method of collection and concentration of the herbicides. The trace-enrichment process was used to effectively remove more than 90% of the herbicides from spiked water samples, as follows:

Spiked water (acidified)	(20 ppb)
↓ 50 mL	
trace enrichment on pre-column of Bondapak C_{18}/ Porasil B (7.6 cm×4mm)	(1 μg)
↓	
elution of herbicides with 1 mL of 75/25 CH_3CN/H_2O (pH 7)	(1 ppm)
↓	
injection of an aliquot of the eluate into HPLC	(100 mL aliquot = 100 ng)

50 mL of the acidified water sample (pH 3.6) spiked to the 20 ppb level for each form of the herbicides, is passed through a pre-column containing Bondapak C_{18}/Porasil B non-polar bonded-phase packing. This results in 1 μg of each form of the herbicides being concentrated on the pre-column. Next, 1 mL of 75/25 acetonitrile/water (pH 7) is passed through the pre-column to elute the herbicides at a concentration of 1 ppm. An aliquot of this eluant is analyzed by HPLC under the conditions given in Figure 6.

This procedure takes approximately 15 minutes, effectively removes the free acid form and the various esters of the herbicides, thus completely eliminating the extraction-derivatization routine. Thus, this procedure meets the general criteria for an improved analytical method in that

it is rapid, capable of multi-residue determination and allows determination of virtually any contaminant level by simply trace-enriching a larger volume of water.

We next applied this straightforward technique to the analysis of a complex agricultural run-off water sample. Analyses of samples of this complexity normally require extractions with several different solvents to ensure removal of residues of widely divergent polarities, sometimes followed by clean-up and derivatization steps. Some of our original work, and the work of Paschal et al. [1] indicates that the trace enrichment process would effectively collect and concentrate a wide spectrum of pesticide (and non-pesticide) residues. Paschal reported recovery efficiencies of 99% for methyl and ethyl parathion in water, using the marcoreticular resin XAD-2 as an adsorption medium.

Figure 7 is the chromatogram of a sample of agricultural run-off water which was spiked at the 20 ppb level with 18 pesticides from four classes: organophosphates, carbamates, chlorphenoxy acids (and esters) and triazine herbicides. One-hundred mL of sample was trace-enriched on a pre-column (SEP-PAK™, Waters Associates) of Bondapak C_{18}/Porasil B; the concentrated organics were eluted with 1 mL of acetonitrile, giving a 100x concentration factor. A 40 μL aliquot of the extract was chromatographed on a reverse-phase column of μBondapak/C_{18}, giving the separation shown in Figure 7. The unspiked sample was then trace-enriched, eluted and analyzed in exactly the same fashion, giving separation and detection of 13 pesticides as shown in Figure 8. The concentration of the pesticides was in the low ppb region, but detection could be extended below this level by trace-enriching a larger volume of water. Another sample from the same geographical region was treated and analyzed in the same way (Fig. 9). Notice that even though there was a considerable increase in the number of peaks in Figure 9, it was still possible to identify five of the pesticides present in the sample again at the low ppb level.

Because of tremendous time-saving in sample preparation and analysis time (one chromatographic run as opposed to multiple GC runs), as well as the ability to analyze thermally labile compounds without fear of decomposition, the use of this type of methodology in rapidly screening water samples for pollutant levels should greatly increase.

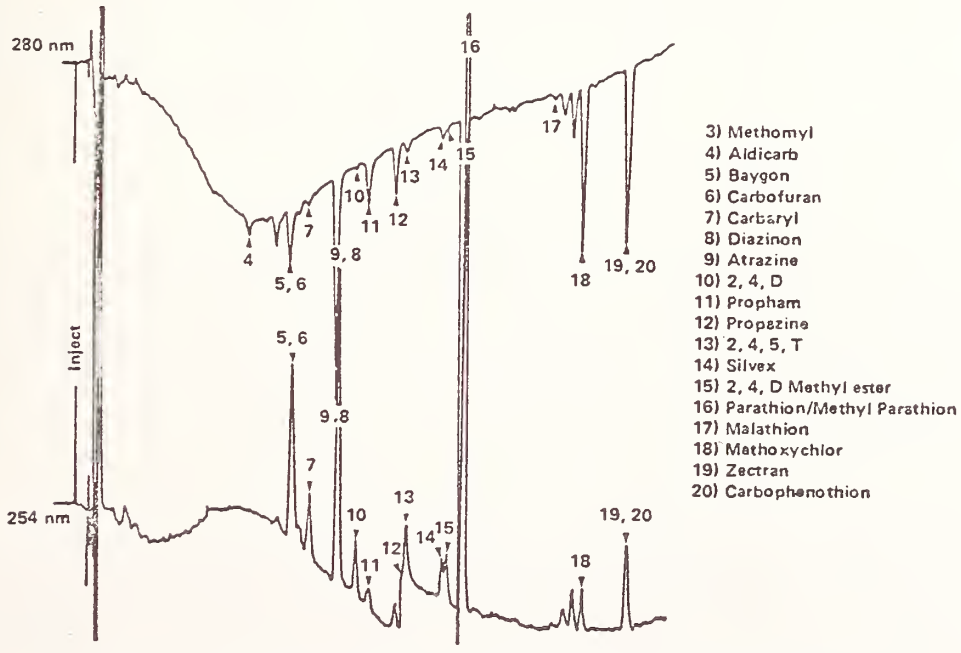

Figure 7. Trace organics in agricultural run-off water, drinking water spiked with standards.

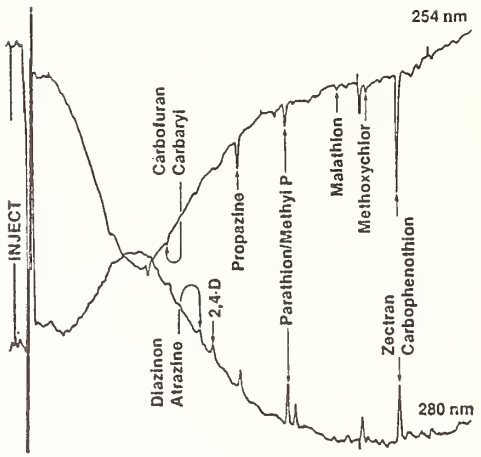

Figure 8. Trace organics in agricultural run-off water, trace enriched agricultural run-off water.

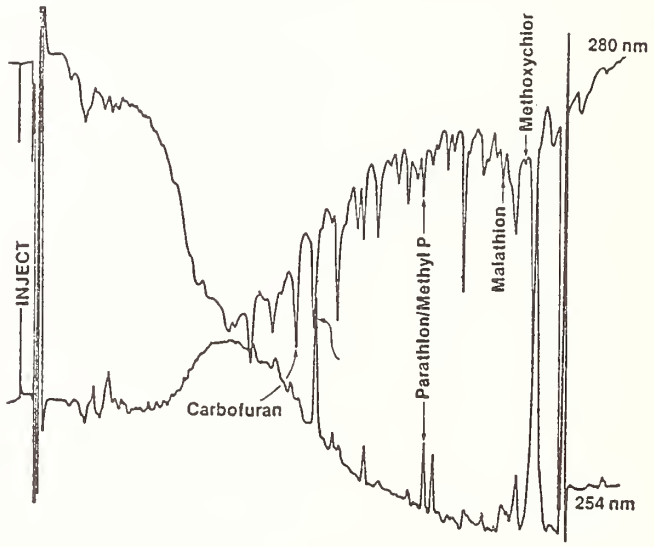

Figure 9. Trace organics in agricultural run-off water, trace enriched agricultural run-off water.

The last example of the use of HPLC and trace-enrichment to the analysis of trace-level organics is that of pentachlorophenol in plant waste effluents. This method was developed as a rapid monitoring technique to determine compliance with effluent discharge permits. Because of the length of time involved in the analysis of PCP by gas chromatography/ecd or by the 4-AAP method, there exists a very real possibility that a plant may be in violation of a discharge permit for some time before the actual PCP levels are determined. The possibility of erroneous results exist with the GC method because of the potential for contamination by PCP on glassware, etc. Also, the 4-AAP method only determines total phenols, rather than specific phenols which may be cited in a permit.

Figure 10 shows the chromatogram resulting from the direct injection of a standard mixture of four phenols (in deionized water). Conditions were selected for the optimum separation of these four phenols (which co-occur) directly on the analytical column, rather than on a pre-column; the concentrated organics were selectively eluted with a reverse-phase gradient (0–70% CH_3CN/H_2O (2% NH_4OAc), 15 min) on a μBondapak/C_{18} column.

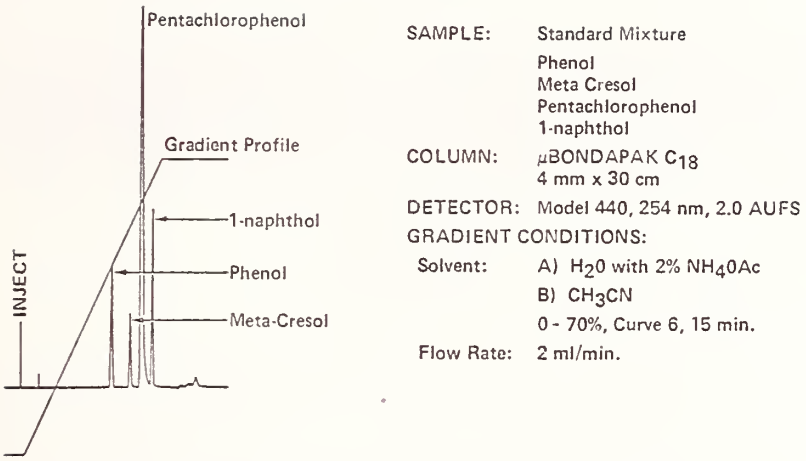

Figure 10. Pentachlorophenol in wastewater, standard analysis.

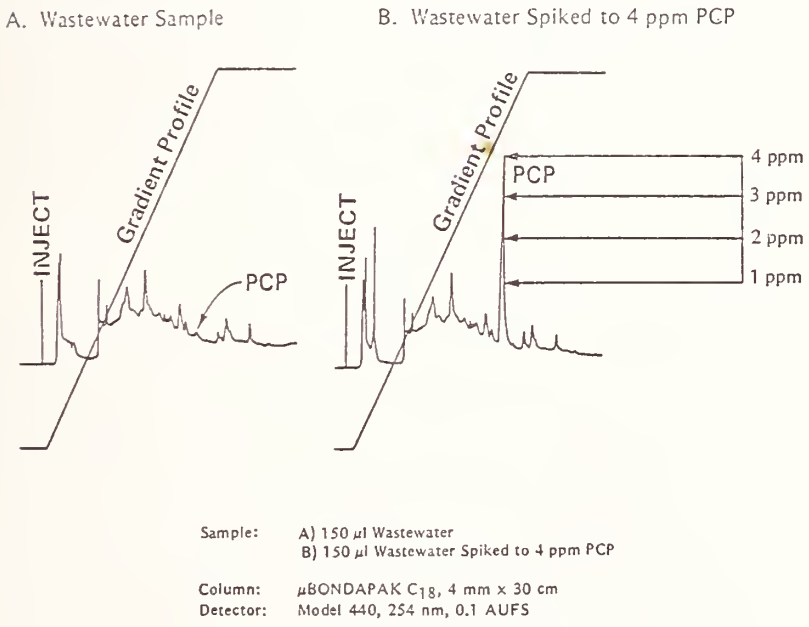

Figure 11. Wastewater sample analysis.

Figure 11 shows the chromatograms of the actual waste-water sample analyzed under the same conditions as the standards. Figure 11A shows an indication of PCP (at very low levels) as determined by its characteristic elution volume. Using the standard addition technique (spiking the waste-water to 4 ppm with PCP, Fig. 11B) allows determination of the peak to be PCP at the (approximately) 0.10 ppm level. By using this rapid HPLC method, compliance with effluent permits can be determined rapidly (approximately 15 min) and with a minimum of interference from other organic materials present.

Summary

This paper has discussed several applications of HPLC to the analysis of trace-level organics in water. Because of the diverse nature of the organics, attempts to totally characterize the organic content of a water sample, or to analyze for a specific compound, usually require lengthy and tedious extraction and analysis schemes. However, the use of the *trace-enrichment* technique coupled with the high resolution capability of HPLC allows the analysis of a wide array of the organics present in water, at trace levels and in a single analysis.

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CO-CONDENSATION WITH *n*-PENTANE. A NEW ROUTE TO CONCENTRATION OF ATMOSPHERIC POLLUTANTS

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An air sampling system has been developed in which gaseous *n*-pentane is introduced into the air stream during collection of the analytical sample. The gases are mixed and then passed through a cold trap maintained at liquid nitrogen temperature during which time the pentane vapor is condensed to the solid. Trace organic constituents of the sampled air stream are trapped in the matrix of solid pentane and thereby concentrated. Flow rates of pentane in the range 5 to 50 cm^3/min have allowed the collection of up to 1 gram of solid pentane for sampling times of up to 1 hour. Depending on the flow rate of the sampled air stream and the original concentration of the gaseous pollutant, concentration factors of up to three orders of magnitude are achieved. As an example, an air stream containing 1 ppm by volume of benzene was sampled at a flow rate of 100 cm^3/min together with a pentane stream flow rate of 10 cm^3/min for a total period of 10 minutes. This resulted in the collection of approximately 100 mg (150 microliters) of solid pentane. This was allowed to warm to room temperature and subsequently analyzed by gas chromatography. The trapping efficiencies for a number of common atmospheric pollutants have been measured and all are greater than 90%. The technique is of particular value in the case of reactive materials or those which react with the commonly used charcoal tubes. Other factors and possible interferences are discussed.

Key words: Air sampling; co-condensation; gas chromatography; trapping.

I. Introduction

Among the more important problems of air pollution measurements are those concerned with atmospheric sampling. Numerous techniques have been described [1], the majority of which involve either adsorption on charcoal [2] or a porous polymer [3] or adsorption in a reagent solution [4]. In the particular case of the former methods, certain classes of compounds are often irreversibly adsorbed, and quantitative recoveries of such compounds as acrolein, epichlorohydrin, isocyanates, and other highly reactive molecules may be very difficult to achieve.

For these reasons, we have sought an alternative general technique for atmospheric sampling. One such method is low temperature condensation in the presence of an inert matrix. The matrix material, generally *n*-pentane, is mixed with the sampled air stream in the vapor phase and then condensed, thus trapping the desired constituents. An enriched solution of the components of the air stream is thereby obtained, which may be analyzed directly, or stored in the frozen state. The method avoids the need for lengthy desorption times and, as is shown, affords considerably higher trapping efficiencies than is possible with charcoal tube sampling, particularly at the low concentrations commonly encountered in organic trace analysis.

II. Experimental

Reagents. *n*-Pentane was Fisher Pesticide Grade and each lot was checked chromatographically to insure the absence of interfering compounds. All other reagents were ACS Reagent Grade and were distilled prior to use.

Apparatus. A Varian MAT 112 Gas Chromatograph-Mass Spectrometer was used for all investigations. Chromatograms were recorded by use of a second ionizer operating at 20 eV with detection by means of a Faraday cup and DC amplifier combination. The column used was 2 m by

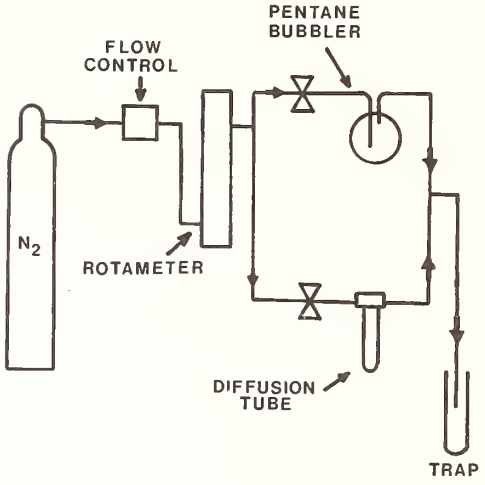

Figure 1. Schematic diagram of the pentane co-condensation apparatus.

3.2 mm of 10% Carbowax 20M-TPA on Chromosorb W with helium used as carrier gas. Injections were made with Hamilton 701N syringe which had been gravimetrically calibrated with mercury. These were found to be reproducible to 2% for a 0.5 μL injection.

Condensation Apparatus. A schematic diagram of the apparatus used is shown in Figure 1. All lines were 3.2 mm Teflon tubing and all connectors were Swagelock Type 304 stainless steel. The carrier gas was Matheson High Purity nitrogen and the flow was controlled by a Brooks constant flow controller. A Brooks rotameter together with two Cajon needle valves served to establish and monitor flow rates in each of the individual lines. All flow rates were measured with a soap bubble flow meter daily and the overall reproducibility of the flow rates was 2%. The sampled air stream containing pentane was condensed in micro traps of 5 mL total volume using either liquid nitrogen or solid CO_2 as the refrigerant.

Charcoal Tubes. The charcoal tubes used were NIOSH Certified Charcoal Tubes, Class X, and contained 150 mg total of charcoal divided into two sections of 100 mg and 50 mg. They were purchased from SKF, Inc., and samples at random were chosen from several different lots. Analyses were performed on both parts of the tube, in separate vials. At no time during this work was any compound found on the rear portion of the charcoal thus indicating that the breakthrough capacity of the charcoal tube had not been exceeded.

III. Results and Discussion

The principle on which the co-condensation technique rests, that of entrainment of a sample followed by simultaneous condensation, necessitates strict requirements for the entrainer. It must have a high vapor pressure at a convenient temperature (25 °C), yet it must be readily condensible with common refrigerants. It should also be easily purified and, above all, it should be inert towards the desired reactive compounds. *n*-Pentane is an ideal choice for the entrainer as it is readily liquified at −78 °C and it also has a vapor pressure of about 0.5 atm at 25 °C [5]. It is also inert towards all but the most vigorous oxidants or halogenating agents. We were able to generate a stream of pentane vapor by a simple bubbler arrangement (Fig. 1) and by a suitable choice of flow rates (ranging from 5 to 20 mL/min) obtain approximately 0.5 to 2 mL of condensate during a period of 30 minutes. A series of initial experiments showed that for collection times of 30 minutes (condensate volume about 0.9 mL) the technique was reproducible to better than 1%. A simple calculation using the ideal gas law also showed that at −78 °C the resulting collection efficiency was better than 98%.[1]

[1] Using PV=nRT, with T=298°, V=300 mL (10 mL/min for 30 min) and P=490 mm, the theoretical amount of *n*-pentane would be 0.57 g (0.91 mL). This is to be compared with 0.55 g (0.88 mL) actually recovered (average of 10 runs).

In order to compare the efficiency of the co-condensation technique with that of charcoal tube sampling, a diffusion tube was inserted into the sampling line (Fig. 1). The basic procedure for comparative experiments was as follows. The flow rates were set in each of the lines to provide approximately 1 μg/min of the compound from the diffusion tube and to allow a total of about 0.9 mL of pentane to be collected in 30 minutes. In certain cases this required cooling the diffusion tube using ice or solid CO_2. The two streams were combined in a tee and led into a micro trap immersed in solid CO_2-toluene slurry (–65 °C). After a period of 30 minutes the traps were removed and stored in solid CO_2. The line from the mixing tee was then connected to a charcoal sampling tube and the pentane flow was vented to the hood. The carrier flow was checked at this point to insure that it had not changed and the sorption of material by charcoal was allowed to proceed for 30 minutes. At the end of this time the charcoal was removed from the tubes, placed in stoppered vials, and solvent added to adsorb the material from the charcoal. The solvents used and the desorption times are listed in Table 1. After a suitable desorption period, aliquots of the solutions so obtained were analyzed by gas chromatography. Aliquots of the *n*-pentane solutions (after allowing time to equilibrate to 25 °C) were analyzed in the same manner. A composite chromatogram together with the analysis conditions is shown in Figure 2. The results of the various analyses are listed in Table 1.

As we expected, the efficiency of sorption and desorption for substances such as toluene or benzene is close to 100%. These materials are relatively inert and would not be expected to be strongly held on most common supports in the presence of solvent. The use of these materials however, serves as a valuable check on the efficiency of the co-condensation technique, where by reference to Table 1 it can be seen that this method also yields recoveries close to 100%.

In the case of reactive materials such as acrolein and epichlorohydrin, the recoveries from charcoal are considerably lower than those obtained from pentane co-condensation. In addition, they are strongly concentration dependent. Figure 3 is a plot of the relative recovery from charcoal as a function of concentration of these two compounds. It can be seen that as the total amount approaches 0.1 μg the recoveries approach zero. We believe that this is due to the interaction of the charcoal with these compounds, as both of them are known to polymerize readily in the presence of trace impurities. The absolute recoveries shown in Figure 3 were obtained by comparison with freshly prepared gravimetric standards. These standards were prepared by a sealed ampoule technique [6] in *n*-pentane, and calibration curves were obtained by using the GC peak areas.

As has been noted, conditions of high humidity severely decreases the sorption efficiency of charcoal tubes [7]. In order to ascertain whether water vapor would affect the co-condensation method, an additional diffusion tube was inserted in the carrier gas line and filled with distilled water. The sample investigated was phenyl isocyanate. Sorption on charcoal was allowed to

TABLE 1. *Trapping effiencies of various methods*

Compound	Desorption solvent	Desorption time[a]	Relative recovery[b]
Benzene	CS_2	60 min	96%
Toluene	CS_2	60 min	98%
Acrolein	CH_2Cl_2[c]	60 min	30%
Acrylonitrile	CH_2Cl_2	60 min	40%
Epichlorohydrin	CH_2Cl_2	60 min	30–80%
Phenyl isocyanate	CH_2Cl_2	120 min	50%, variable

[a] Chosen on the basis of preliminary experiments conducted at various times in order to ascertain the optimum time for maximum recovery.

[b] Relative recoveries are expressed as the GC peak area from charcoal divided by those from co-condensation and are for equal total amounts of compound sampled.

[c] Containing 1% isopropanol.

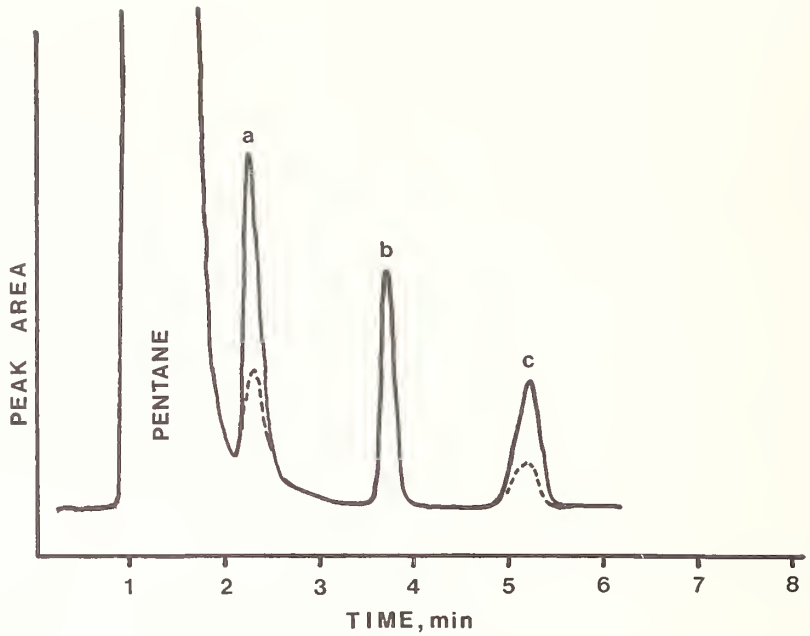

Figure 2. Composite chromatogram of compounds examined. The dashed lines indicate recoveries from charcoal and solid lines from co-condensation. The same total amount was sampled in each case. a=acrolein, b=acrylonitrile, c=epichlorohydrin

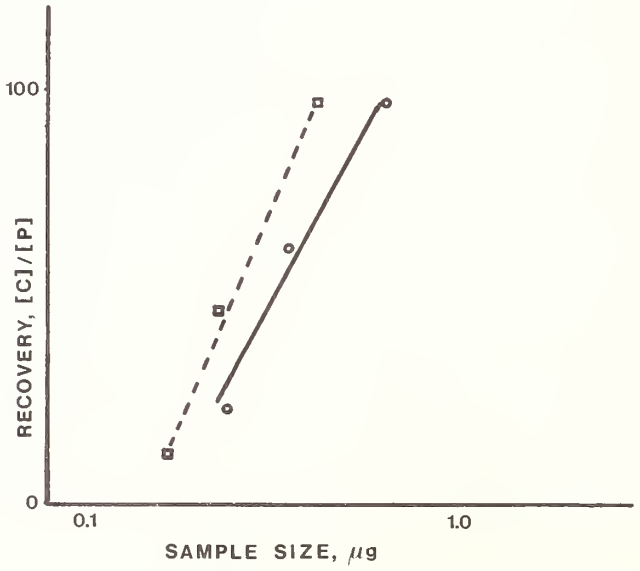

Figure 3. Relative recoveries of charcoal trapping vs. pentane co-condensation for the same total amount of compound. ------- acrolein, —— ephichlorohydrin

proceed as before and a series of pentane condensations was also collected. Upon analysis, poor and highly variable yields were found for phenyl isocyanate collected on charcoal, whereas the pentane co-condensation method afforded reproducible results. Upon examination of the traps containing pentane, the water was seen as micro-droplets at the bottom of the trap. We attribute the poor yields from charcoal to either displacement of the isocyanate by water vapor or to reaction of the isocyanate with water, a process which occurs readily to form ureas. Presumably the droplets in the traps are incapable of reacting with the components in the pentane and thus the yields are more reproducible.

IV. Conclusion

A concentration technique of general applicability for trace reactive pollutants in the atmosphere has been developed. The co-condensation method avoids the problem of reactivity with, or irreversible adsorption on, solid supports. Also, by use of the former technique, an enriched solution of the sample is obtained, which may be analyzed directly, thus avoiding the need for lengthy desorption times. Finally, the technique minimizes exposure of the sample to water which may be a problem in cases of those molecules (epoxides, isocyanates, etc.) known to be reactive towards this substance.

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ANALYSIS OF ORGANIC AMBIENT AEROSOLS

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Methods for the determination of organic compounds in atmospheric aerosols are described. The amount of carbon in organic compounds is determined from the difference between the total carbon content of the aerosol determined by the gamma ray analysis of light elements technique and the elemental carbon (soot) content determined by reflectance. The detailed composition of the organic species in the aerosol is determined by gas chromatography-mass spectrometry.

Key words: Air pollution; ambient aerosols; computer controlled gas chromatography/mass spectrometry; GRALE; organic analysis; reflectance.

I. Introduction

The measurement of the chemical composition of atmospheric aerosols (particles), especially fine particles with diameters less than 3.5 μm, is of great interest because they are associated with adverse health effects and visibility degradation. In a typical eastern U.S. city these particles were found to contain 18% carbon by weight expressed as methylene groups ($-CH_2-$) as is shown in Figure 1 [1]. The chemical composition of the carbonaceous particles is predominantly organic compounds and soot, a substance composed mainly of elemental carbon [2]. Although much effort has been expended to understand the sources and composition of other constituents of atmospheric fine particles such as sulfur compounds, much less is known about the nature of the carbonaceous aerosol. Yet the organic compounds are thought to be a major health hazard, and the soot is thought to be an important contributor to visibility degradation.

It is clear from previous work that hundreds of particulate organic compounds exist in the atmosphere [3] and their relative abundance may vary greatly with time and location. The types of compounds identified in atmospheric aerosols can be subdivided by their acidity as shown in Table 1.

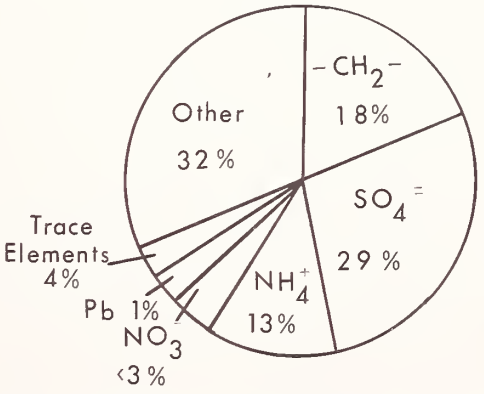

Figure 1. Fine particle mass balance in Charleston, West Virginia.

TABLE 1. *Organic compounds identified in atmospheric aerosols*

Acidity	Identified compounds
Neutral	Aliphatic–predominantly long chain hydrocarbon for C_{10} to C_{28}
	Aromatic–includes polycylic aromatic hydrocarbons (PAH)
	Oxygenated–aldehydes, ketones, epoxides, peroxides, quinones and lactones
Acidic	Long chain of fatty acids Dicarboxylic acids Phenols (nonvolatile)
Basic	Polynuclear azaheterocyclic hydrocarbon (aza arenes)

The relationship between organic atmospheric aerosols and health has not been definitely established. However, certain epidemiological evidence such as the two-fold increase in the incidence of lung cancer among city dwellers compared to rural residents has led to the suspicion that such a link exists [4]. The identification of polynuclear aromatic hydrocarbons (PAH) in urban aerosols, many of which have been established as carcinogens, has heightened the concern about the health effects of organic aerosols. In addition, some of the components of the basic fraction of organic aerosols, such as dibenz[a,j]acridines, dibenz[a,h]acridine and some alkylated benzacridines, have been shown to be carcinogens or co-carcinogens.

In the past few years several methods have been used to analyze the organic constituents of atmospheric particulate matter. The methods generally involve solvent extraction of organic material collected on a filter followed by various analytical techniques such as ultraviolet absorption [5], fluorescence spectrometry [6], liquid chromatography [7], gas chromatography [8] and mass spectrometry (MS) [9]. Other workers [10,11] have collected particles directly on a gas chromatography column for subsequent GC analysis. This method eliminates tedious Soxhlet extractions which are difficult to reproduce and are time-consuming. Gas chromatography combined with mass spectrometry (GC/MS) has recently been used very successfully in the study of airborne particles [2,11–14]. Qualitative and quantitative information on particulates has also been obtained by computer-controlled high resolution mass spectrometry [15].

In this paper two approaches are described to determine the organic compounds in ambient atmospheric fine particles. The amount of carbon in organic compounds is determined from the difference between the total carbon content of the aerosol and the amount of carbon present as soot (elemental carbon). The total carbon content is determined by the gamma-ray analysis for light elements technique (GRALE). GRALE is based on the determination of gamma rays emitted during proton emission [16]. The soot content is determined by the reflectance of light from the aerosol [2].

The detailed composition of the organic fraction is determined with a gas chromatograph-mass spectrometer computer system (GC/MS/com) after direct transfer of the organic components from glass fiber filters onto the GC column. Heating the glass fiber filters in a stream of heated helium after insertion into a modified GC inlet overcomes many of the problems associated with solvent extraction while minimizing the loss or decomposition due to handling methods. This technique utilizes the power of GC/MS analysis without the need for prior solvent extraction of the aerosol from the collection medium. Furthermore, this method allows the collection of atmospheric aerosols on the same type glass fiber filter used for analysis of other chemical species.

II. Experimental Procedures

A. Aerosol Sampling

Filter samples of atmospheric aerosols were collected on Pallflex E-70 type glass fiber filters with a detachable cellulose backing using a TWOMASS automated two-stage tape sampler [17]. Alternatively, other two-stage samplers such as the EPA dichotomous virtual impactor [18] could be used. Samples were collected on the six-story roof of the Washington University chemistry laboratory in July 1977 and February 1978.

B. Total Carbon Analysis by the GRALE Technique

Fine particle aerosol filter samples were analysed for carbon using the gamma-ray analysis of light elements (GRALE) technique [16]. This nondestructive technique is based on the measurement of gamma-ray emission induced by the proton bombardment of aerosol samples. The gamma-ray energy is, in general, unique to a particular nuclide and thus can be used as a signature for the chemical element. Elemental concentrations are obtained in units of $\mu g/cm^2$ and are not affected by the chemical form of the elements. Filter samples were irradiated with 7 MeV protons for 800 s in the external beam facility of the Washington University Sector Focused Cyclotron. Gamma-ray spectra are analysed immediately after each irradiation with an on-line computer. Details of the method are given by Macias et al. [16]

C. Aerosol Soot Analysis by Reflectance

The nondestructive quantitative analysis of soot is determined rapidly from the reflectance of the aerosol deposit. Particulate soot is composed of carbon with small amounts of other compounds absorbed on the surface. This technique assumes that soot is the predominant species which is dark in color in the fine particle aerosol and therefore the darkness of the deposit is due primarily to soot.

A reflectance photometer was used to analyse samples mounted on 5×5 cm slide mounts. Light from a power stabilized 12 V tungsten-filament map was collimated and focused onto the 0.3 cm^2 aerosol deposit with a 2.3-cm plano-convex lens. The reflected light 90° to the incident beam was focused with a similar lens onto the surface of an N/P silicon solar cell (Centerlab Semiconductor, Globe-Unions, Inc.). The output of the photodetector was measured with a digital voltmeter. The method has been extensively calibrated with pure elemental carbon standards and atmospheric aerosol standards as described in detail elsewhere [2].

D. Organic Aerosol Analysis by GC/MS

The heating chamber inlet used for the GC/MS analysis of organic compounds in atmospheric aerosols is shown in Figure 2. The filter sample is placed in the center of the heating tube. Helium carrier gas for the GC/MS is passed through the chamber and over the filter continuously. The inlet system consists of a 6.3 mm i.d. stainless steel tube with a 3.2 mm i.d. stainless steel tube welded on to it for carrier gas entry. A lavite insulator is slipped over the stainless steel tube and is threaded to accommodate a heating wire. The leads of the heating coil are attached to a variac. The heating conditions of the chamber can be varied by changing the voltage from the variac. The lower end of the chamber passes through a stainless steel block which fits into the injector block of the GC. The GC column fits directly into the bottom of the heating chamber.

The stainless steel chamber is heated to 450 °C in 3 minutes. The heating cycle has been adjusted to minimize pyrolysis of the volatile organic components while still permitting rapid transfer to the GC column. Initial experiments on a few long chain hydrocarbons indicate high transfer efficiency (80–90%) and minimal decomposition. The sample injector block temperature is

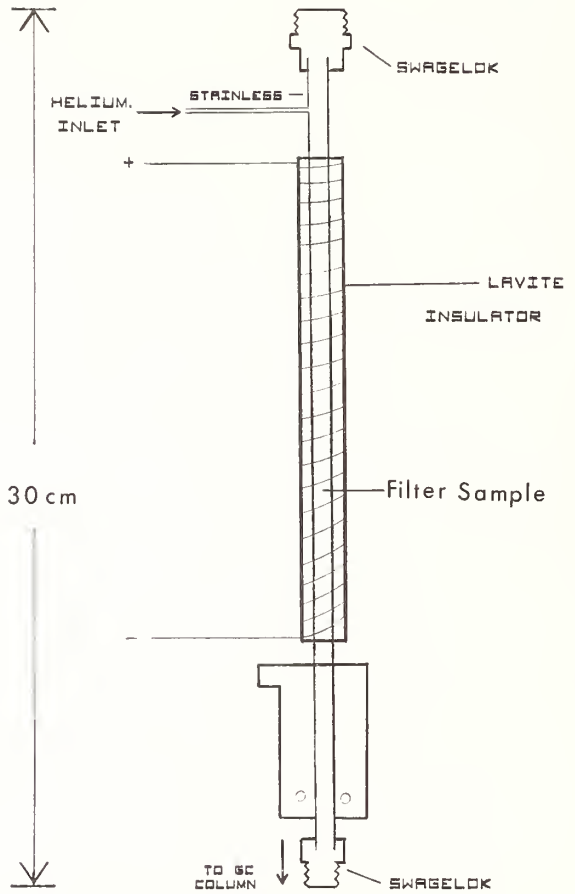

Figure 2. Schematic of GC/MS heated inlet system.

maintained at 300 °C. The GC column is 2 mm diameter glass, 3.6 m long, packed with 1% Dexsil 300. Helium carrier gas flows through the column at a rate of 30 mL/minute. The GC column oven is programmed from 40° to 300 °C at a rate of 10 °C/minute. Temperature programming begins at the same time the filter is heated. The final temperature is held for 20 minutes.

The transfer efficiency from a filter inserted in the modified inlet system versus direct injection into the GC/MS has been determined using 100 ng samples and found to be greater than 85% for the following PAH's: naphthalene, biphenyl, benzo quinoline, pyrene, anthraquinone, 2-3 benzfluorene, chrysene, benz[a]pyrene and perylene.

III. Results

Atmospheric fine particles were sampled during a 4-day air pollution episode in July 1977 in St. Louis. The total mass concentration, the total carbon concentration, and the soot concentration expressed as elemental carbon are plotted for each 3 h sample in Figure 3. The total organic carbon content of the aerosol can be determined from the difference between the total and elemental carbon concentrations. The organic carbon and elemental carbon concentrations are plotted as a function of time in Figure 4. During this sampling period the organic carbon

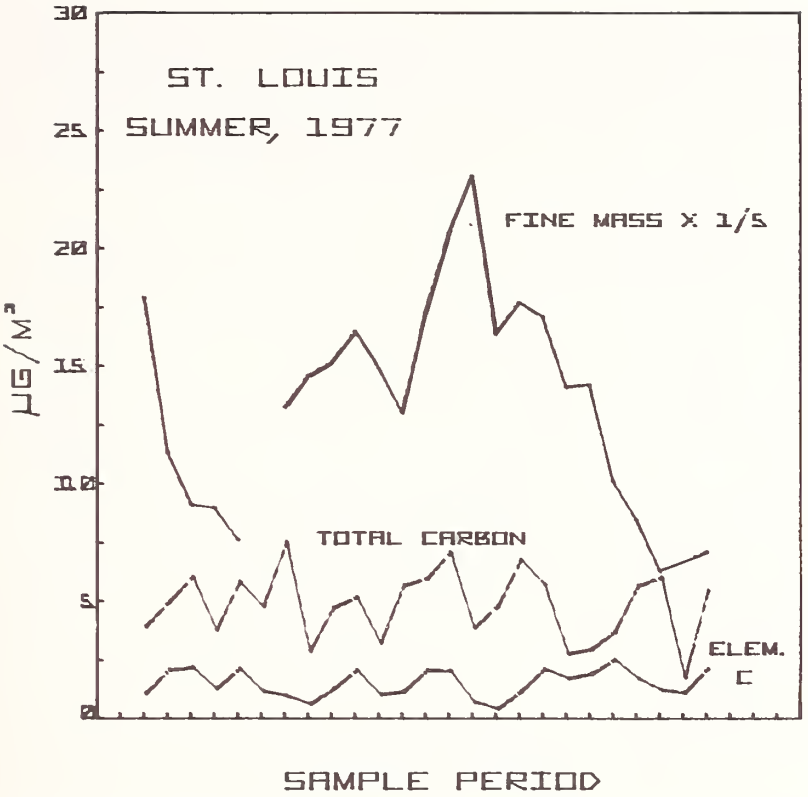

Figure 3. Atmospheric fine particle mass total carbon and elemental carbon (soot) concentration during a 4-day air pollution episode in St. Louis in July 1977.

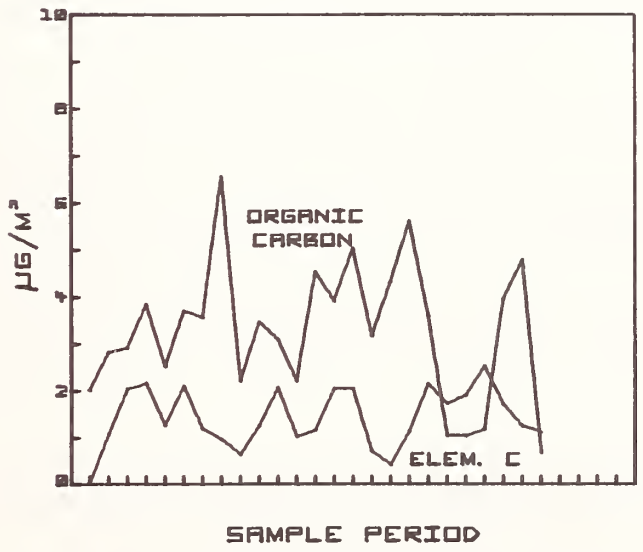

Figure 4. The organic and elemental carbon (soot) concentration plotted as a function of time during the July 1977 air pollution episode.

amounted to 65% of the total fine particle carbon. It can be seen in Figure 5 that the organic and soot carbon fractions do not correlate indicating that they come from different sources.

The GC/MS total ion current of an atmospheric aerosol sample collected for 3 hours in St. Louis is shown in Figure 6. The following compounds are identified in the figure: naphthalene, diphenyl, phenanthrene, benzpyrene, benzophenanthrene, chrysene, and benzfluorene. The

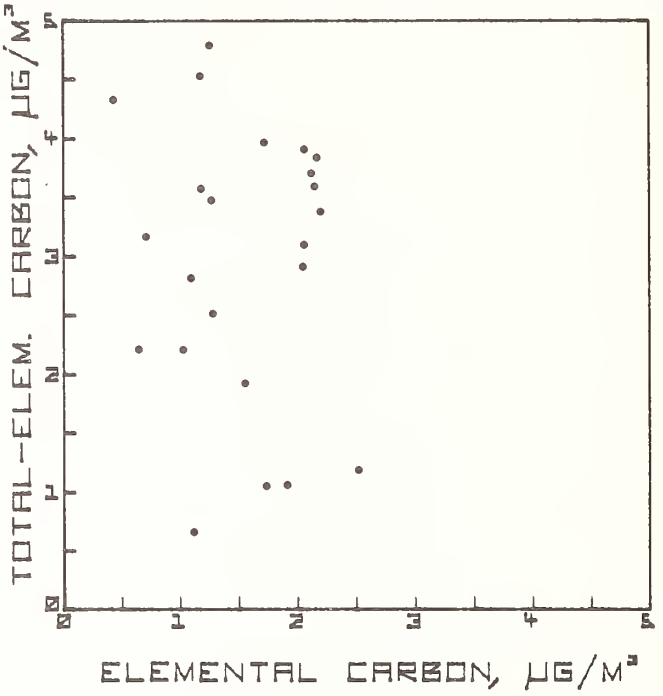

Figure 5. Cross correlation plot of organic and elemental carbon concentration during the July 1977 air pollution episode.

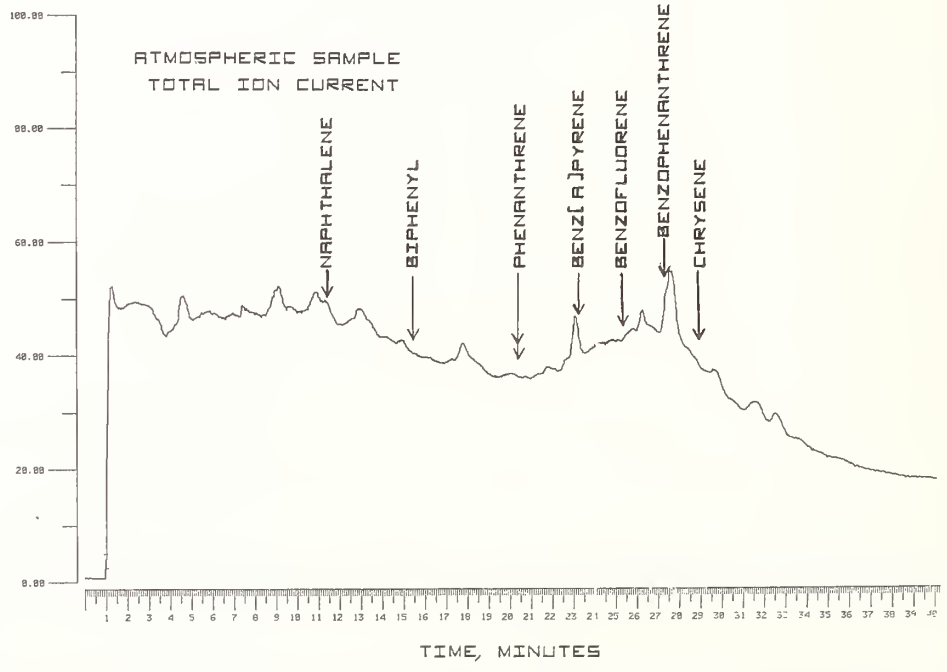

Figure 6. GC/MS total ion current for an atmospheric aerosol sample collected for 3 hours in St. Louis.

method obviously has sufficient sensitivity for measuring these PAH compounds even for short collection times. Examination of the concentration of these compounds with time indicates large temporal fluctuations. Therefore it seems feasible to use this method for aerosol source characterization using organic source fingerprint information.

IV. Acknowledgment

We wish to thank Dr. R. Delumyea and Mr. L. C. Chu for their help in various phases of this work. This work has been supported in part by the USEPA. The use of the NIH Mass Spectrometry Resource at Washington University is gratefully acknowledged.

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POLYNUCLEAR AROMATIC HYDROCARBONS ASSOCIATED WITH COAL COMBUSTION

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Samples of coal tar exudate, coke and stack fly ash from coal combustion were subjected to several extraction procedures, including a novel high-pressure liquid CO_2 extraction. Extracts were analyzed on wall coated open tubular (WCOT) glass capillary columns; selected extracts were also subjected to GC/MS analysis utilizing a glass capillary as the inlet to the mass spectrometer, and a number of polynuclear aromatic hydrocarbons were identified on the basis of computer-matched fragmentation patterns, supported by retention data.

Key words: Coal combustion products; glass capillary gas chromatography; high pressure extraction; polynuclear aromatic hydrocarbon.

I. Introduction

Polynuclear aromatic hydrocarbons (PAH) constitute one group of volatile components associated with coal combustion and coal tars. When fossil fuels are heated to combustion temperatures, the increased temperature would be expected to cause a more rapid release of these materials to the environment. Some of the PAH have been indicated as carcinogens in animals, and while carcinogenicity in humans has not been clearly demonstrated, there is evidence to indicate that a relationship may exist [1,2]. Hence there is great interest in the development of rapid, sensitive and accurate methods for measuring, qualitatively and quantitatively, PAH in environmental samples.

Some of the procedures that have been used for their analysis employ lengthy extractions, followed by fractionation processes such as liquid-solid chromatography to simplify the complex extraction mixtures. The individual aromatic compounds in these simpler fractions can then be identified and measured by ultraviolet spectrophotometry. These standard procedures are capable of yielding data that are quite accurate, but large amounts of sample are required and the methods are quite time consuming. Gas chromatographic techniques would seem to offer advantages, in that they can be applied to smaller samples and they require shorter analysis times, but the resolution of these complex samples containing a wide range of compounds embracing many groups of closely related isomers would require gas chromatographic systems of very high efficiency. The gas chromatographic separation of PAH is further complicated because they frequently occur in environmental samples at concentrations so low that their detection is seriously hampered; some type of sample pre-concentration is necessary. Some PAH's exhibit such low vapor pressures that relatively high temperature separation is necessary; furthermore some exhibit strong affinities for adsorptive surfaces necessitating the use of well-deactivated columns in highly inert analytical systems.

Glass capillary gas chromatography seems well fitted to these requirements. The investigator moves from a limit of 5,000 theoretical plates in the typical packed column to 100,000 or 200,000 *effective* theoretical plates in an open tubular glass capillary column; hence, as a first advantage, he has greatly increased his powers of resolution. Secondly, he also has the possibility of much shorter analysis times. Thirdly, although a smaller amount of any given compound is chromatographed, band broadening is very much less (i.e., sharper peaks). Consequently this smaller quantity emerges to the detector at a higher concentration per unit of time, with the result that the glass capillary column, properly installed, can exhibit about a 100-fold increase in sensitivity over the packed column. And fourth and finally, glass is a relatively inert surface, but even in glass there are degrees of inertness and this is of concern because several workers have commented on the fact that PAH are not highly stable. Losses result from delayed analysis, and rearrangements and degradations of some PAH under a variety of conditions have been demonstrated [3,4]. The different degrees of inertness displayed by different glass capillary columns depends, among other things, on the deactivation procedures employed in their manufacture and the method of installation in the chromatograph, and these will influence the reactivity toward sensitive compounds. Active sites in either the column or the system can lead to asymmetric or tailing peaks, rearrangements or peak subtraction.

II. Results and Discussion

Figure 1 shows a test chromatogram consisting of two injections on a well-deactivated SE 54 (1% vinyl, 5% phenyl, methyl silicone) column. Especially noteworthy is the complete absence of adsorptive tailing on the alcohol and nicotine peaks. It is perhaps also worth noting that nicotine can be a very effective test compound. Nicotine fails to negotiate some columns that give a neutral reaction with the classical 2,6-dimethyl aniline, 2,6-dimethyl phenol acidity test mixture. To obtain chromatograms with distinct, symmetrical, non-tailing peaks from mixtures of active compounds, it is necessary to start with a particularly well-deactivated column, to straighten the ends of the column, deactivate the straightened ends with polyethyleneglycol solution, and install the column with one straightened end in the high-velocity zone of the glass inlet splitter and the other straightened end in the high-velocity zone of the detector. A similar test mixture should then be injected to ensure that active sites generated during column installation have been deactivated.

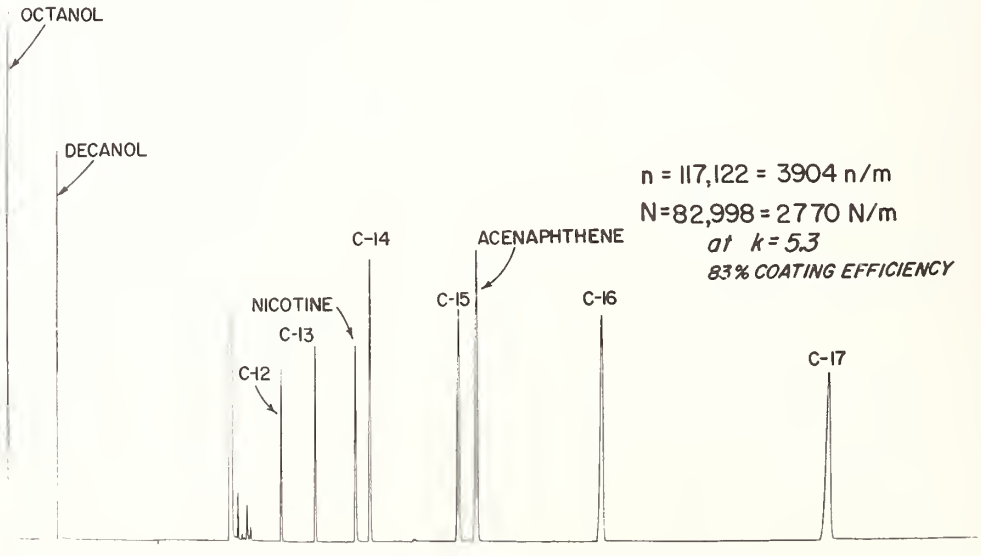

Figure 1. Test chromatograms on a well-deactivated column properly installed in an inert system. 30 m×0.25 mm J&W glass capillary column, coated with SE 54. Helium carrier gas at 21 cm/s, 150 °C isothermal.

Procedures for column straightening, deactivation and installation have been described in some detail [5].

Figure 2 shows a chromatogram of a test mixture of PAH on a 10 m×0.25 mm i.d. glass capillary column, and Figure 3 shows a chromatogram of a molten coal tar exudate collected from the oven door threshold at a metallurgical coke manufacturing facility. The exudate was placed in a Soxhlet extractor, extracted with methanol for 24 hours and the extract concentrated in a rotary evaporator. The identifications, which are consistent with their retentions, are based on computer-matched mass spectral fragmentation patterns when the mixture was analyzed in a Hewlett-Packard 5992 gas chromatograph mass spectrometer fitted with a glass capillary column. The computer was unable to match spectra from many of these peaks with the reference spectra in its library storage. No identities have been assigned to those peaks.

Figure 4 shows a chromatogram of a grab sample of coke from the stockpile of the oven, subjected to 24 hour Soxhlet extraction with 1:1 benzene-methanol, and concentrated in a rotary evaporator. Again, identifications were based on the computerized correlation of mass spectral fragmentation patterns obtained with the Hewlett-Packard 5992 GC/MS, using the retention behavior as confirming evidence.

Table 1 lists the retention characteristics of several PAH compounds. It is perhaps worth noting that on WCOT columns, retention indices are influenced not only by the liquid phase and the column temperature, but also by the deactivation treatment—if any—received by the column. Isothermal separations at temperatures 25 °C above and below the reported values were used to calculate the $\Delta I/10$ °C values [6]. With values of this magnitude, it is obvious that changes in the rate of temperature programming or in the length of the column will affect the relative positions of the individual PAH compounds. If the mixture also contains aliphatic hydrocarbons or compounds with other functional entities, the relative elution order may also be affected by these variables.

During the combustion of coal for electric power generation, fly ash particles composed largely of fused aluminosilicate are formed [7,8]. These enter the effluent stream together with the volatile constituents. Most of the particles are collected within the industrial plant, but a small percentage negotiate the electrostatic precipitators and are released through the stack [9]. These are the smaller particles and exhibit a relatively large surface area/weight ratio; they may well tend to adsorb and act as a carrier for the volatile emissions in that gaseous effluent including a number of polynuclear aromatic hydrocarbon compounds.

The fly ash used in this study was collected at 100 °C from the stack downstream from the electrostatic precipitator of a power plant burning low sulfur high ash coal. It was a fine respirable fraction, with a volume median diameter of 2.2 μm. It was first subjected to lengthy Soxhlet extraction with cyclohexane, followed by solvent partitioning using a procedure described by Liberti et al. [10]. Figure 5 shows the cyclohexane fraction, and Figure 6 the nitromethane fraction from the solvent partitioning.

Because some of these compounds are relatively labile, we are also interested in finding simpler, more rapid and less stringent methods for their isolation. Column chromatography has been used to fractionate fly ash extracts prior to analysis. This suggested our first approach, in which we treated the fly ash as a pre-loaded chromatographic support, placing it in a small chromatographic column where it was subjected to sequential elution with 50 mL portions of benzene, dichloromethane, and tetrahydrofuran, followed by 100 mL of methanol. Figure 7 shows chromatograms of the four eluates. The high degree of baseline rise with programming can be attributed to the fact that this column was lab-constructed from lime-soda glass. Methyl silicones coated on boro-silicate glass exhibit greater temperature stability, longer life and a lower degree of baseline rise with temperature programming.

In a separate study, we found need for low-temperature solvent-free extracts of certain materials. We achieved this with a pressure chamber permitting the use of CO_2 with a standard Soxhlet. Full description of this unit, on which a patent application is pending, will be published elsewhere; a diagram is shown in Figure 8. In practice the sample, ocean or sea sediments,

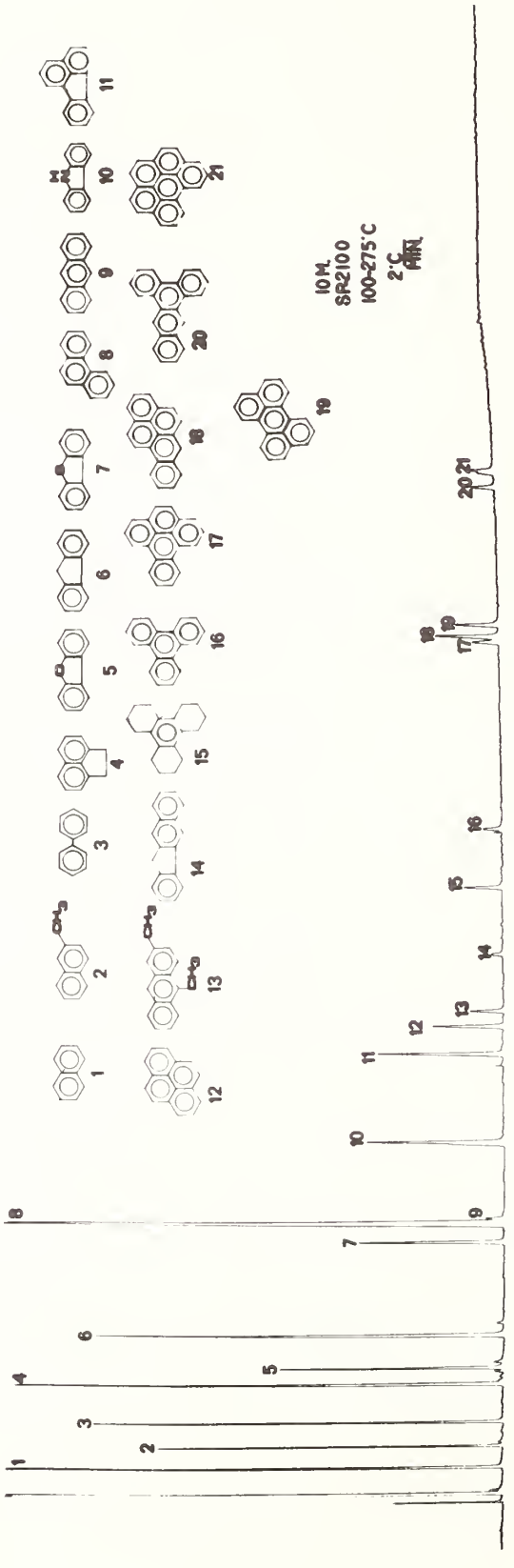

Figure 2. Separation of a mixture of PAH standards. 10 m×0.25 mm J&W glass capillary column coated with SP 2100 and programmed from 100° to 275 °C at 2 °C/min.

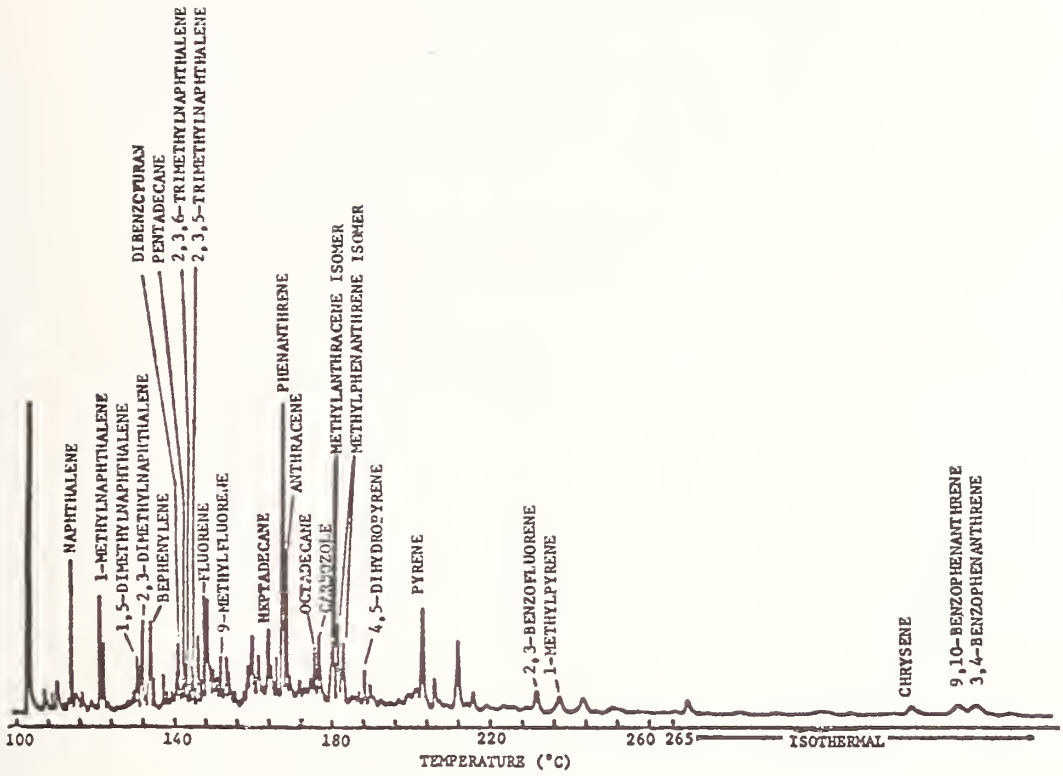

Figure 3. Chromatogram of a Soxhlet extract of a coal tar exuded from a coke oven. Column and conditions as shown in Figure 2.

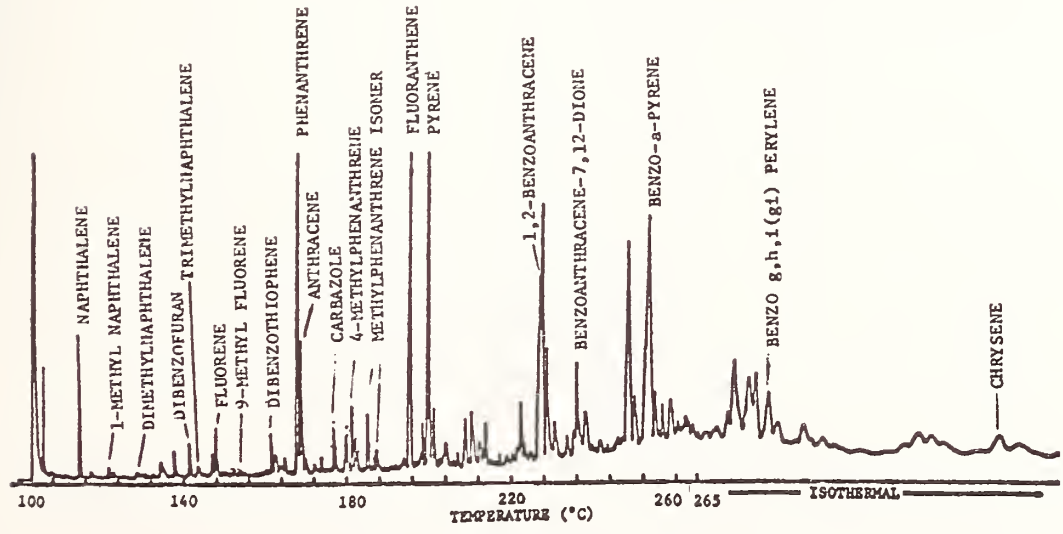

Figure 4. Chromatogram of a Soxhlet extract of a coke sample. Column and conditions as shown in Figure 2.

TABLE 1. *Retention characteristics of selected PAH on SP 2100*

Peak[a]	Compound	Programmed I[b]	Elution Temp, °C	I	ΔI/10°C[c]
1	naphthalene	1156	107	$1179_{125°}$	5.6
2	2-methylnaphthalene	1269	110	$1284_{125°}$	6.2
3	biphenyl	1349	115	$1352_{125°}$	5.8
4	acenaphthene	1438	122	$1466_{150°}$	11.4
5	dibenzofuran	1469	126	$1500_{150°}$	8.2
6	fluorene	1529	131	$1556_{150°}$	10.6
7	dibenzothiophene	1672	149	$1704_{150°}$	10.8
8	phenanthrene	1711	152	$1732_{150°}$	10.0
9	anthracene	1718	153	$1740_{150°}$	10.6
10	carbazole	1835	167	$1836_{175°}$	6.0
11	fluoranthene	1970	183	$1995_{175°}$	13.8
12	pyrene	2013	188	$2038_{175°}$	15.6
13	2,10-dimethyl anthracene	2038	191	$2104_{200°}$	13.2
14	2,3-benzofluorene	2130	202	$2184_{200°}$	15.4
15	dodecahydrotriphenylene	2245	214	$2280_{200°}$	15.6
16	triphenylene	2346	225	$2380_{200°}$	14.8
17	benzo-e-pyrene	2703	260	$2731_{225°}$	15.0
18	benzo-a-pyrene	2713	261	$2745_{225°}$	23.6
19	perylene	2737	263	$2829_{250°}$	26.4
20	1,2,3,4-dibenzoanthracene	3034	275[d]	$3087_{250°}$	24.4
21	benzo-g,h,i(g,i) perylene	3072	275[d]	$3121_{250°}$	33.4

[a] See Figure 2.

[b] Observed on a 10 m×0.25 mm J&W 2100, programmed from 100° to 275 °C at 2 °C/min and held; helium carrier gas at an average linear velocity of 28 cm/s.

[c] Obtained from isothermal data at 25 °C above and below reported value. $\Delta I/10\ °C = I_{\text{higher temp}} - I_{\text{lower temp}}$ (See reference 6).

[d] Elutes during 275 °C isothermal hold at end of program.

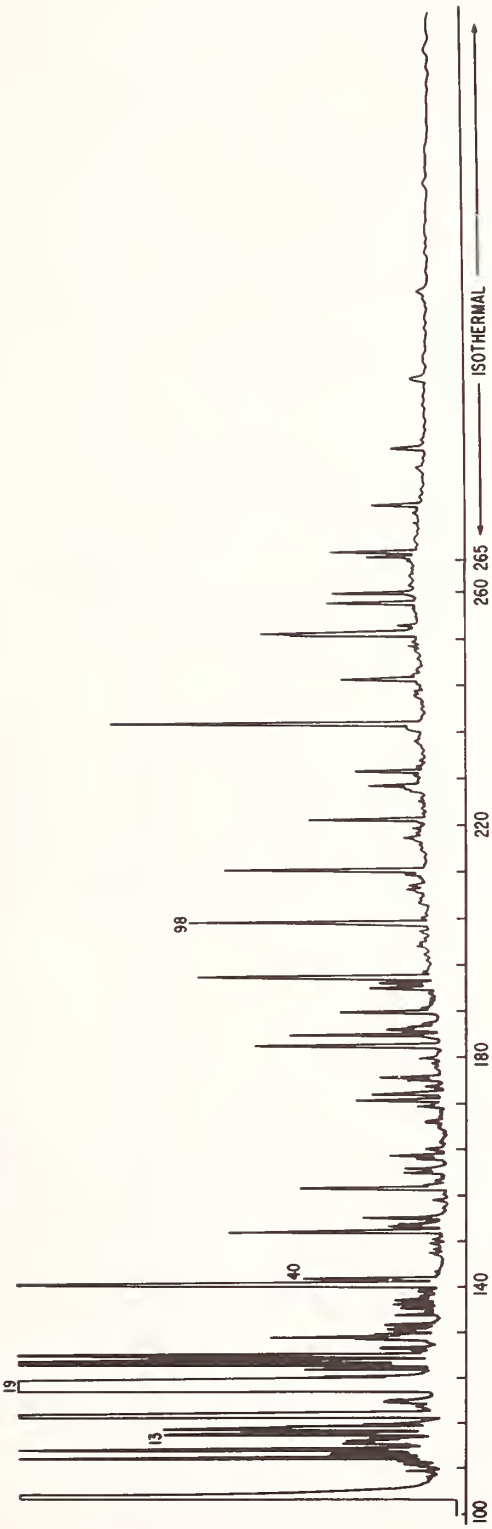

Figure 5. Chromatogram of the cyclohexane fraction (from cyclohexane-nitro methane partitioning) of a cyclohexane extract of coal fly ash. Column and conditions as shown in Figure 2.

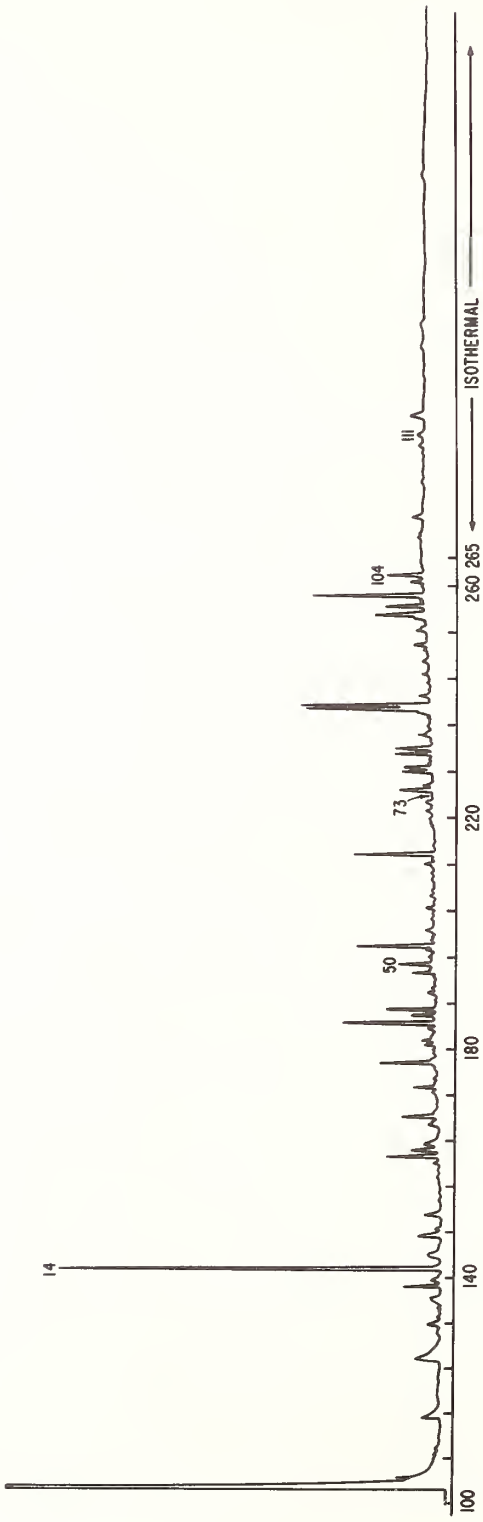

Figure 6. Chromatogram of the nitromethane fraction (from cyclohexane-nitro methane partitioning) of a cyclohexane extract of coal fly ash. Column and conditioning as shown in Figure 2.

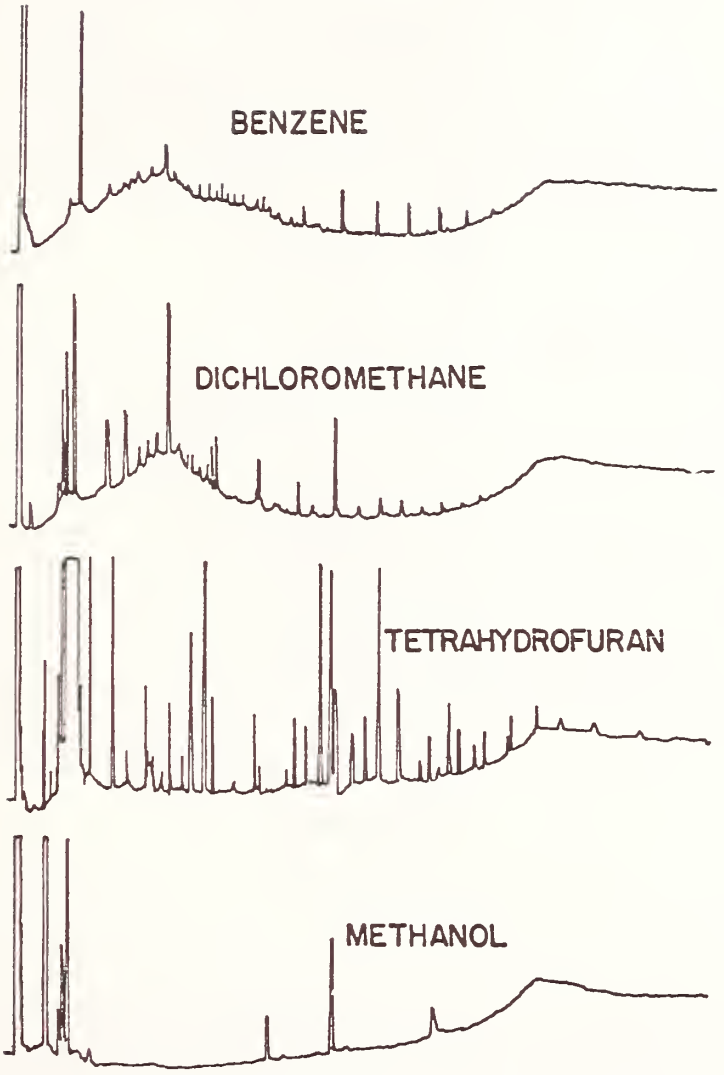

Figure 7. Chromatograms of sequential eluates from coal fly ash. Column and conditions as shown in Figure 2.

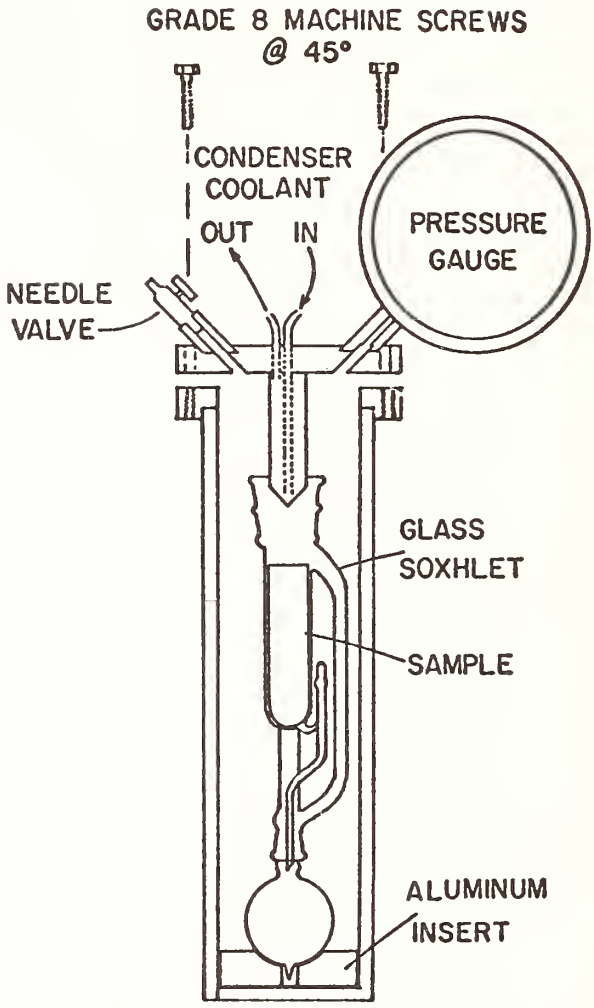

Figure 8. Diagram of a high-pressure chamber and condenser assembly, having a standard glass Soxhlet extractor. See text for details.

shellfish (which can be remarkably efficient bioconcentrators), activated carbon or resins that have been used to adsorb organics from vapor or water streams, porous polymers used to collect insect pheremones or other vapor entrained samples including polynuclear aromatic hydrocarbons in air, food materials for flavor analysis, or, in this case, fly ash, is placed in the extraction thimble. A predetermined quantity of dry ice is added outside the assembly. While the lid is being applied, a CO_2 blanket rises displacing air from the chamber. When the condenser is flooded with ice water, pure CO_2 drips into the extractor. At the end of the operation, the apparatus can be chilled to a point as low as –70 °C, where the vapor pressure of the extracted materials is extremely low. The needle valve is then opened very slightly, and an hour or two later the apparatus is opened to yield a solvent-free extract. As an interesting aside, when working with aroma assessment or biological assay, strips of filter paper can be placed in the flask, and are impregnated with the extractable substances. These can then be used in sniff testing by flavor panels or bioassay of insect pheremones without the interference of solvent odors.

Figure 9 shows a comparison of a 3 h standard Soxhlet extraction of fly ash using benzene, compared to the high pressure CO_2 extraction for the same period of time. While at first glance it would appear that benzene was a superior extractant, this is due to concentration differences in the two samples injected. On a dry-weight basis the benzene extracted *ca.* 1 mg of material from 1 g of

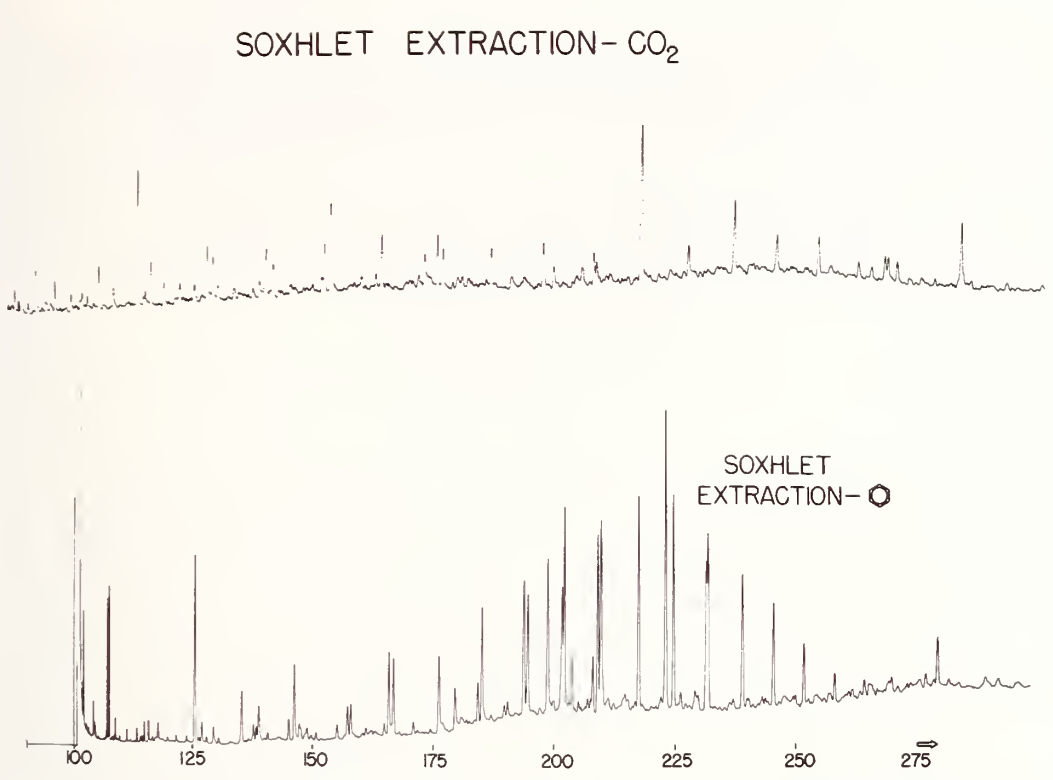

Figure 9. Chromatograms obtained from (top) high pressure Soxhlet extraction, using carbon dioxide as the extractant, and (bottom) standard Soxhlet extraction, using benzene as the extractant. See text for extraction details. Column and chromatographic conditions as detailed in Figure 2.

fly ash while the CO_2 extracted *ca.* 5 mg from 1 g of fly ash. There are also qualitative differences between the two. Whether these are due to differences in extraction efficiencies or to changes engendered during extraction has not yet been ascertained.

It should be emphasized that these are preliminary results of a continuing study. We don't yet know enough about the materials associated with fly ash, nor do we know what happens to them on exposure to atmospheric conditions including ambient chemicals and sunlight, or what their public health significance may be. We have concluded that further study is required to conclusively identify the organic components, and that part of these efforts should be concerned with finding milder methods of sample preparation and establishing more rapid and definitive methods of analysis.

III. Acknowledgments

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DIURNAL AND SPATIAL VARIATIONS OF ORGANIC AEROSOL CONSTITUENTS IN THE LOS ANGELES BASIN

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Two-hour and 14-hour hi-vol aerosol samples were collected simultaneously at Pasadena, Pomona and Riverside, California on 4 successive days in July 1975. Simultaneous ozone and visibility measurements were made for correlation with aerosol constituents. Samples were analyzed for primary (C_p), secondary (C_s), and elemental carbon (C_e) by selective solvent extraction-carbon analysis. In addition, high resolution mass spectroscopy was used to provide detailed analysis of organic constituents and to evaluate the selective extraction procedure. The composition of the carbonaceous material at the three sites was found to be similar with $C_s/C_p \geqslant 2$. The C_e, as estimated by insoluble carbon, was the most abundant carbon form. Adipic and glutaric acids were among the more abundant aerosol constituents of probable secondary origin. Elevated morning levels of C_s, dicarboxylic acids and acid nitrates as well as low morning Br/Pb ratios gave evidence of the retention of secondary organic aerosol from preceding days. Cyclic olefins appear to be the principal secondary organic aerosol precursor. Primary organics show evidence of a motor vehicle origin plus additional unidentified sources. Comparison between 2-hour and 14-hour samples reveals evidence of both loss of organics by volatilization and increased collection efficiency for organics with increased particle loading.

Key words: Diurnal variations; elemental carbon; mass spectroscopy; organic aerosol constituents; primary organic carbon; sampling errors; secondary organic carbon; solvent extraction; spatial variations.

I. Introduction

A previous paper [1] reviewed various techniques which have been applied to characterization of atmospheric carbonaceous particulate matter and described a new procedure for such characterization. The procedure described employed a combination of solvent extraction and carbon determinations. It was postulated that cyclohexane was a selective solvent for the extraction of "primary" particle phase organics (i.e., those injected into the atmosphere in the particle state), while total organics could be approximated as those solubilized by successive extraction with benzene and 1:2 v/v methanol-chloroform. Insoluble carbon was used to estimate the elemental carbon present. "Secondary" organics (i.e., those formed as a result of chemical reactions in the atmosphere) were determined by subtracting primary from total organics, all expressed as carbon. Insoluble carbon also includes carbonates, if present, as well as carbon in various polymeric forms (e.g., pollen, spores, rubber particles). Samples were analyzed for carbonates but not for carbon in rubber or viable particles. Thus the results cited for elemental carbon were upper-limit values.

The present paper reports on the validation of the selective extraction approach and its application to samples collected simultaneously at Pasadena, Pomona and Riverside, within California's South Coast Air Basin (SCAB) (Fig. 1). Results of analyses employing high resolution mass spectroscopy thermal analysis (MSTA) [2,3] on the same samples are also reported.

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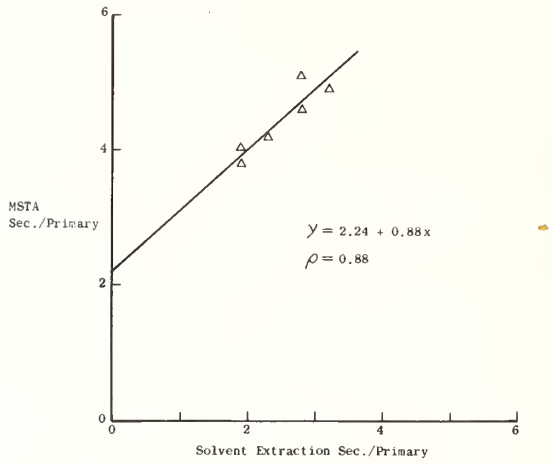

Figure 1. Correlation between organic analysis by selective solvent extraction and MSTA.

In addition to analyses for carbonaceous species, the samples were analyzed for lead and bromine. The ratio of these elements can be used to assess the age of an air mass when motor vehicle exhaust is the principal source of both elements [4]. Such information was employed in interpreting diurnal and spatial variations of carbonaceous materials. Ozone was monitored at each site to provide additional evaluation of the correlation between mean daytime ozone concentrations and secondary organics [1].

At each site two hi-vol filter samplers were used, one sampling for 14 hours and the second, for 7, 2-hour periods. This design permitted assessment of diurnal and spatial variations of primary and secondary organics and elemental carbon in the SCAB. A comparison of analyses made on short and long term samples provided measurement of sampling errors for carbonaceous materials. A more detailed account of this work is available elsewhere [5].

II. Results and Discussion

A. Validation of the Selective Solvent Extraction Technique

The extraction efficiency for atmospheric particulate carbon of the solvents used are compared in Table 1. Cyclohexane extracted significantly less carbon compared with the other solvent systems. To evaluate the hypothesis that cyclohexane is selective for the extraction of primary organics, results of the MSTA of cyclohexane extracts were compared with MSTA for direct analysis of the same filter samples without extraction (Table 2). MSTA provides information on individual compounds and in some cases, on classes of compounds. The sum of alkanes plus alkenes and alkylbenzenes was used as a model for primary organics while the dicarboxylic acids and difunctional nitrates plus nitrites were used as model secondary organics. Comparing the MSTA of cyclohexane extracts and filter samples, cyclohexane enhanced the recovery of alkanes

Table 1. *Mean extraction efficiency of solvents for carbonaceous material in atmospheric samples*

Solvent	% of total C in extract
Cyclohexane	17
Benzene	25
Benzene plus MeOH-$CHCl_3$	57

TABLE 2. *Comparison of cyclohexane soluble organics and direct analysis of filter samples by MSTA* ($\mu g/m^3$)

Episode	Site	Alkanes + alkenes		Alkylbenzenes		Hexanedioic acid		Pentanedioic acid		Organic nitrates + nitrites[a]	
		C_6H_{12}	filter	C_6H_{12}	filter	C_6H_{12}	filter	C_6H_{12}	filter	C_6H_{12}	filter
July 9	Pasadena	1.6	0.76	0.13	0.38	1.0	1.4	0.0	1.6	0.16	0.39
July 9	Pomona	1.3	0.76	0.16	0.41	0.11	1.6	0.05	1.4	0.047	0.22
July 9	Riverside	0.89	0.37	0.13	0.15	0.47	0.88	0.15	0.84	---	0.17
July 10	Pasadena	1.2	0.63	0.16	0.38	1.0	1.8	--	1.3	---	0.17
July 10	Pomona	1.4	0.68	0.09	0.32	0.29	1.1	--	1.4	0.022	0.090
July 10	Riverside	0.60	0.32	0.16	0.20	0.18	1.0	--	0.96	---	0.032

$$\text{Mean Ratio} \quad \frac{\text{Total secondary organics}^b}{\text{Total primary organics}^c} = 3.4 \text{ (filters); } 0.56 \text{ (cyclohexane extract)}$$

[a] The sum of acid nitrates and nitrites and aldehyde nitrates and nitrites.
[b] Hexanedioic acid + pentanedioic acid + organic nitrates and nitrites.
[c] Alkanes + alkenes + alkylbenzenes.

plus alkenes relative to direct filter analysis. Alkylbenzenes recovered in cyclohexane were about half of the level on the filters but their concentration was substantially lower relative to alkanes plus alkenes. On the average cyclohexane extracted about 40% of the hexanedioic acid and a small fraction of the pentanedioic acid and difunctional nitrates and nitrites. The ratio, total model secondary organic indicators/total model primary organics for the filters was about 6 times higher than in the cyclohexane extracts.

Additional indications of the selectivity of cyclohexane is seen in Figure 1 which plots the ratio of secondary to primary organics by solvent extraction against the ratio of total model secondary organics to total model primary organics by MSTA of 14-hour samples. A high correlation coefficient ($\rho = 0.88$) is observed. The positive intercept probably reflects the omission of important classes of primary organics (e.g., phthalates) from the set chosen as models. We conclude that while cyclohexane is not perfectly selective for primary organic aerosol constituents, it provides a useful upper limit to the primary organics.

B. Composition of 14-Hour Samples

Table 3 lists the results obtained by the solvent extraction-carbon analysis technique for 14-hour samples. Insoluble carbon, as a measure of elemental carbon, was the largest C constituent averaging somewhat more than 40% of the total C. Secondary organic carbon was 2–3 times more abundant than primary. In Riverside, which can receive pollutants formed during transport from locations to the west, there were somewhat more secondary organics in two of the four trials. In three of the four sampling days, the abundance of primary organic carbon was slightly less at Riverside although the differences are relatively small. In all cases the proportion of secondary organic carbon was somewhat lower at Pomona compared with the other sites.

Table 4 shows MSTA results for 14-hour filter samples for the two days of highest ozone levels. Results are shown both in $\mu g/m^3$ and relative to the concentration of total alkanes plus alkenes in the same sample. Dicarboxylic acids are seen to be more than twice as abundant as the model primary organics. The relative concentration of secondary organics at Riverside is higher than at the other sites on both days. Similar to the solvent extraction-carbon analysis results, the proportion of secondary organics was least at Pomona on both days.

TABLE 3. *Composition of 14-hour carbonaceous samples by solvent extraction-carbon analysis*

(% of total carbon)

Episode	Site	Sol. C	Primary C	Secondary C	Elemental C
July 9	Pasadena	58.2	17.5	40.7	41.8
	Pomona	54.1	18.5	35.6	45.9
	Riverside	58.5	15.3	43.2	41.5
July 10	Pasadena	57.8	13.7	44.1	42.2
	Pomona	48.5	16.8	31.7	51.5
	Riverside	58.5	15.3	43.2	41.5
July 11	Pasadena	67.7	19.9	47.8	32.4
	Pomona	58.6	20.8	37.8	41.4
	Riverside	57.6	15.4	42.2	42.4
July 12	Pasadena	55.8	20.4	35.4	44.2
	Pomona	50.1	16.5	33.5	50.0
	Riverside	42.8	14.1	38.6	47.2
	Mean	56.5	17.0	39.5	43.5

TABLE 4. *Analysis of 14-hour filter samples by MSTA*[a]

		Total alkanes + alkenes		Alkylbenzenes		Hexanedioic acid		Pentanedioic acid		Organic nitrites + nitrites		Σ secondary[c]	
Episode	Site[b]	μg/m^3	Relative	μg/m^3	Relative	μg/m^3	Relative	μg/m^3	Relative	μg/m^3	Relative	μg/m^3	Relative
July 9	Pa	0.68	1.0	0.13	0.19	1.4	2.1	1.6	2.4	0.39	0.6	3.4	5.0
July 9	Po	0.67	1.0	0.16	0.24	1.6	2.4	1.4	2.1	0.22	0.3	3.2	4.8
July 9	Riv	0.28	1.0	0.13	0.46	0.88	3.1	0.84	3.0	0.17	0.6	1.9	6.8
July 10	Pa	0.52	1.0	0.16	0.31	1.8	3.5	1.3	2.5	0.17	0.3	3.3	6.3
July 10	Po	0.59	1.0	0.09	0.15	1.1	1.9	1.4	2.4	0.090	0.15	2.6	4.4
July 10	Riv	0.23	1.0	0.16	0.70	1.0	4.4	0.96	4.2	0.032	0.14	2.0	8.7

[a] Concentration given in μg/m^3 and relative to the concentrations of total alkanes + alkenes in the same sample.
[b] Pa = Pasadena; Po = Pomona; Riv = Riverside.
[c] The sum of hexanedioic, pentanedioic acids and organic nitrates + nitrites.

C. Diurnal Variations of Aerosol Constituents

The diurnal variations of carbonaceous materials on July 9 and 10, 1975, obtained by the solvent extraction-carbon analysis and MSTA techniques (on filter samples), are shown for Pomona in Figures 2 and 3. July 9th was the first day of a relatively polluted episode with ozone maxima up to 0.38 ppm. On July 9 the peak in secondary organics by solvent extraction followed that for O_3 while primary organic carbon showed two weak maxima and elemental carbon peaked earlier in the day. MSTA showed a similar pattern, two weak maxima for total model primary and a single afternoon maxima for total model secondary organics. The lead concentration peaked in early

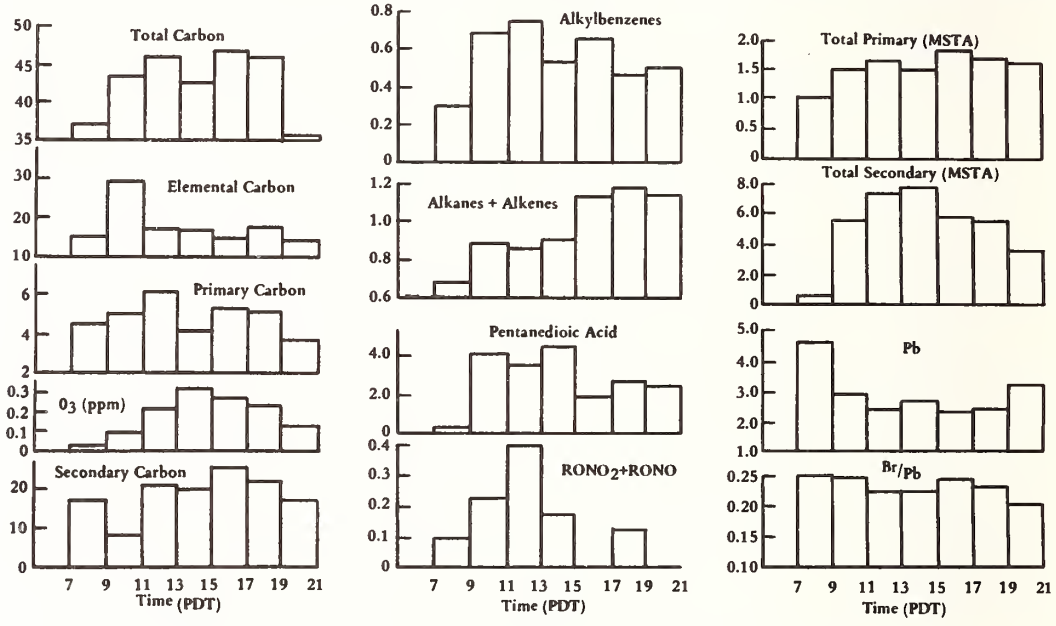

Figure 2. Diurnal variations for aerosol constituents, Pomona, July 9, 1975.

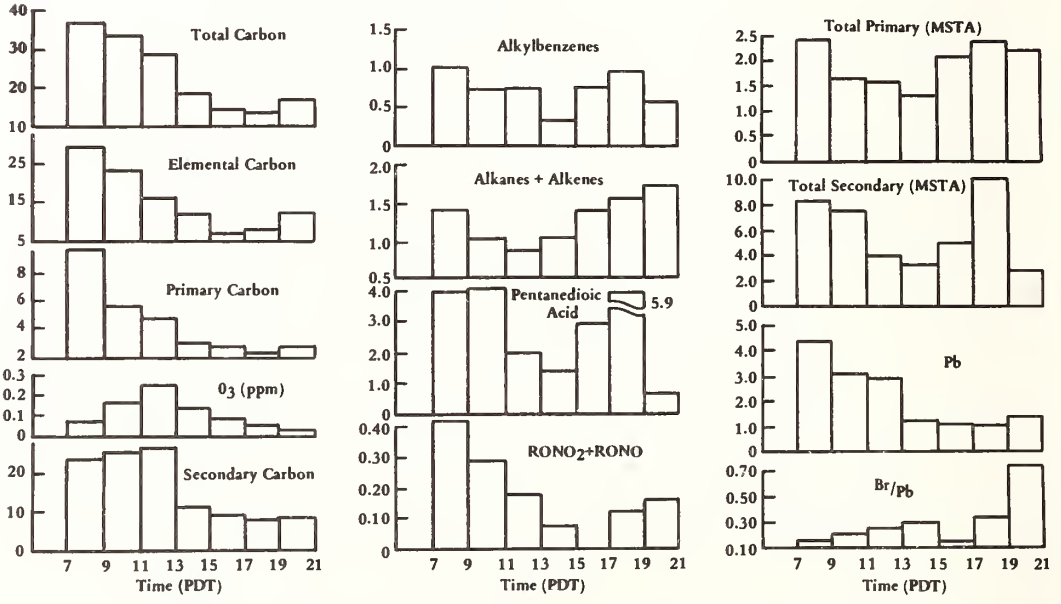

Figure 3. Diurnal variations for aerosol constituents, Pomona, July 10, 1975.

morning, consistent with the early morning traffic peak, low windspeed and the expected low mixing height. The Br/Pb ratio was fairly constant throughout the day which contrasts greatly with results for the following day. On July 10th, both primary and secondary organic particulate levels were greatly elevated in the early morning. While the morning Pb level was high, the low morning Br/Pb ratio suggests that relatively aged aerosol was being sampled. The high Br/Pb ratio observed during the evening, 0.7, reflects either non-automotive sources of Br or analytical error in this sample. Results at Pomona are similar to those observed in Pasadena and Riverside on these two days.

D. SAMPLING ERRORS

Table 5 compares the 14-hour sample results with those calculated from the corresponding 7, successive, 2-hour samples for total carbon (C_t), elemental carbon (C_e), cyclohexane (CEC), benzene (BEC) and methanol-chloroform (MCC) soluble carbon. The calculated 14-hour average values for C_t consistently exceeds those observed at all sites. Of the fractions contributing to the

TABLE 5. *Comparison of observed and calculated 14-hour values[a] Calculated/observed 14-hour analyses[b,c]*

Episode	Site	C_T	CEC	BEC	MCC[d]	C_e
July 9	Pasadena	1.11	.73	.52	1.60	1.11
		(.04)	(.13)	(.07)	(.18)	(.15)
July 9	Pomona	1.21	.75	.60	1.98	1.15
		(.04)	(.13)	(.08)	(.22)	(.13)
July 9	Riverside	1.18	.63	.53	1.52	1.29
		(.04)	(.11)	(.07)	(.17)	(.16)
July 10	Pasadena	1.12	1.02	.59	1.59	1.08
		(.04)	(.18)	(.08)	(.18)	(.15)
July 10	Pomona	1.10	.79	.67	1.85	.92
		(.04)	(.15)	(.09)	(.21)	(.11)
July 10	Riverside	1.26	.66	.68	1.65	1.21
		(.05)	(.13)	(.09)	(.18)	(.15)
July 11	Pasadena	1.28	.64	.54	1.45	1.78
		(.05)	(.13)	(.07)	(.16)	(.29)
July 11	Pomona	1.35	.77	.87	1.72	1.34
		(.05)	(.14)	(.11)	(.19)	(.19)
July 11	Riverside	1.16	.60	.59	1.58	1.15
		(.04)	(.12)	(.08)	(.18)	(.16)
July 12	Pasadena	1.34	.54	.65	1.68	1.46
		(.06)	(.12)	(.09)	(.19)	(.19)
July 12	Pomona	1.09	.67	.59	1.80	.93
		(.04)	(.13)	(.08)	(.20)	(.12)
July 12	Riverside	1.34	1.12	.58	1.94	1.36
		(.05)	(.15)	(.08)	(.22)	(.15)
	Mean Ratio	1.21	.74	.62	1.70	1.23

[a] Calculated from 7, successive 2-hour samples collected simultaneously with the 14-hour sample.
[b] C_T=total carbon; CEC=cyclohexane soluble C; BEC=benzene soluble C; MCC=methanol-chloroform soluble C; C_e=elemental C estimated by insoluble carbon, C_t−(BEC+MCC)
[c] One sigma value shown below each ratio.
[d] Following extraction for BEC.

total carbon, MCC and C_e showed similar ratios. Ratios $>1.C$ for carbonaceous materials may reflect the loss of more volatile constituents not strongly adsorbed on other materials during the prolonged (14-hour) sampling, consistent with Della Fiorentina's observations [6,7]. This hypothesis can serve to rationalize the high ratio for MCC (1.7). However, a ratio of 1.2 for C_e cannot be explained by volatilization and may be indicative of other sources of error. Thus, only ratios >1.2, (e.g., 1.7 for MCC) are here considered to be indicative of losses of organics due to volatilization.

Ratios <1.0 may reflect both sampling and analytical errors. If gas phase organics are adsorbed on previously collected, non-volatile materials (e.g., soot) the efficiency of such collection of gas phase organics should increase with increased particulate loading. This would result in greater levels of carbon from this source on 14-hour samples than calculated from the 2-hour samples. Since the atmospheric concentration of hydrocarbons in the gas phase appears to be substantially greater than that of polar organic materials, the collection of gas phase organics by adsorption on particulate matter would be expected to enhance the BEC and CEC fractions. Possible sources of analytical errors leading to ratios <1.0 were considered as part of quality assurance studies reported elsewhere [5]. The results suggest these errors to be of minor importance.

III. Conclusions

We conclude that the solvent extraction-carbon analysis approach for estimating primary and secondary organics is a useful technique and correlates reasonably well with results by mass spectroscopy. For four days in July 1975 in California's South Coast Air Basin the carbonaceous fractions in order of abundance were elemental C > secondary organic C > primary organic C. The extent to which polymeric forms of carbon (e.g., in spores, pollen, tire dust) contributed to the estimate for elemental carbon remains unclear. The period July 9-10, 1975, represented a stagnation episode during which aerosols were retained in the SCAB from one day to the next. The indicators of such aerosol retention are elevated concentrations of secondary organics preceding the diurnal ozone peak and low Br/Pb ratios for early morning aerosol samples. Because of the possible retention of aerosols from one day to the next, pollutant transport and/or dilution may be dominant factors in determining concentrations of secondary organics rather than degree of conversion of precursors. Accordingly, high correlations between concentrations of secondary organic materials and indicators of smog intensity (e.g., ozone) may not be observed. Finally, hi-vol sampling for particulate organics is subject to both positive and negative errors. Improved sampling procedures are needed to obtain measures of ambient carbonaceous particulate matter which are less subject to error.

IV. Acknowledgments

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TRACE ANALYSIS IN RESPIRATORY CARCINOGENESIS

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Carcinogenic polynuclear and N-heterocyclic aromatic hydrocarbons are found in urban air in traces of 10^{-9} to 10^{-8} g/m^3. Their quantitative determination is made by using ^{14}C-labelled internal standards in distributions between solvent pairs, followed by GLC and/or HPLC separations and identification by UV and MS. Volatile carcinogens in tobacco smoke include hydrazines ($2–4\times10^{-8}$ g/cig), vinyl chloride (0.6 to 2×10^{-8} g/cig), certain nitroalkanes (10^{-7} to 10^{-6} g/cig) and N-nitrosamines (10^{-9} to 10^{-8} g/cig). Development and application of specific analytical methods for the quantitative analyses will be presented. The highly reactive hydrazines are derivatized in the smoke trap with pentafluorobenzaldehyde; vinyl chloride is derivatized by bromination. The derivatives of the trace compounds, as well as the nitroalkanes, are enriched by chromatography and analyzed by GLC with a ^{63}Ni-ECD. Volatile and nonvolatile N-nitrosamines are determined by GLC with a thermal energy analyzer (TEA) or by HPLC/TEA, respectively (detection limit 10^{-10} g).

During cigarette smoking significantly greater quantities of carcinogenic N-nitrosamines are formed in-between puffs, than are produced in the mainstream during puffing. Therefore, it was suspected that smoke pollnted indoor environments would reflect these contaminants. Analyses of various indoor atmospheres revealed concentrations up to 2×10^{-7} g/m^3 of N-nitrosamines depending on the degree of tobacco smoke pollution. A portable collection device and analytical methods for the determination of these other indoor pollutants will be described.

Key words: Aza-arenes; hydrazines; nitroalkanes; nitrosamines; sidestream smoke; thermal energy analyzer; tobacco smoke; vinyl chloride.

I. Introduction

During the last 40 years a sharp increase in the incidence of cancer of the respiratory tract occurred in developed countries (Fig. 1) [1,2]. Epidemiological studies have implicated three factors in the overall increase of lung cancer: tobacco smoking, especially cigarette smoking, urban pollution and, to a minor extent, the emergence of new industrial environments [3–5]. Human data provided the main impetus for the bioassays and chemical analytical studies in tobacco and air pollution carcinogenesis and occupational cancer of the respiratory tract [6–8].

II. Tobacco Smoke

The main single factor for the increased lung cancer rate is cigarette smoking [3–5]. Bioassays have clearly shown that the total smoke aerosol and especially its particulate matter are carcinogenic to the experimental animal, thus supporting the human findings [6]. Fractionation experiments together with bioassays have demonstrated that certain polynuclear aromatic hydrocarbons serve as the major tumor initiators and that certain neutral and weakly acidic components are cocarcinogens [9]. These compounds by themselves, however, cannot account for the total carcinogenic activity of cigarette smoke; thus other carcinogens must be present.

In the case of the gas phase of cigarette smoke, studies have shown that fractionation experiments lead to artifacts. It was thus the task of the analytical chemist to examine the smoke for those volatile carcinogens, which, on the basis of theoretical considerations, might be present

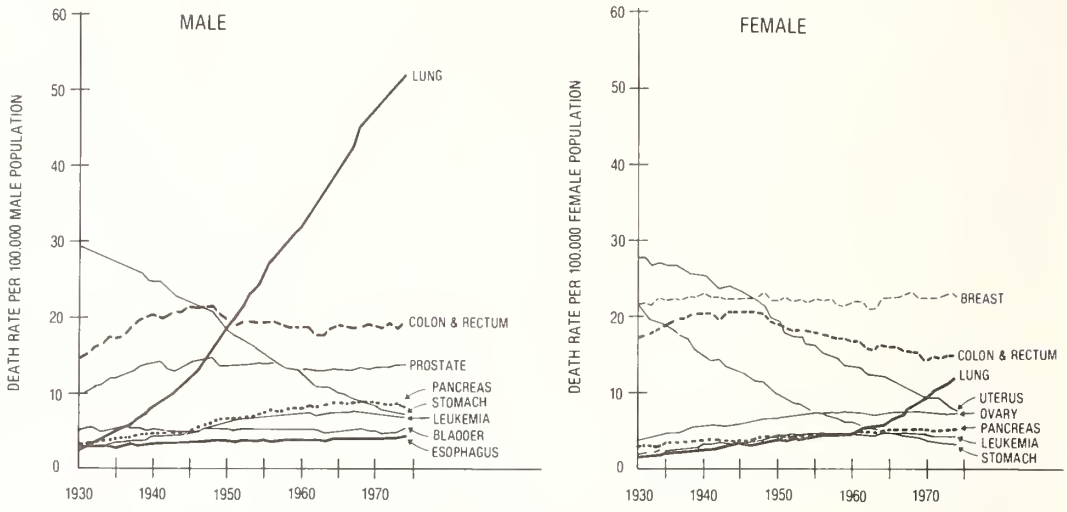


Figure 1. Age-adjusted cancer death rates for selected sites, United States.

TABLE 1. *Suspected volatile carcinogens in tobacco smoke*

$CH_2=CHCl$	VINYL CHLORIDE
$R_{R'}\!>\!N\text{-}NH_2$	HYDRAZINES
$CH_3\text{-}CH(NO_2)\text{-}CH_3$	2-NITROPROPANE
$NH_2\text{-}CO\text{-}OR$	URETHANES
$R_{R'}\!>\!N\text{-}NO$	N-NITROSAMINES

in the smoke. These were primarily chlorinated hydrocarbons, especially vinyl chloride, nitrohydrocarbons, hydrazines, urethanes and N-nitrosamines (Table 1).

III. Vinyl Chloride (VC)

The relatively high reactivity of VC and the low concentration of chloride in tobacco made it likely that VC could be present only in minute quantities (<100 ng/cigarette) in fresh smoke and that special precautions and a highly sensitive detection technique were required. For the actual analysis, we smoked 30 cigarettes through a glass fiber filter and subsequently through an activated charcoal column [10]. After smoking, the charcoal was brominated and the resulting dibromo-VC was enriched by chromatography. In the final step, VC was determined by GLC with a ^{63}Nickel-EC-detector. The detection limit was 15 pg (15×10^{-12} g). The final GLC (Fig. 2) served also for the chemical characterization by mass spectrometry. We found that the amount of VC in the smoke is dependent on the concentration of chloride in the tobacco and that commercial

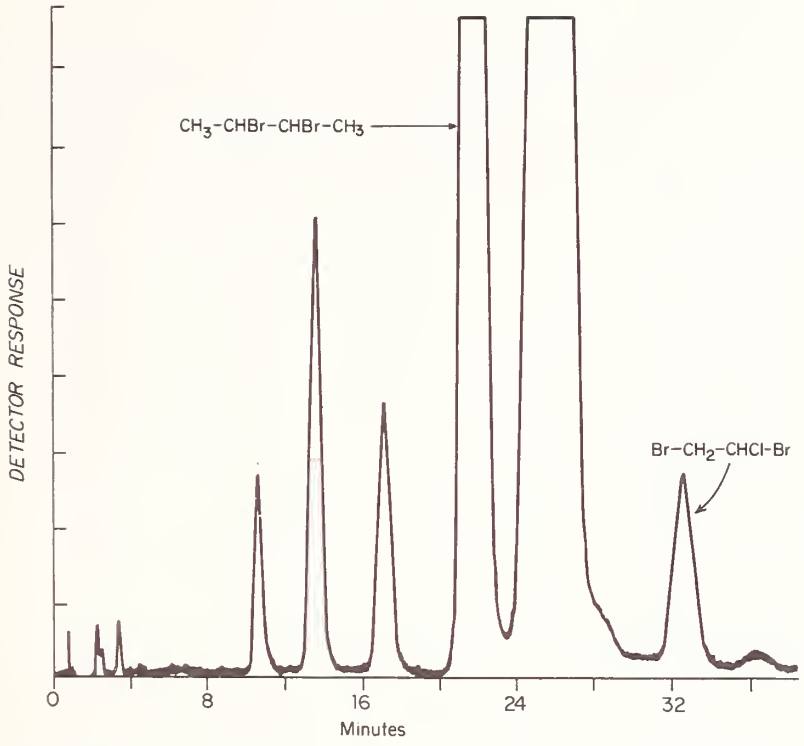


Figure 2. Gas chromatogram of a concentrate of vinyl chloride as 1,2-dibromol-chloroethane from cigarette smoke.

cigarettes contained between 1–30 ng of VC (Table 2) or maximally 30 ppb. Based on human data and on results from animal studies we concluded that these minute amounts of VC do not contribute to the carcinogenicity of tobacco smoke to any measurable degree.

IV. Hydrazines

Another type of carcinogenic volatiles present in trace amounts in tobacco smoke are the hydrazines. As has been discussed at this conference earlier by I. Schmeltz, the hydrazines also had to be trapped directly from the fresh smoke in order to avoid reaction with other smoke constituents [11].

V. Nitrohydrocarbons

Some years ago, certain secondary nitrohydrocarbons were reported to be carcinogenic to the experimental arimal [12,13]. This fact and the observation that thermally activated nitrogen oxides generated from nitrates in the tobacco serve as scavengers of C,H-radicals with the resulting formation of nitroalkanes [14] made it desirable to develop a quantitative method for this class of volatile compounds. For the analysis the smoke is directed through a glass fiber filter which traps nitroalkanes. From the steam distillate of the particulates the primary and secondary nitroalkanes are enriched by extraction from the weakly acidic portion (Fig. 3). They are subsequently analyzed by GLC with an ECD and characterized by mass spectrometry (Fig. 4) [15]. The secondary nitroalkanes with carcinogenic potential were identified as 2-nitropropane and 2-nitro-n-butane. As expected, the smoke yield of nitroalkanes is dependent on the nitrate content of the tobacco (Table 3). Based on these analytical data we have now initiated mutagenicity assays with Ames tester strains and bioassays in small mammals in order to evaluate the possible contribution of secondary nitroalkanes to the carcinogenic potential of cigarette smoke.

TABLE 2. *Vinyl chloride in cigarette smoke*

PRODUCT*		LENGTH (mm)	BUTT LENGTH (mm)	VINYL CHLORIDE (ng/cig.)
Reference cigarette IRI		85	23	12.4
Commercial cigarettes				
Cigarette A	F	85	23	5.6
Cigarette B	F	85	23	14.1
Cigarette C	F	85	23	11.4
Cigarette D	NF	70	23	11.9
Cigarette E	NF	70	23	15.8
Cigarette G	F	85	23	10.9
Cigarette H	NF	85	23	12.2
Little cigars				
Little cigar A	F	85	23	14.4
Little cigar B	F	85	23	27.3
Charcoal filter cigarettes**				
Cigarette A with F		85	35	5.1
Cigarette A with F cut off		60	10	15.3
Cigarette B with F		85	35	1.3
Cigarette B with F cut off		60	10	4.0
Marijuana Cigarette		85	23	5.4

*F: Filter; NF: non-filter

** Cigarette column smoked: 50-mm

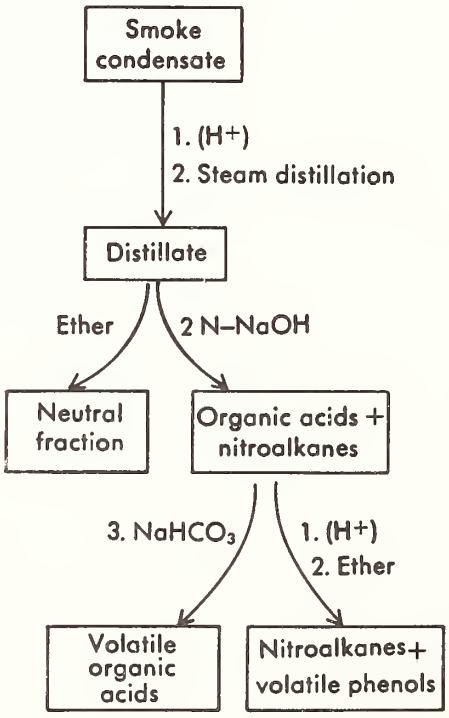


Figure 3. Analytical scheme for the analysis of nitroalkanes in cigarette smoke.

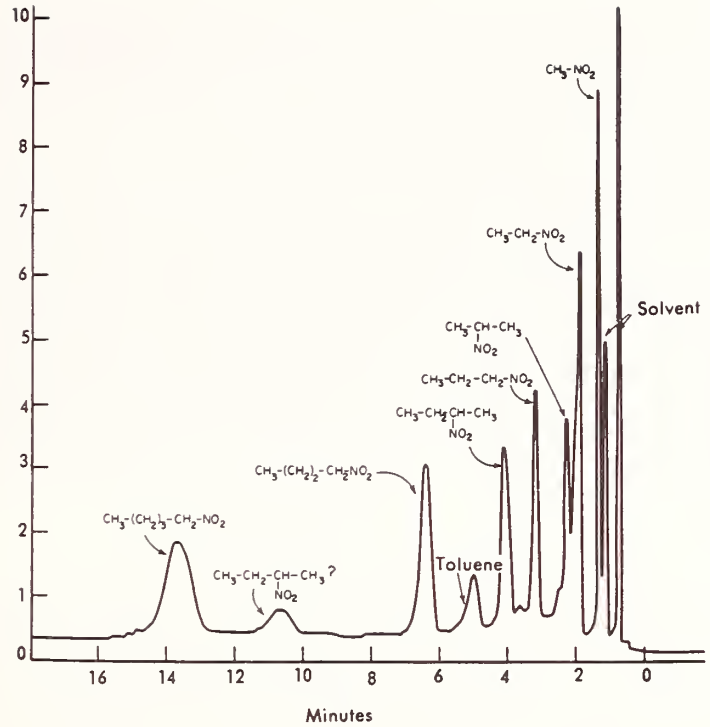


Figure 4. Gas chromatogram of a concentrate of nitro-compounds from cigarette smoke.

TABLE 3. *Six nitroalkanes in the smoke of various cigarettes (μg/cig)**

Cigarette	% NO_3	Nitromethane	Nitro-ethane	2-Nitropropane	1-Nitropropane	1-Nitro-n-butane	1-Nitro-n-pentane
Virginia	0.24	0.186	0.270	0.220	0.183	0.189	trace**
Blend A	0.35	0.523	1.08	1.08	0.728	0.713	0.215
Blend B	0.5	0.596	1.07	1.19	0.703	0.773	0.239
Burley I	2.15	0.709	1.48	1.43	1.10	0.940	0.295
Burley II	2.6	0.730	1.33	1.54	1.18	1.20	0.315
Burley V	2.75	0.790	1.48	1.49	0.80	0.70	0.330
Burley III	3.46	0.885	1.71	1.79	1.26	1.23	0.320
Burley IV	4.62	1.05	2.20	2.18	1.42	1.41	0.390
Blend B + 8.3% $NaNO_3$	6.1	1.45	2.58	2.42	1.58	1.58	0.515
Blend B + 5% $Cu(NO_3)2$	3.8	0.962	1.79	1.80	1.36	1.10	0.415

* All cigarettes were 85 mm long without filter tips and were smoked under standard conditions. The data for Blend A are average values from 5 analyses; all other results are average values from 2 analyses. For each analysis 200 cigarettes were smoked.

** Less than 0.05

VI. N-Nitrosamines

In 1956 Magee and Barnes reported strong carcinogenic activity for dimethylnitrosamine (DMN) in rats [16]. Since then many studies reported on carcinogenic activity of N-nitrosamines with available hydrogens on the carbon alpha to the nitrosamine nitrogen [17]. Even microgram amounts of DMN or diethylnitrosamines induce tumors in mice, rats or hamsters.

These bioassay data prompted extensive analytical studies for volatile nitrosamines in the environment. Most of these studies, however, were restrained by the elaborate enrichment procedures required for the quantitative determination of these environmental carcinogens. The development of the highly sensitive and specific thermal energy analyzer (TEA) by Fine et al. [18] has significantly simplified the quantitative determination of the nitrosamines. The volatile

nitrosamines from the smoke are concentrated, separated and detected by GLC—TEA. The mechanism of detection of the nitrosamines involves pyrolytic decomposition to nitrogen oxide which is then oxidized by ozone to excited NO_2 (Fig. 5). The excited NO_2 decays to its ground state with the emission of light in the near-IR region which is then detected by a photomultiplier tube. The detection limit for DMN is about 50 pg (50×10^{-12} g).

For the analysis of nitrosamines in unaged smoke, the cigarettes are smoked under standard conditions, and the freshly generated aerosol is trapped in an ascorbic acid solution at pH 4.5 [19]. Figure 6 shows the analytical procedure for the determination of volatile nitrosamines (VNA) in cigarette smoke. Table 4 summarizes results for VNA in the mainstream smoke (MS) of cigarettes, the smoke which is inhaled (Fig. 7). The results did not only show a significant difference in the yield of nitrosamines but indicated also a good correlation with the nitrate concentration in tobacco. We found also that filter tips containing cellulose acetate can selectively reduce VNA in the smoke up to 80% whereas charcoal filter tips do not remove VNA selectively.

An important finding was that sidestream smoke (SS), the smoke generated between puffs, contains at least 20 times more DMN (600 to 1770 ng/cig) and 10–40 higher amounts of the other VNA (Table 5).

VII. Indoor Pollution

Since we found significantly higher concentrations of VNA in SS compared to MS, the question arose as to the possible presence of these carcinogens in indoor environments heavily polluted by tobacco smoke. The availability of the highly sensitive TEA enabled us to investigate this question. We developed a portable air pollution sampler which was housed in an attache case to permit undisclosed collection of polluted indoor air. Table 6 summarizes our findings for dimethylnitrosamine. From these data we calculated that a nonsmoker who remains for 1 hour in the most heavily polluted places measured may expose his respiratory tract to quantities of volatile nitrosamines equivalent to those in the mainstream smoke of 9–19 nonfilter cigarettes or 17–35 filter cigarettes [20]. We realize, however, that a person rarely stays in such polluted rooms for longer periods of time and that the inhalation of undiluted cigarette smoke may be different from normal breathing; moreover, in polluted rooms one inhales practically only highly diluted volatile smoke constitutents as compared to the heavy aerosol with "tar" one inhales during smoking. Additional chemical analytical studies and above all, detailed epidemiological studies are needed before one can evaluate the overall effect on man of long term exposure to environments polluted with tobacco smoke.

VIII. Urban Pollutants

In our analytical studies of urban air we were so far unable to detect measurable amounts of VNA. This parallels studies by others. An exception are the analyses of polluted air near a chemical plant in Baltimore, Md. where DMN was found [21,22]. In addition to the analysis of urban air for carcinogenic polynuclear aromatic hydrocarbons, which is important in terms of the carcinogenic activity of pollutants observed in bioassays [7], we were interested in the occurrence of trace amounts of aza-arenes in urban air as well as in cigarette smoke [27]. Our interest in aza-arenes in polluted air is related to several observations. First, the basic portion of urban pollutants is mutagenic in certain bacterial tester strains [24,25] and induces tumors in newborn mice [26]. Furthermore the two ring aza-arene, quinoline, and its derivatives are mutagenic [27] and carcinogenic [28]. For the analysis the basic portion of the particulate matter is fractionated by HPLC and the individual fractions are analyzed by GLC-MS and reversed phase HPLC (Fig. 9) [29]. The amounts of aza-arenes isolated from two air samples from New York City are summarized in Table 7. In general, it appears that aza-arenes are present in polluted air in about 1/10 of the concentration compared to the corresponding polycyclic hydrocarbons. This study on aza-arenes in urban air represents another example for the determination of minute amounts of

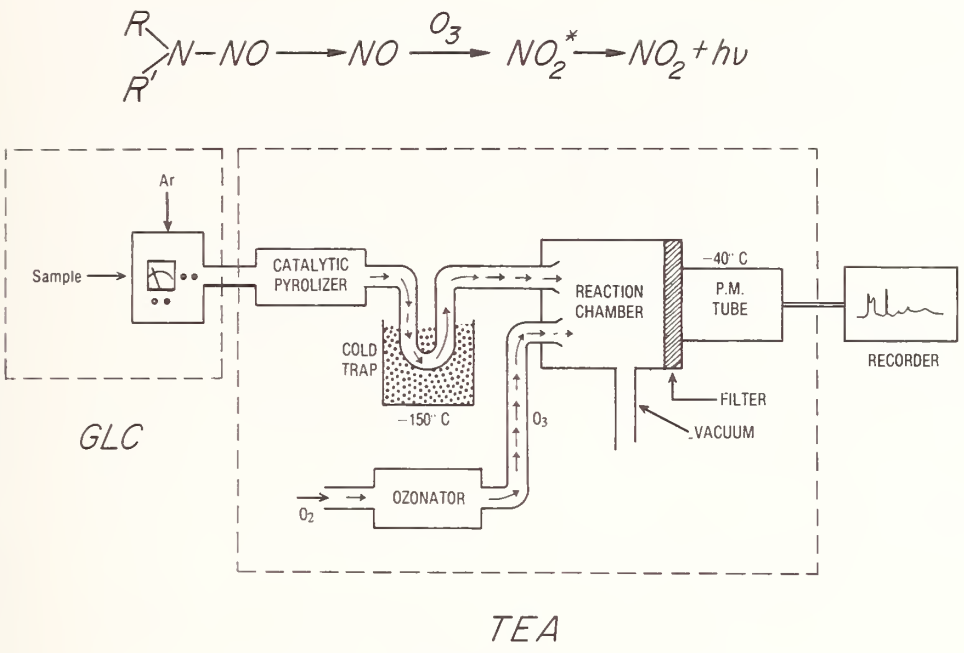


Figure 5. Diagram of thermal energy analyzer.

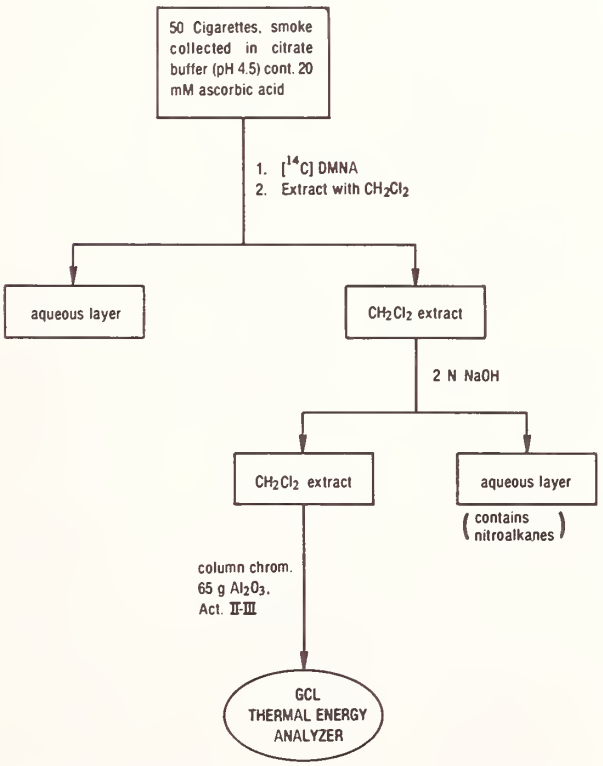


Figure 6. Analytical procedure for the determination of volatile nitrosamines in cigarette smoke.

TABLE 4. *Volatile nitrosamines in the mainstream smoke of cigarettes (ng/cig)*

	DMN	MEN	DEN	NPY
Cigarette A without filter	13	1.8	1.5	11
Cigarette B without filter	43	5.9	3.8	22
Cigarette C without filter	65	7.7	N.D.	34
Little cigar with filter	43	4.8	1.1	19
Cigarette D with filter	5.7	0.4	1.3	5.1
Catterton, High NO_3	97	8.0	4.8	42
Catterton, Low NO_3	20	1.2	2.3	4.1
Robinson, High NO_3	81	4.9	3.6	32
Robinson, Low NO_3	10	0.7	1.8	2.7
Bright tobacco	13	<0.1	1.8	6.2
Burley tobacco	76	9.1	2.5	52

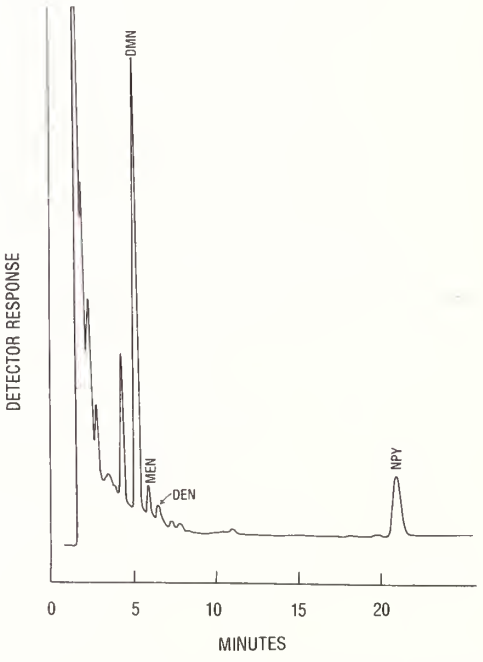


Figure 7. Typical gas chromatogram of volatile nitrosamines in mainstream cigarette smoke.

TABLE 5. *Volatile N-nitrosamines in the sidestream smoke of commercial cigarettes (ng/cig)*

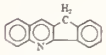
	DMN	MEN	DEN	NPY
Cigarette A	680	9.4	53	300
Cigarette B	823	30	8.2	204
Cigarette D	736	10	73	387
Cigarette E	1040	10	63	213
Little Cigar	1770	75	29	612

TABLE 6. *Dimethylnitrosamine in polluted indoor air*

Environment	Flow Rate (l/m)	Volume collected (l)	Concentration[a] (ng/l)	Exposure per hour[b] (ng)
Train I (Bar Car)	4	360	0.13	62-110
Train II (Bar Car)	2	180	0.11	53-62
Bar	2	360	0.24	115-200
Sports Hall	2.75	495	0.09	43-76
Betting Parlor	3	270	0.05	24-42
Discotheque	3	675	0.09	43-76
Large room of bank	2	600	0.01	5-9
Suburban residence[c]	3	720	<0.005	–
Urban residence[c]	3	720	<0.003	–
Blank	–	–	<0.001	–

[a] Isolated amounts
[b] Respiratory rate 8-14 l/min
[c] Non-smokers residence

TABLE 7. *Concentration of aza-arenes in New York City suspended particulate matter*

No.	Aza-arenes	Name	Mol wt	Concn in ng/1000 m³ Sample 1	Sample 2
1		Quinoline	129	69	22
1a		Methylquinolines	143	35	33
1b		Dimethylquinolines	157	48	44
1c		Ethylquinolines	157	14	22
1d		3C-quinolines	171	10	ND[a]
2		Isoquinoline	129	180	140
2a		5 or 8 methylisoquinoline	143	310	170
2b		Other methylisoquinolines	143	76	70
2c		Dimethylisoquinolines	157	62	ND
2d		Ethylisoquinolines	157	160	68
2e		3C-isoquinolines	171	28	ND
3		Acridine	179	41	40
3a		Methylacridines	183	7	ND
4		Benzo(h)quinoline	179	10	13
5		Benzo(f)quinoline	179	11	10
6		Phenanthridine	179	22	18
7		Benzo(f)isoquinoline	179	110	34
8		4-Azafluorene (5-H-indeno-[1,2-b]-pyridine)[b]	167	5	5
9		11H-indeno(1,2-b)quinoline	217	Trace	Trace
10		4-Azapyrene (benzo (lmn)-phenanthridine)	203	21[c]	22[c]
11		1-Azafluoranthene (indeno-[1,2,3-ij]isoquinoline)	203	5[c]	5[c]
12		Benzothiazole	135	14	20
13		Caffeine	194	3400	7000

[a] ND: not determined. [b] Name according to Patterson's Ring Index. All structures are also drawn according to the ring index. [c] Includes other isomers.

carcinogens in the respiratory environment with the aid of modern chemical-analytical instrumentation.

IX. Summary

Examples were presented for the analysis of trace amounts of carcinogens in respiratory environments. These included vinyl chloride, hydrazines, nitrohydrocarbons and volatile N-nitrosamines in tobacco smoke, N-nitrosamines in polluted indoor air and aza-arenes in urban air.

X. Acknowledgment

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A NEW APPROACH TO THE TRACE ANALYSIS OF MONO- AND DI-HALOGENATED ORGANICS, AN ANALYSIS OF METHYL CHLORIDE IN THE ATMOSPHERE

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The intentional addition of oxygen to the nitrogen carrier gas of a constant-current Electron Capture Detector (ECD) is shown to provide a useful extension of ECD analysis capabilities. The ECD sensitivity to compounds having few and particularly only one chlorine atom is greatly increased. As an example of this use, an analysis of methyl chloride in ambient air is reported. Oxygen doping is also shown to be useful for compound identification in ECD analyses, since the magnitude of the response enhancements caused by the addition of a definite amount of oxygen to the carrier gas varies greatly with compound type and is reproducible for a given compound. A systematic study of oxygen doping for the ECD analysis of CH_3Cl, CH_2Cl_2, $CHCl_3$, CCl_4, and CH_3Br along with the accompanying effects on baseline frequency and noise is reported from which the selection of oxygen concentration and detector temperature can be made to cause the most favorable signals from sample components.

Key words: Carrier gas doping; electron capture detector; methyl chloride; monochlorinated organics.

I. Introduction

The effect of oxygen contamination in the carrier gas of a Gas Chromatograph-Electron Capture Detector (GC-ECD) has been previously described several times and has been universally considered an undesirable complication in analysis procedures [1–3]. We have recently shown [4], however, using a constant-current ECD, that a large increase in the response to *n*-butyl chloride is caused by oxygen doping of nitrogen carrier gas, and have suggested that the intentional doping of carrier gas with oxygen may hold some promise for the improved ECD analysis of monochlorinated organics.

It is suspected that the response enhancement occurs because, in addition to dissociative electron capture, reaction 1, which is slow

$$e^- + RCl \rightarrow R + Cl^- \quad (1)$$

for *n*-butyl chloride, that if oxygen is added the following set of reactions may also occur:

$$e^- + O_2 \rightleftharpoons O_2^- \quad (2)$$

$$O_2^- + RCl \rightarrow Cl^- + \text{neutrals} \quad (3)$$

Since the chemical sum of reactions 2 and 3 is similar or equivalent to reaction 1, the presence of oxygen can provide an additional and faster mechanism for electron attainment by the monochlorinated organic molecule in which O_2^- serves as a catalyst. A more detailed discussion of thermodynamic and kinetic aspects of this proposed mechanism can be found in the previous study [4].

We wish to report here a more detailed study of this effect on the ECD response to a series of halogenated methanes, CH_3Cl, CH_2Cl_2, $CHCl_3$, CCl_4 and CH_3Br; and then apply the method to the analysis of methyl chloride in the atmosphere. This latter task provides a challenging test of the technique since, due to its low concentration and poor ECD sensitivity, published evidence of the detection of methyl chloride in ambient air samples of low volume has been previously presented only once—by a specialized GC-MS system [5].

II. Experimental

The gas chromatograph used is a Varian 3700 Aerograph with constant-current, pulse-modulated operation of a ^{63}Ni detector. A 10-ft. by 1/8-in. stainless steel column packed with 10% SF-96 on Chromosorb W was used. For preliminary studies the column temperature was 26 °C. For air analyses the column temperature was programmed from −50 °C to +10 °C. Data was normally collected on a strip chart recorder. When peak areas were desired, an Autolab minigrator was used.

The normal carrier gas flow rate was modified to include additional devices to allow gas sampling and controlled oxygen doping. These are shown in Figure 1. The pressure of the nitrogen carrier gas was maintained at 20 psig (the normal flow rate control valves were bypassed). If valves A, B, and C are all off (bypass position), carrier gas goes directly to the GC. Gas samples are introduced by flushing the sample loop C with it and turning valve C on. The sample loop was of 2-mL volume (1/8 in. stainless tubing) for preliminary studies and of 20-mL volume (1/4 in. stainless tubing) for atmospheric analysis. Variation of oxygen concentration was accomplished by the combined use of a 5-L stainless sphere (B) and another loop (A) of 13 mL volume (1/4 copper tubing). With this loop, 20 psig aliquots of purified air were added to the carrier stream via valves A. With valves B open, each aliquot of air introduced increased the O_2 concentration in the dilution sphere by 0.5 ppth (volume ratio). Any desired concentration of O_2 in multiples of 0.5 ppth was established by quickly refilling and expelling the oxygen loop into the dilution sphere. In a period of 0.5 h the established oxygen concentration would decrease by less than 10% at which time the initial concentration was reestablished by adding small amounts of air via one of the valve pair A. The magnitude of the ECD baseline allowed a continuous monitor of the carrier gas oxygen concentration. The carrier gas was ultra high purity nitrogen (Matheson) and was first passed through activated charcoal and 13X molecular sieve filters. The air was zero-grade (Matheson) and was passed through a 13X molecular sieve filter. The air was first analyzed by the ECD system to ensure that it contained an acceptably low amount of contaminants which might cause ECD responses.

Standards were prepared by the successive dilution of pure halogenated methanes into air tight glass vessels containing nitrogen gas. In the preliminary studies the prepared contents of a 20-L carboy were transferred to the 2-mL sample loop using a 100-mL ground glass syringe

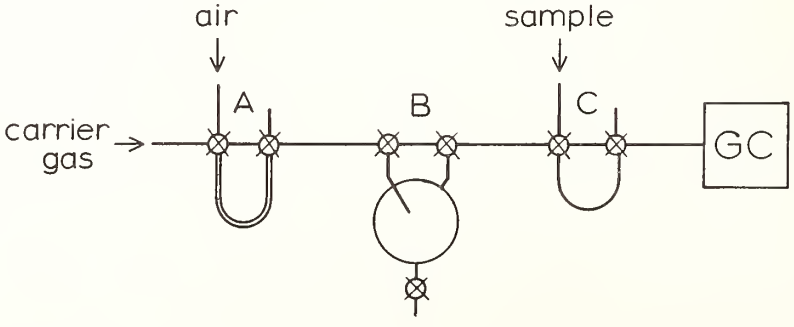

Figure 1. Carrier gas flow system for the controlled addition and mixing of oxygen with the carrier gas and for gaseous sample introduction.

equipped with a Hamilton shut-off valve. The column temperature was 26 °C. All of the chlorocarbons were chromatographically resolved from each other and could, therefore, be run simultaneously. Typical chromatograms are shown in Figure 2. Since the CH_3Br peak overlapped partially with that of CH_3Cl, these were not run simultaneously.

For the atmospheric analyses, the 2-mL sample loop was replaced by a 20-mL loop. Also the GC oven was temperature programmed from −50° to 10 °C. The timing of analysis events is as follows: an air sample is captured on the roof of the chemistry building using a 100-mL glass syringe with a lock, and is flushed through the 20-mL sample loop. Sample valve C is opened, injecting the air sample onto the column. At −50 °C the contents of interest in the sample are immobilized at the head of the column. The oven remains at −50 °C for 45 seconds allowing sample oxygen to pass out. Then the column temperature is increased at a rate of 89°/min (maximum rate) to 10 °C where the oven is maintained until the completion of the analyses. Between analyses the oven is held at 60 °C for 10 minutes to remove the less volatile components of the air sample from the column.

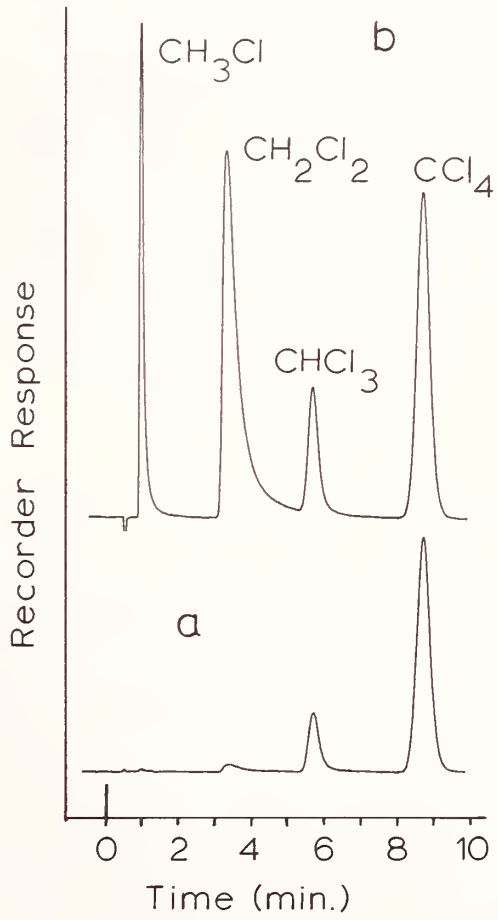

Figure 2. Typical chromatograms in studies of oxygen's effects on ECD responses to the chloromethanes. (a) Normal ECD conditions of pure nitrogen carrier gas. (b) Same standard run with 2.0 ppth oxygen in carrier gas. Detector temperature is 300 °C. Concentrations are 620 ppb CH_3Cl, 960 ppb CH_2Cl_2, 68 ppb $CHCl_3$, and 17 ppb CCl_4. Sample loop 2 mL. Oven 26 °C.

III. Results and Discussion

A comparison of the relative responses of the halomethanes under the condition of no oxygen added indicates the expected trend [6] that an increase in ECD sensitivity is caused by the successive addition of chlorine atoms to methane. Also methyl bromide responds considerably better than does methyl chloride. For example, at a detector temperature of 300 °C, the relative ECD response of CH_3Cl, CH_2Cl_2, CH_3Br, $CHCl_3$, and CCl_4 were 1.0, 2.9, 8.9, 470, and 15,000, respectively. The addition of oxygen to the carrier gas, however, causes the response of the lower chlorinated species to increase much more dramatically than it does the highly chlorinated molecules. This is illustrated in Figures 2 and 3. In Figure 2, typical chromatograms from which these data were obtained illustrate the effect of 2.0 ppth oxygen on the peaks in the analysis of a mixture of the four chlorinated methanes at a detector temperature of 300 °C. While the CCl_4 peak is relatively unaffected, the CH_3Cl peak is increased 95 times by the oxygen presence. In Figure 3 the response factors of all compounds at 300 °C are plotted against oxygen concentration. At the highest oxygen doping used, 5.0 ppth, the response enhancements observed for CH_3Cl, CH_2Cl_2, $CHCl_3$ and CCl_4 are 307, 57, 4.9, and 1.8, respectively. These are inversely related to their normal ECD sensitivities and are consistent with the proposed mechanism comprised of reactions 1–3 (see ref. 4).

Since oxygen doping may improve the ECD analysis of CH_3Cl, in particular, in Figure 4 the effect of detector temperature on the response enhancements of methyl chloride is shown. It would appear from this that the lower temperatures might be best if the largest oxygen-induced responses to CH_3Cl are desired. In choosing the optimum analysis condition, however, the factors of baseline frequency and baseline noise must also be considered. In Figure 5 is shown the effect on baseline frequency of temperature for the various levels of oxygen doping. The increase in baseline frequency which accompanies oxygen addition is undoubtedly due to the oxygen electron capture equilibria reaction 2. Since this reaction is exothermic, it is disfavored by higher temperatures. Thus at the lower temperature of 200 °C, oxygen's effect on the baseline frequency is seen to be too great for analysis purposes. The loss of linear response due to instrumental saturation begins to occur at about 100 kHz with our instrument. At 200 °C this point is approached with the addition of 1.5 ppth O_2. At the higher detector temperatures, however, the baseline frequency is

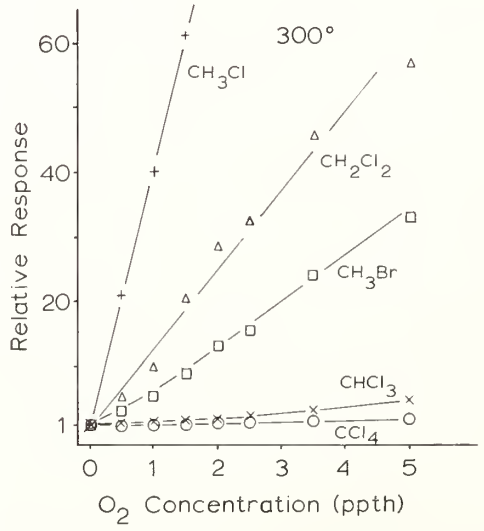

Figure 3. The effect of oxygen doping of the carrier gas on the ECD response to several halogenated methanes at a detector temperature of 300 °C.

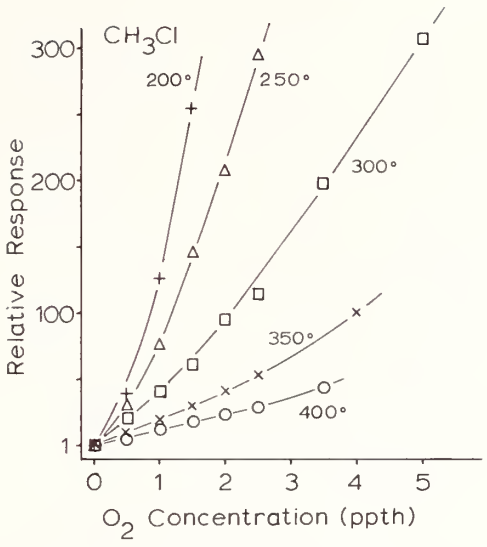

Figure 4. The effect of detector temperature on the oxygen-induced response enhancements of methyl chloride.

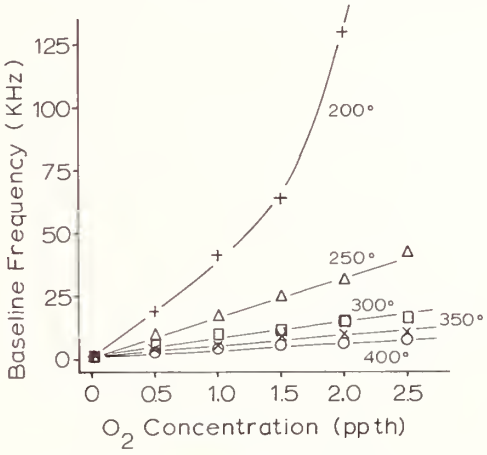

Figure 5. The effect of oxygen doping on the ECD chromatogram baseline at several detector temperatures.

not increased so drastically and a sizeable range of linear response to sample compounds still remains even with the addition of relatively large amounts of oxygen.

The effect of oxygen doping on baseline noise is shown in Figure 6. Baseline noise roughly parallels baseline frequency and, therefore, Figure 6 closely resembles the pattern of Figure 5. Again, the higher detector temperatures are preferable with respect to providing minimum baseline noise with oxygen doping.

The combined effects of response enhancements and baseline noise are shown in Figure 7 for CH_3Cl where the response enhancement divided by noise (proportional to signal-to-noise), is plotted against oxygen concentration for all temperatures. The clear result of this treatment is that for methyl chloride analysis, neither of the detector temperature extremes, 200 °C and 400 °C, are the best choices. Rather, any one of the middle temperatures will provide the best analysis condition causing an increase in signal-to-noise response of methyl chloride of about one order of magnitude. Although 250 °C appears best in Figure 7, it has the disadvantage of higher baseline frequency (Figure 5) and, therefore, a smaller linear dynamic range. For this reason 300 °C or 350 °C may be the best general choice of detector temperature for methyl chloride analysis.

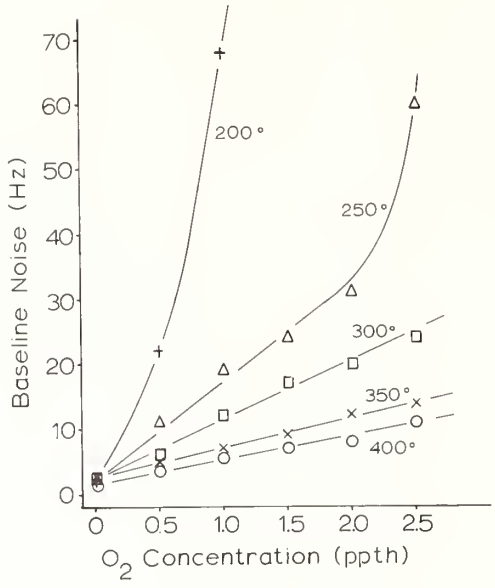

Figure 6. The effect of oxygen doping on baseline noise (peak-to-peak) at several detector temperatures.

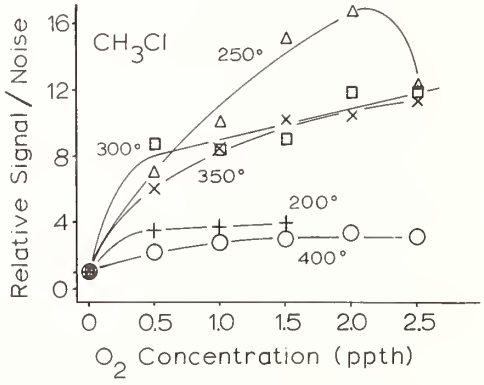

Figure 7. Relative signal-to-noise ratio expected for the ECD analysis of methyl chloride as a function of oxygen doping and detector temperature.

To illustrate the effect oxygen doping will have on the peaks of all of the subject compounds, the relative signal-to-noise or noise-to-signal (whichever is >1) of each as a function of oxygen doping at 250 °C is shown in Figure 8. It is seen that for those compounds for which response enhancements are not caused by oxygen doping, a loss in signal-to-noise is to be expected. As will be shown in the air analysis described below, this effect can be useful in de-emphasizing interfering peaks.

Since the basis of the response to methyl chloride described here is a new one, it is important to demonstrate that the ECD responses caused by it will be linearly related to methyl chloride concentration. The calibration curve shown in Figure 9 shows that this requirement is met for the nearly 3 orders of magnitude in response available between the limit of detection and the 100 kHz limit for linearity imposed by the instrument itself.

We have applied this technique to the analyses of methyl chloride in real air samples. The details of this procedure have been described in the Experimental section. Examples of the chromatographs obtained with and without oxygen doping are shown in Figure 10. Chromatograms a, b, and c were obtained by the normal ECD method, without oxygen doping. A sharp negative

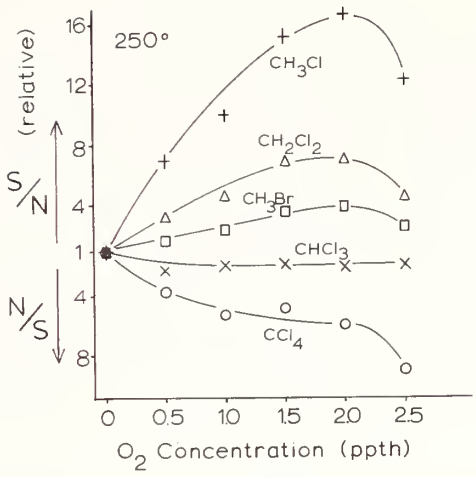

Figure 8. Relative signal-to-noise or noise-to-signal ratio (whichever is >1) for all compounds studied as a function of oxygen doping at 250 °C.

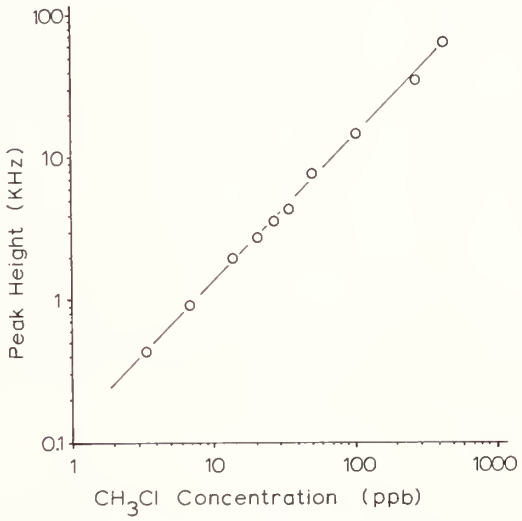

Figure 9. ECD calibration curve for methyl chloride using oxygen-doped carrier gas. Conditions: 2 mL sample loop, column temperature 26 °C, detector temperature 300 °C, 2.0 ppth oxygen in nitrogen carrier gas.

spike and the first time marker indicate the point at which the 20-mL sample loop is opened. About 45 seconds is allowed for the sample oxygen to pass through the column during which the disturbance it causes is clearly seen in chromatogram a. At the point of the second time marker, the column temperature is rapidly raised to 10 °C. Several peaks then appear, the two largest of which in chromatogram a are due to CF_2Cl_2 and $CFCl_3$. Chromatogram b shows the methyl chloride peak from a standard of concentration 7.7 ppb (parts per billion). The third time marker, at about 1.5 min denotes the expected retention time of CH_3Cl. The air sample, chromatogram a, has a peak near the same retention time, but would lead to the unsuspected conclusion that the air contained about 8 ppb CH_3Cl which is about 10 times greater than the value previously reported, 0.6 ppb [5]. Based on the sensitivity demonstrated by chromatogram b, only a barely perceivable peak for CH_3Cl might have been expected of the atmospheric sample. Chromatogram c shows that the peak under consideration in the air sample is not, in fact, CH_3Cl since a doublet is observed upon mixing the air sample with an equal volume of the methyl chloride standard. Chromatograms d, e, and f were obtained with 2.0 ppth oxygen in the carrier gas and a detector temperature of

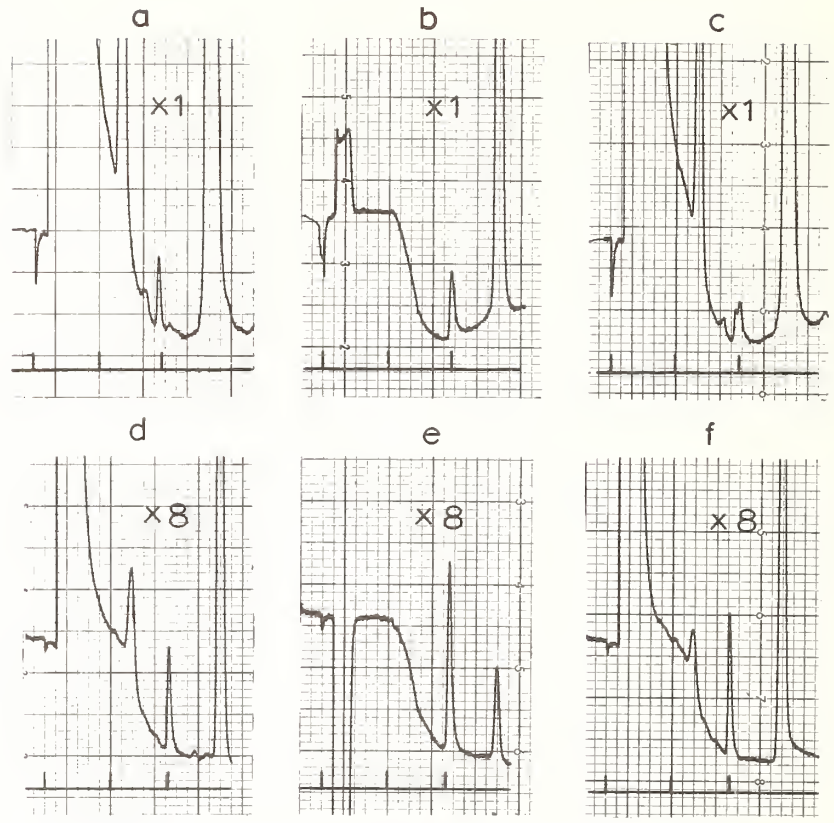

Figure 10. Chromatograms of real air analyses. a–c are under normal ECD conditions of pure nitrogen carrier gas. d–f are with 2.0 ppth oxygen added to the carrier gas. The detector temperature is 300 °C. The time markers indicate the following events: first, the sample value is opened; second, 45 seconds later the rapid temperature program of the GC oven from –50 to 10 °C is commenced, and third, methyl chloride eludes at about 1.5 minutes. Samples: (a) ambient air, (b) standard with 7.7 ppb CH_3Cl, (c) equivolume mixture of a and b, (d) ambient air, (e) standard with 1.5 ppb CH_3Cl, (f) equivolume mixture of d and e.

300 °C. The recorder is now on an attenuation X8 greater than previously. Under this condition we might expect an enhancement of the CH_3Cl peaks of about 95 (Fig. 4). Correcting for the lower concentration of the 1.5 ppb standard used and the attenuation change, chromatogram e is in good agreement with this expectation. Again a peak is observed in the air analysis, chromatogram d, at the retention time of CH_3Cl. In this case, however, chromatogram f of the mixture of standard and sample indicates only one peak suggesting that we have now identified CH_3Cl in the air sample. The peak observed in chromatogram a has apparently been lost in chromatogram d by the attenuation and noise increase while a new peak reflecting CH_3Cl has been brought out. The concentration of methyl chloride in the atmospheric sample deduced from chromatograms d and e is 0.8 ppb, in very reasonable agreement with the previously reported value. Repeating this analysis for 6 different air samples, each taken just prior to measurement from the roof of the chemistry building, an average value of 0.72 ppb with a standard deviation of 0.18 ppb was obtained. In comparing this method for methyl chloride measurement with the GC-MS study [5], the demonstrated signal-to-noise obtained here with an ECD is significantly superior and has been obtained with much simpler and inexpensive instrumentation.

In conclusion we have demonstrated that the increased response of a constant-current ECD to monochlorinated organics which accompanies the intentional addition of oxygen to the carrier gas can be useful in the analysis of these substances. The alternate use of pure nitrogen and oxygen doped carrier is easily done and may be used as a qualitative index of compound identity.

For example, in Figure 2, the magnitude of peak height increase caused by oxygen doping is indicative of the identity of each chlorinated methane. In using negative ion-molecule reactions to produce ECD responses, the high degree of specificity to electro-negative species, a valuable feature of the ECD, is maintained. It is relatively easy to envision many other analysis problems involving normally poorly responding electron capture compounds to which the general method demonstrated here could be gainfully applied.

IV. Acknowledgment

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

ANALYTICAL FACILITIES FOR THE ANALYSIS OF TRACE ORGANIC VOLATILES IN AMBIENT AIR

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During the past 4 years the Research Triangle Institute has developed state-of-the-art capabilities for the analyses of wide varieties of organic volatiles in ambient air. The facility in which analyses are carried out is described and the philosophies and criteria utilized in developing the methodology are discussed in this paper. In particular, the instrumental approach, GC column selections, and sampling methodology are specified. RTI capabilities include 5 gas chromatographs with 11 detectors, which have been modified to accommodate analyses ranging from ppm to ppt levels. Analyses can be performed on hydrocarbons ranging from C_1-C_{12}, halocarbons, sulfur-containing compounds, and photochemical byproducts such as ethyl nitrate and peroxyacetyl nitrate. Calibration procedures, generation of accurate standard materials, and quality control programs associated with ambient monitoring programs are also discussed.

Key words: Complete analysis scheme; gas chromatography; hydrocarbons; permanent gas analysis; trace organics in air.

I. Introduction

To generate good quantitative analyses for ambient air trace organic volatiles, four major areas need to be considered: (1) What type of instrumentation is necessary to perform the desired analyses, (2) What type of sampling technique will be employed to collect the materials of interest, (3) What kind of standardization and quality control will be required to maintain a high reliability factor in the data, and (4) What level of personnel is required to analyze the samples. Each of these factors will affect the quality of the data generated.

The capabilities and philosophies of Research Triangle Institute (RTI) in each of these areas will follow.

II. Discussion

A. Instrumentation

The first major area of interest is the type of instrumentation needed. The type of instrumentation required will depend on the types of compounds to be analyzed. RTI has the capability for measurement of trace hydrocarbons from C_1-C_{12}, halocarbons, trace sulfur-containing compounds, as well as capabilities for permanent gases analyses. Since the hydrocarbon analysis includes such a wide range of compounds and maximum specificity is required, these analyses have been segmented into four categories. A list of these compounds is presented in Table 1.

Three gas chromatographs are utilized to measure these four groups of compounds. The methane analyses are made on a modified Perkin-Elmer 3920. A schematic drawing of the pneumatics is shown in Figure 1. The system was designed to accommodate samples at various pressures. The system operates on a pressure differential basis. The sample loop and connection are evacuated to $4.0–5.0\times10^{-3}$ torr, and the sample is introduced by opening the valve on the container. The sample loop is filled until the pressure reaches $4.0–5.0\times10^{-1}$ torr. The difference

TABLE 1. *List of the hydrocarbon analyses performed by RTI's EMd*

Compounds analyzed	Type of analysis	Detector	Column
CH_4	1 mL direct injection	FID	Durapak® Phenyl-isocyanate
C_2-C_5 aliphatic hydrocarbons	100–250 mL Cryogenic concentration	FID	Durapak® Phenyl-isocyanate
C_5-C_{12} aromatic hydrocarbons	100–250 mL Cryogenic concentration	FID	60 m OV 101 SCOT
C_6-C_{12} aromatic hydrocarbons	100–250 mL Cryogenic concentration	FID	60 m NBMA SCOT

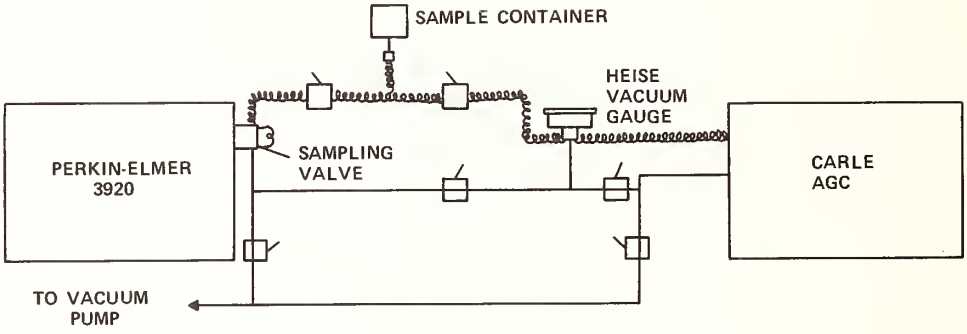

Figure 1. Instrument pneumatics for CH_4 analyses and permanent gases.

in pressure is recorded and the sample is injected into a 2.5 mm×3.2 mm stainless steel column packed with 100 to 120-mesh Durapak® phenylisocyanate. To separate the air peak from the methane, the column is maintained at 0 °C. The chromatogram is displayed on a 255 linear recorder.

The three other hydrocarbon analyses, C_2-C_5 light hydrocarbons, C_5-C_{10} aliphatics, and C_6-C_{10} aromatics utilize the same fundamental principle. Sample air is withdrawn from the sampling container and cryogenically concentrated at liquid oxygen temperature. The air sample is passed through a 30 cm×1.6 mm nickel tubing packed with 60 to 80-mesh silanized glass beads. Trace organics are collected in the trap and volatilized by immersing the trap in a 185 °C silicone oil bath. The concentrated sample is then injected onto the analytical columns. The schematic of the instrument's valving system is shown in Figure 2.

The C_2-C_5 light hydrocarbon analysis system consists of a Perkin-Elmer model 900 gas chromatograph equipped with a 1.8 m×3.2 mm stainless steel column packed with 100 to 120-mesh Durapak® phenylisocyanate. The column is maintained at 18 °C±0.5 °C in a cold water bath. The analysis is terminated after 30 minutes. Upon elution of *n*-pentane, the 10-port valve is turned to backflush the column of higher molecular weight compounds. The backflushing is completed when the FID response is returned to baseline.

The analysis for C_6-C_{10} aromatic compounds is handled by the same Perkin-Elmer model 900 gas chromatograph used for the C_2-C_5 analysis. The aromatic compounds, however, are separated on a 60 m SCOT MBMA capillary column.

In the C_5-C_{10} aliphatic analysis system a Perkin-Elmer model 3920B gas chromatograph equipped with a 60 m SCOT OV-101 capillary column is used. This capillary column was chosen for its excellent separation of aliphatic compounds in the C_5-C_{12} molecular weight range. The analysis is started by injecting the sample onto the column at 30 °C. This temperature is

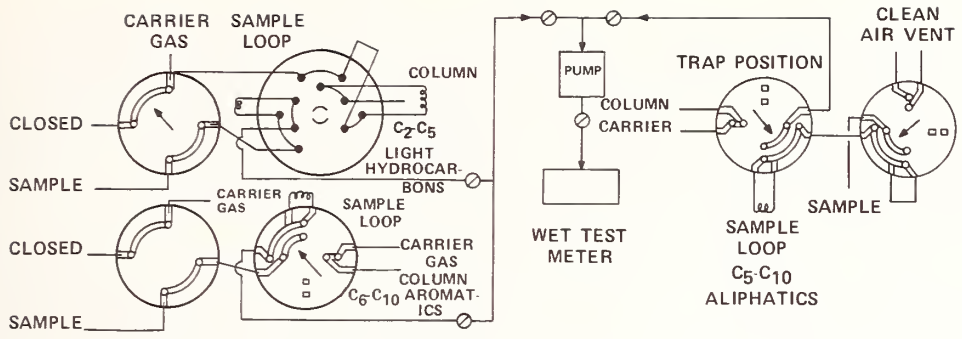

Figure 2. Schematic diagram of the C_2-C_5 light hydrocarbons, C_5-C_{10} aliphatic, and C_6-C_{10} aromatic valving system.

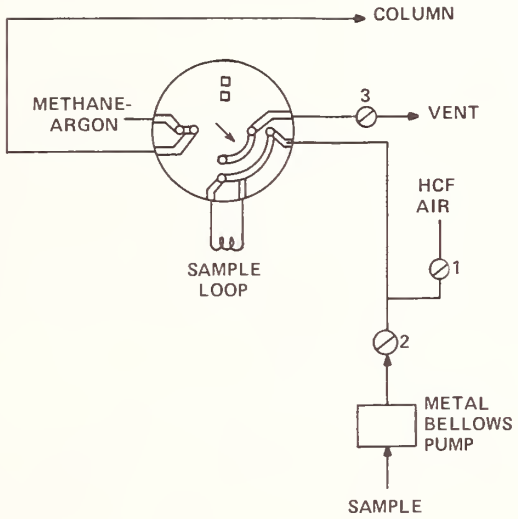

Figure 3. Schematic diagram of the halocarbon valving system.

maintained for 8 minutes, then the oven is temperature programmed at a rate of 4 °C/min up to a 100 °C. The column is held at this temperature until the analysis is completed.

The analysis time for both the C_6-C_{10} aromatics and the C_5-C_{12} aliphatics is 60 minutes. The analysis time for the C_2-C_5 light hydrocarbons can vary depending on the size of the backflush peak. From the description of the hydrocarbon system, it is obvious that a strong emphasis is placed on the use of the capillary columns. These columns are necessary since the number of compounds that occur in ambient air can be very high and maximum resolution is needed to identify and quantify each compound.

Analyses of halocarbons at ambient concentration levels are difficult. The concentration levels are so low that even the very sensitive constant current electron capture detectors are pushed to their most sensitive scale to obtain quantitative numbers. Another problem in halocarbon analysis is the care which must be taken so that the sample is not contaminated with laboratory air. To alleviate such problems, RTI has installed a sophisticated valving system which prevents such contamination problems. A schematic drawing of the system is shown in Figure 3.

The halocarbon analyses are performed on a Perkin-Elmer 3920 equipped with a constant electron capture detector. Direct injection of 2 mL of sample is sufficient to quantitate a background concentration of the most commonly analyzed halocarbons. The halocarbons are separated on a 1.8 m glass column, 2 mm ID packed with Chromosorb W. HP, coated with 10% DC 200. The chromatograms are visually displayed on a linear recorder model 255.

The organic sulfur-containing compounds are analyzed on a two column 3920 Perkin-Elmer gas chromatograph equipped with a flame photometry detector. A 1.8 m×3.2 mm OD Teflon (FEP) column packed with Chromosil 330 provides separation of the low molecular weight mercaptans and disulfides. An 80 m Carbowax 20M glass SCOT column permits the separation of higher molecular weight mercaptans and thiophenes. The valving system is constructed of Hastaloy C Valco sampling valves and the interconnecting tubing is either Teflon or glass-lined stainless steel.

In an environmental laboratory, capabilities to perform permanent gases analyses are of utmost importance. Examples of such analyses are: background CO_2 measurement of SO_2 calibration mixtures for environmental flame photometric instruments, and percent composition of O_2-N_2 in zero air. The permanent gases analyses are performed on a Carle gas chromatograph equipped with a thermistor bead thermal conductivity detector. The gas chromatographic system is able to analyze H_2, O_2, N_2, CO, CO_2, and H_2O. Minimal detectability for this instrument is approximately 50 ppm.

With the many gas chromatographic systems described above and other systems employed for industrial hygiene analyses, source assessment, and photochemical byproduct analyses, a laboratory data system is necessary to handle the volume of data. RTI's laboratory handles several hundred samples per month, and in many cases four to five gas chromatographs are run simultaneously. Our facility has a 3352B data system with 24K of core memory, a paper tape photoreader, and a dual loop capability with up to 15 analog inputs per loop. The data is printed out on either a DEC Writer II or a TTY 33 teletype. The data system can handle both qualitative identification and quantification of the analyses previously described. The data was further validated by visual inspection of the chromatograms and the computer printouts and then entered on a larger computer for further data interpretation.

B. Sampling Methodology

The second data of interest is the type of sampling methodology to be employed. Through the years various research groups have used a multitude of sampling techniques. Some are better than others, some are very selective, some have severe shortcomings. RTI has had experience with many of the sampling techniques. Table 2 is a summary of the various techniques RTI has used in sampling ambient air volatile organics.

With any monitoring technique, there are obvious advantages and disadvantages of the various sampling methods that can be employed. The method employed for sampling should be evaluated for both its capability to accomplish the work and for its cost effectiveness. RTI has

Table 2. *Summary of the sampling technique used in past studies*

Techniques	Compounds monitored
Tedlar film sampling bags	CO, C_1-C_5 HC's
Teflon film sampling bags	Organic sulfur compounds
Glass bulbs	CO, C_1-C_7 HC's Organic sulfur compounds Permanent gases
Stainless steel container	C_1-C_{12} HC's Halocarbons
Tenax GC	C_6H_6
Silanized mole sieve	C_2H_2-C_4H_{10}
Charcoal	Vinyl chloride

selected treated stainless steel containers for use in collecting samples for analysis of trace organic volatiles. This decision was made after a thorough evaluation of various sampling containers in which stainless steel containers were found to be the best for the collection of C_2-C_{12} hydrocarbon species [1]. This technique also allows collection of samples for the analysis of methane, carbon monoxide, and halocarbons. A schematic diagram of the sampling system is shown in Figure 4. This system is all stainless steel except for the seal in the metal bellows pump, which is Teflon (TFE). The system was constructed to minimize the residence time of the sample air in the tubing coming from the sampling manifold. This system permits the collection of either grab samples or integrated samples.

Silanized glass containers are employed for the collection of sulfur containing organic compounds. The glass bulbs are evacuated to 1×10^{-3} torr. Sample is introduced into the bulb by simply opening the Teflon stopcock to the environment to be sampled. This method can only provide short term samples or grab samples (with the use of a glass critical orifice).

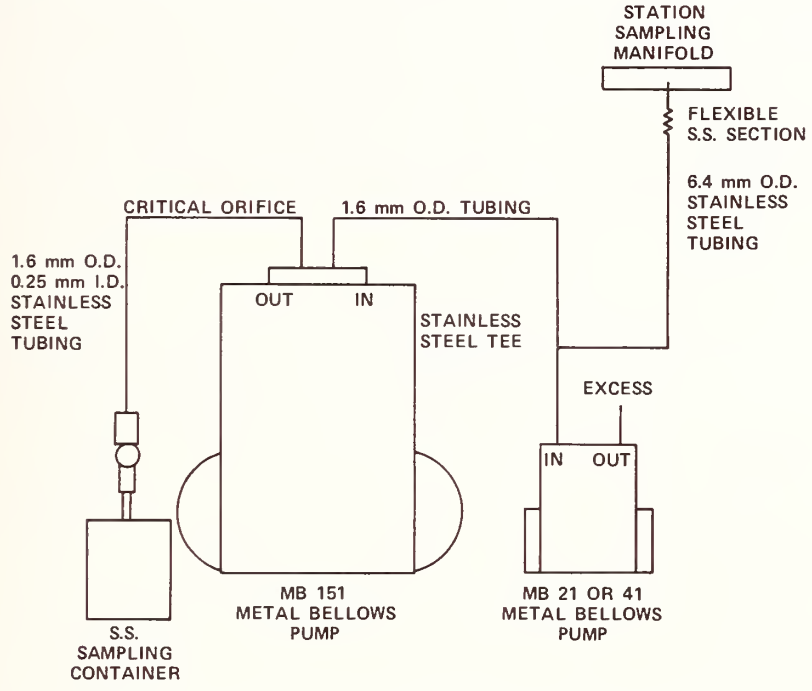

Figure 4. Schematic of RTI's pumping system used to collect field samples.

C. Standardization and Quality Control

If good instrumentation and sampling techniques are a primary requirement for generation of quality data, calibration capabilities and quality control of the laboratory are even more important. The RTI EMD approach is as follows: Each instrument has to be calibrated with the particular compound to be analyzed. The use of one compound to calibrate the analysis of 50–60 compounds is not adequate. Since only two organic compounds are available from National Bureau of Standards (NBS) as primary standard, other alternatives must be used to calibrate the instruments.

The use of permeation tubes and diffusion tubes can help generate some of the standard mixtures necessary. RTI EMD has a permeation tube system that can hold up to four permeation devices, but for a routine check of 50–60 compounds, RTI has generated standards in the 30–40 ppm range that contain between 15–20 components. The standards are prepared in gravimetrically

standardized stainless steel tanks (35 L). The liquids are vaporized and introduced into the evacuated tanks. Evacuation of the tanks facilitates faster mixing of the various components. The mixture is then pressurized to 34±0.1 atm with zero nitrogen. Compounds ranging from C_5-C_{12} have been prepared by this method and have been found to be quite satisfactory. The mixture is left to equilibrate and then analyzed. The concentration of some of the components are checked against permeation tubes or standards prepared in gravimetrically calibrated glass bulbs.

To calibrate the gas chromatographs, the standards are introduced onto the analytical column through a gas sampling valve with a gravimetrically calibrated sample loop. In many cases, purchased gas sample loops are not exactly of the specified volume, and it is RTI's policy to gravimetrically check the internal volume. However, calibrating the gas chromatograph in the ppm range does not necessarily mean that the analytical technique utilized to concentrate and analyze the sample is functioning with a 100 percent recovery. To check the overall performance of the analytical system, standards at the ppb level have to be accurately generated. To accomplish such tasks, RTI EMD has built what it calls a "double dilution system" (see Fig. 5). The principle is fairly straightforward. Standard mixtures usually in the 10–50 ppm range are diluted with catalytically cleaned air. The resulting mixture of 0.1–0.2 ppm is pumped through a critical orifice into the second stage, where it is further diluted to low ppb levels. The entire system is made of glass or stainless steel. The flow rates are checked with a 1 L/revolution wet test meter. The pollutants are measured with a soap bubble flow meter. With this system, RTI EMD can quantitatively prepare gas standards at any concentration level desired.

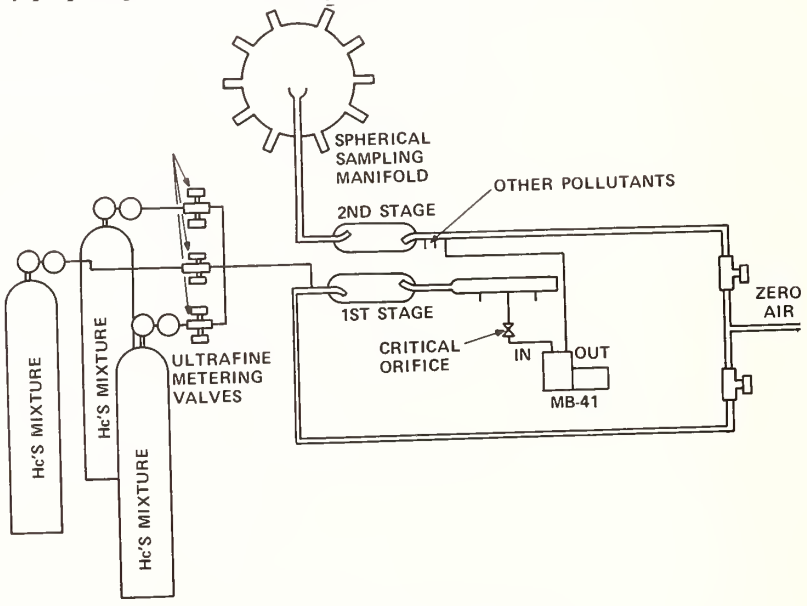

Figure 5. Double dilution system.

To insure that analytical procedures meet our specifications, a strict quality control program needs to be in effect. The quality control program associated with our ambient air hydrocarbon measurement is threefold. Monthly, each detector is checked for its absolute response with primary and gravimetrically prepared gas mixtures. Approximately 20 percent of all samples analyzed are quality control (QC) samples, which consist of zero air samples and standard gas mixtures prepared from the double diluted system. The standard samples contain 20–50 components at the ambient air concentration level which are analyzed twice per week to check the retention time of the components of interest. Some of the mixtures and zero air QC samples are analyzed and sent along with other sampling containers to the sampling site before being returned

to the laboratory for analysis. Additional quality control steps include repetition of 5 percent of the total samples as well as 2–5 percent of duplicate samples collected. This type of quality control program brings credibility to the overall sampling and analytical analyses.

D. PERSONNEL

To maintain such a facility, reliable and highly trained personnel are required. It has been proven in RTI experiences that a person with a B.S. in chemistry and some understanding of gas chromatographic techniques can be trained to perform the analyses. Two to three months of experience are usually required to bring the person to full potential. More experienced personnel may require less training. It may take 5–6 months to train a person to handle most of the analyses.

III. Summary

The analytical instrumentation, the sampling methodology, the calibration and quality control procedure, and the level of personnel required to analyze the samples are important factors that must be considered when attempting to make reliable measurements of volatile organics in ambient air. Each of these areas were discussed in some detail.

From the discussion it can be seen that each area has a bearing on the other and poor performance in one area can only mean deterioration of the overall quality of the data. It is the laboratory's responsibility to improve each area discussed in order to provide the theoretician with highly reliable data.

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

ANALYSIS OF TRACE LEVELS OF PETROLEUM HYDROCARBONS IN MARINE SEDIMENTS USING A SOLVENT/SLURRY EXTRACTION PROCEDURE

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A solvent slurry technique for the extraction of hydrocarbons in marine sediments has been developed based on dewatering with methanol and extraction on a ball-mill tumbler with 2:1 dichloromethane/methanol. After filtration and preliminary chromatographic cleanup, the extract was fractionated into saturated and unsaturated hydrocarbons using column adsorption chromatography. Both fractions were analyzed by gas chromatography with high resolution glass capillary columns. The procedure affords quantitation of individual hydrocarbons at part-per-billion levels in sediments.

Key words: Extraction; hydrocarbons; marine; petroleum; sediments.

I. Introduction

Many schemes exist for sediment extraction, with the exhaustive Soxhlet method [1] generally considered the most efficient, although perhaps not the most convenient. Alternatives to the Soxhlet procedures have been employed by various researchers. Farrington and Tripp [2] showed that refluxing sediment for 3 hours with 1:1 benzene/0.5 N KOH in methanol was as efficient as Soxhlet extraction using the same solvents. Rohrback and Reed [3] reported that agitation on a shaker table was only 4% less efficient than Soxhlet, but we encountered stable emulsions in applying the technique. To avoid emulsions, Warner [4] suggested extracting sediments with an aqueous diethyl ether solvent slurry on a ball-mill tumbler. We found this convenient for processing numbers of samples [5,6], although subsequently some hydrocarbon yields were found lower than by Soxhlet.

The tumbling procedure described here, using methanol (CH_3OH) and dichloromethane/methanol (CH_2Cl_2/CH_3OH) solvents, maintained the convenience of ambient solvent slurry extraction and gave yields comparable to exhaustive Soxhlet extraction [7]. Sediments were dewatered with CH_3OH and extracted with CH_2Cl_2/CH_3OH solvent slurry on a ball-mill tumbler. The total extract was chromatographed on a silica gel column to isolate two fractions containing primarily (a) saturated and (b) unsaturated hydrocarbons. These fractions were then analyzed by high resolution glass capillary gas chromatography (GC).

II. Experimental

A. Materials

1-liter bottles: Wheaton #219180
Teflon bottle caps: custom-machined from 38 mm o.d. Teflon rod
600-mL beaker
500-mL Teflon wash bottle: Nalge #2403-0500
150-mL coarse, fritted-glass filter: Corning #36060
1-liter separatory funnel with Teflon stopcock: Corning #6402
500-mL Erlenmeyer flask
Chromatography column, 19 mm i.d.: Kontes #K-420280, size 232
Chromatography column, 10.5 mm i.d.: Kontes #K-420280, size 213
500-mL Erlenmeyer flask, 24/40 STJ
Distilling column: Kontes #K-50300, size 121
25-mL concentrator tube: Kontes #K-570050, size 2525
Teflon chips (ebullators): Bel-Art Products #41001, Pequannock, NJ
Evaporative concentrator: Kontes #K-569251, size 3-19
Vials: Wheaton #223682
Vial caps: Hewlett-Packard #5080-8766 or 5080-8713
Capillary GLC columns: Supelco Inc. and Quadrex Corp.

B. Reagents

Methanol: MCB Spectroquality MX0475, SG2859, Lot 7G13
Dichloromethane: Mallinckrodt Nanograde, #3023
Hexane: Burdick and Jackson non-spectro grade, distilled in glass
Distilled water, carbon-filtered and distilled in glass
Silica gel, 100-200 mesh: MCB #SX144-06
Sand: MCB #SK78, CB1045
Copper granules: Mallinckrodt #4649
Hexamethylbenzene (GC internal standard), *n*-decylcyclohexane (alkane recovery standard) and 1,3,5,-triisopropylbenzene (aromatic recovery standard), Chemical Samples Co.
Aliphatic and aromatic reference compounds were purchased from: Aldrich, Analabs, Applied Science Labs., and Chemical Samples Co.

C. Apparatus

Ball-mill tumbler, model 8-RA, Scott-Murray Manufacturing, 8511 Roosevelt Way, N.E., Seattle, WA (frame, roller bars, motor only)
Water bath: custom-made
Tube heater block: Kontes #K-720003
Tube heater control unit: Kontes #K-720001
Gas chromatograph: Hewlett-Packard #5840A, with flame ionization detector and automatic liquid sampled (#7661A)

D. Extraction Procedure

The general extraction scheme is shown in Figure 1. A 100 g sample of sediment was weighed into a 1-liter bottle, and 50 mL of CH_3OH were added. Sediment dry weight was determined using 10–20 g samples [5,6]. The recovery standards were added to every sample (including blank samples). An aliquot containing all of the compounds to be determined was quantitatively added to a second blank to determine losses due to handling. The bottles were

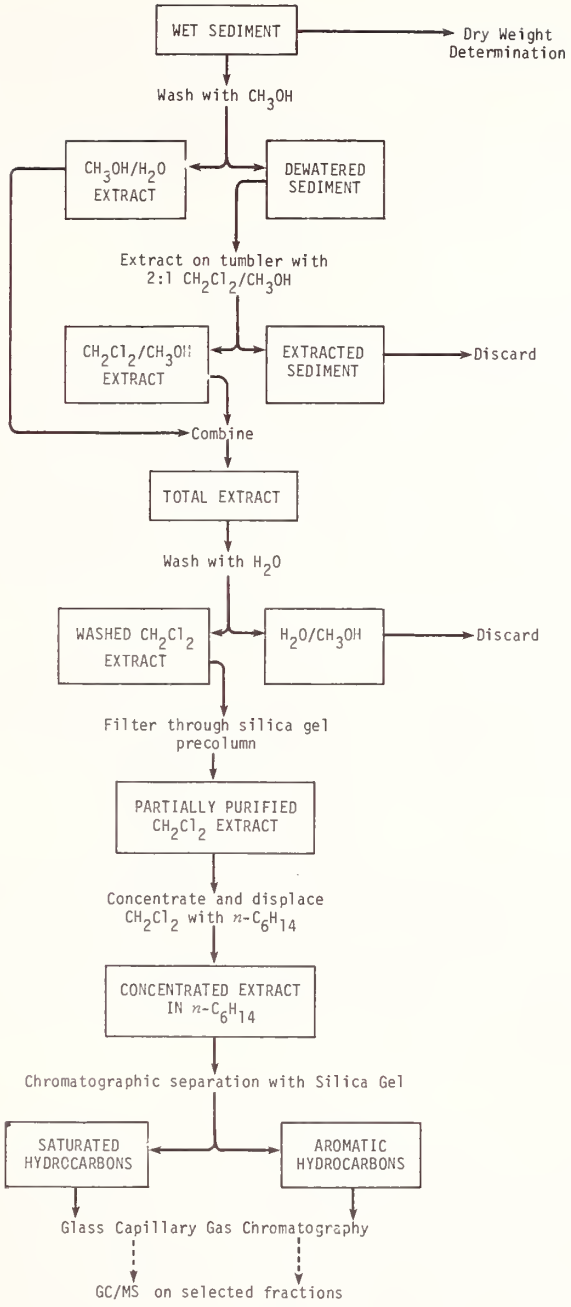

Figure 1. Extraction scheme.

gently agitated by hand to dewater the sediment. The methanol was decanted into a 600-mL beaker and the dewatering step repeated. After adding 100-mL of CH_2Cl_2/CH_3OH (2:1), the bottles were sealed with Teflon screw caps and rolled on a ball-mill tumbler for 16-hours (overnight) at *ca.* 75 rpm. The extract was decanted into the 600-mL beaker containing the methanolic extracts. The sample was rinsed with CH_2Cl_2 and again decanted into the 600-mL beaker. The extraction step was repeated for 6 hours, then again for 16 hours (overnight); all extracts were combined.

The combined extracts were filtered through a fritted-glass filter into a 1-liter separatory funnel, and the beaker and filter were rinsed twice with CH_2Cl_2. The filtrate was gently swirled for 2 min with 500 mL of distilled water to partition the CH_3OH between the aqueous and CH_2Cl_2 phases. After the phases separated, the lower CH_2Cl_2 phase was drained into a 500-mL Erlenmeyer flask. The aqueous phase was back-extracted with 20 mL of CH_2Cl_2, which was added to the previous CH_2Cl_2 phase. After discarding the aqueous phase, the aqueous extraction and the CH_2Cl_2 back-extraction steps were repeated.

E. Extract Clean-Up

The total CH_2Cl_2 extract was filtered through a 19-mm i.d. chromatography column containing 20 mL of silica gel in CH_2Cl_2 covered with a 1-cm layer of sand. The column was eluted with one bed volume of CH_2Cl_2 and the total eluates were collected in a 500-mL Erlenmeyer flask equipped with a 24/40 outer joint. This step removed residual CH_3OH, H_2O, particulates, and gel-forming polar materials which could plug the silica gel column used for chromatography as described below. The flask, equipped with a distilling column, then was placed in a waterbath and the eluate concentrated to about 15 mL. The concentrate was transferred to a 25-mL concentrator tube, an ebullator added, an evaporative concentrator attached, and the extract further concentrated on a tube heater to *ca.* 1 mL. Two mL of hexane were added and the extract was reconcentrated to *ca.* 1 mL.

The extract was chromatographed on a silica gel column according to the procedure reported by MacLeod et al. [5,6]. Two fractions containing, respectively, saturated and unsaturated hydrocarbons were collected in 25-mL concentrator tubes. The fractions were concentrated on the tube heater to 1 mL, transferred to sample vials, and 4 μg of hexamethylbenzene (GC internal standard) were added. The vials were sealed for later GC analysis.

F. Gas Chromatography

The vial contents were automatically sampled and analyzed by GC. High resolution glass capillary columns were employed in the splitless injection mode [8]; see Figure 2 for column parameters and operating conditions. The *n*-alkanes from decane thru hentriacontane (n-$C_{10}H_{22}$-$C_{31}H_{64}$), plus pristane and phytane were routinely quantitated. The aromatic hydrocarbons listed in Table 1 were also routinely quantitated. The detection limit was 1 ng/μL injected; the signal to noise ratio was greater than 10:1.

III. Discussion

Room temperature extraction of hydrocarbons from sediments by the solvent-slurry technique on a ball-mill tumbler was convenient [5,6], but yields were lower than when Soxhlet techniques were used. Several solvent systems and extraction times were evaluated to improve the yields. The highest and most consistent hydrocarbon recoveries were obtained by dewatering twice with methanol followed by the extraction procedure described above. Earlier, we reported that filtering tissue extracts through silica gel was useful in removing polar, gel-forming substances which would otherwise plug chromatographic columns when developed with petroleum solvents [5]. Now, this preliminary cleanup procedure is extended to the sediment extract, where it also serves to remove residual H_2O, CH_3OH, and particulates.

Although reagents and solvents used were of the highest purity available, the best methanol from several sources contained contaminants which could not be removed by redistillation. Therefore, the commercial product that showed the least interference was used. "Distilled in glass" and "pesticide" grades were satisfactory for the other solvents.

The bottle and vial caps were also sources of contamination. Bakelite bottle caps with liners of Teflon bonded to rubber and glued into the cap were found to contaminate extracts through

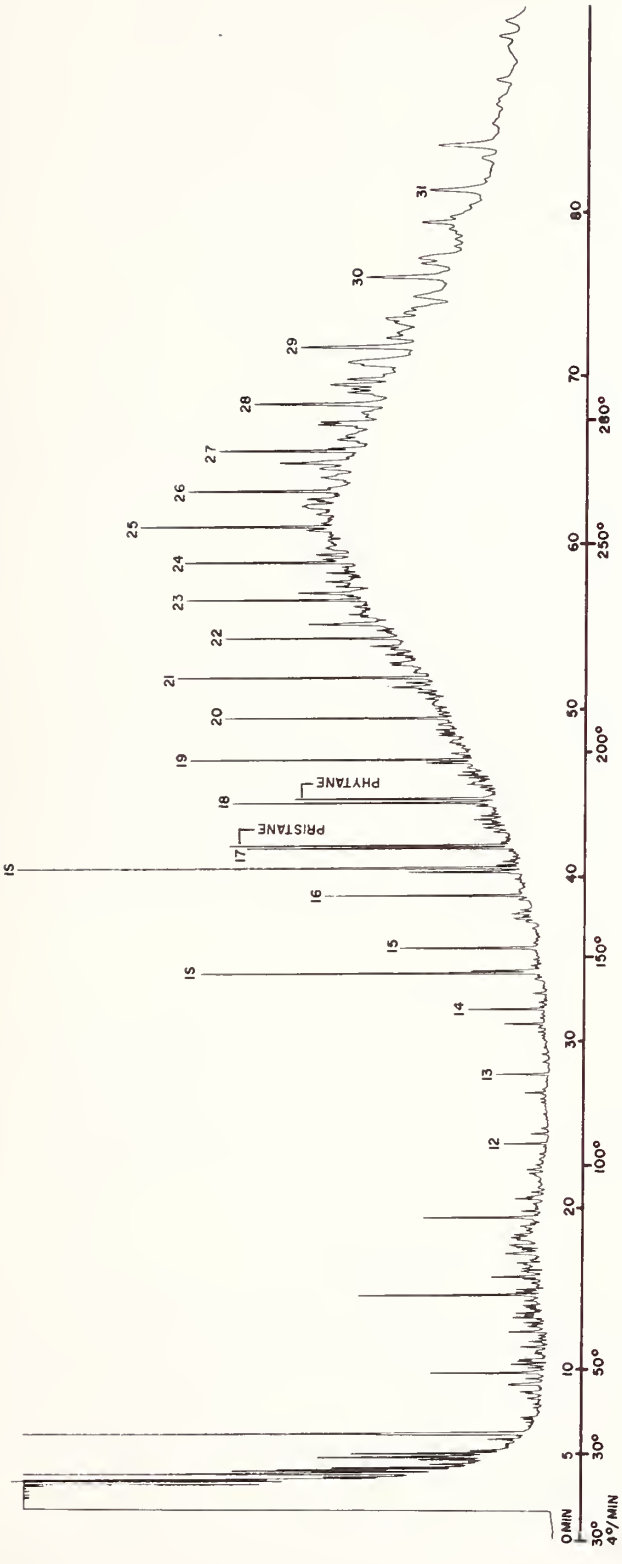

Figure 2. Gas chromatogram of the saturated hydrocarbon fraction from an extract of Duwamish River sediment, Port of Seattle.

Hewlett-Packard 5840A GC, with FID. 30 m×0.25 mm SE-54 WCOT column. Helium carrier pressure 22 psi. 2μL splitless injection (with delay of 18 sec until split (20:1) valve opened). Numbered peaks refer to *n*-alkanes of corresponding chain length.

TABLE 1. *Compounds quantitated in the second silica gel eluate*

o-xylene	fluorene
isopropylbenzene	dibenzothiophene
n-propylbenzene	phenanthrene
indan	anthracene
1,2,3,4-tetramethylbenzene	1-methylphenanthrene
naphthalene	fluoranthene
benzothiophene	pyrene
2-methylnaphthalene	benz[a] anthracene
1-methylnaphthalene	chrysene
biphenyl	benzo[a] pyrene
2,6-dimethylnaphthalene	benzo[e]pyrene
2,3,5-trimethylnaphthalene	perylene

leaching of the rubber backing by the extraction solvent. Use of all-Teflon caps prevented this problem. In the case of the GC vials, the cap septa had a Teflon film bonded to silicone rubber. After the septa were pierced with a syringe needle, the sample solvent leached contaminants into the sample. As a backup, samples were split and sealed into two vials—one for automatic injection and one for reserve. Contamination from both sources gave a characteristic pattern of *n*-alkanes from C_{19} to C_{29} resembling commercial paraffin wax.

Quality assurance steps incorporated into the analytical procedure included: (a) the addition of an alkane and aromatic recovery standard to every sample, (b) analysis of a reagent blank, and (c) analysis of a reagent blank with added standard compounds. The recovery of added *n*-decylcyclohexane was 82%±8% standard deviation ($n=22$); of added 1,3,5-triisopropylbenzene 72%±8% ($n=23$). Average recovery of added *n*-alkanes (decane thru hentriacontane) was 83%. Recovery of individual *n*-alkanes ranged from 75% (*n*-nonacosane) to 97% (*n*-pentacosane).

Five replicate aliquots of a homogenized contaminated sediment were analyzed to assess the reproducibility of the method. Figure 2 shows the glass capillary gas chromatogram for the saturated hydrocarbons. Precision for the alkanes reported ranged from ±2% (pristane) to ±18% (*n*-heptacosane), relative standard deviation.

IV. Acknowledgments

We are grateful to T. I. Scherman, P. G. Prohaska, R. L. Dills, D. D. Gennero, M. Y. Uyeda, D. D. Duncan, and D. G. Burrows for technical assistance. This study was supported by the Energy Resources Program of the National Oceanic and Atmospheric Administration with funds from the Environmental Protection Agency. Reference to a company or product does not imply endorsement by the United States Department of Commerce to the exclusion of others that may be suitable.

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

AUTOMATIC ANALYSIS OF ORGANIC POLLUTANTS IN WATER VIA GC/MS

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The use of gas chromatograph/mass spectrometer (GC/MS) systems for the routine analysis of organic pollutants in water samples has grown significantly during the last few years. However, these systems have had several disadvantages; namely, the need for a highly skilled operator and the need for elaborate sample preparation prior to analysis. A GC/MS system will be described which eliminates these disadvantages.

The recent development of microcomputer controlled GC/MS systems allows simplification of operation for routine analysis. The ability to preprogram gas chromatograph and mass spectrometer conditions as well as to format data output reduces the need for operator attention. The instrument is programmed to automatically tune itself and set up proper GC and mass spectrometer conditions. The instrument will accept raw water samples and automatically prepare those samples for analysis. The subsequent data acquisition and data analysis is carried out automatically. A final report is prepared without the need for operator attention. Several analysis schemes which demonstrate these automatic techniques will be shown.

In addition, results of a real time qualitative and quantitative analysis scheme will be shown. This system automatically defines qualitatively and quantitatively organic compounds as they elute from the GC column. The sensitivity of this method will be demonstrated down to the 60 parts-per-trillion level.

Key words: Gas chromatography/mass spectrometry; microcomputer; routine analysis; trace organics in water.

I. Introduction

The use of gas chromatograph/mass spectrometer (GC/MS) systems for the routine analysis of organic pollutants in water samples has grown significantly during the last few years [1,2]. However these systems have had several disadvantages; namely, the need for a highly skilled operator and the need for elaborate sample preparation prior to analysis.

The recent development of microcomputer controlled GC/MS systems allows simplification of operation for routine analysis. The ability to preprogram gas chromatograph and mass spectrometer conditions as well as to format data output reduces the need for operator attention. The calculator controlled GC/MS can be programmed to automatically tune itself and set up proper gas chromatograph and mass spectrometric conditions. The instrument will accept raw water samples for the analysis of volatile organic compounds. Subsequent sampling, analysis and data reduction can be carried out under control of the calculator. It is thus possible to make GC/MS analysis of volatile organic compounds in water semiautomatic. Indeed the operator need only insert his water sample, and answer four questions asked by the calculator. The sampling process and analysis is completely controlled by the calculator. The analysis of nonvolatile organic compounds can be carried out in this manner, however the liquid/liquid sampling procedure has not been automated.

II. Experimental

A Hewlett-Packard 5992A GC/MS system was used for these analyses. A modified purge and trap apparatus [3] was constructed using pneumatic valves to direct the flow of the helium purge and carrier gases. The valves and trap heater were controlled by the calculator of the

GC/MS system. Data was stored on a Hewlett-Packard 18939A Flexible Disc accessory storage device.

III. Results and Discussion

The analysis scheme is carried out in the following manner. The operator is queried as to which of the following analyses is needed:

Volatile Organic Compounds
Acid Extract
Base/Neutral Extract
Pesticides

The calculator then informs the operator of the proper GC column to be used for the particular analysis requested. The operator then is requested to enter the concentration range of the sample and the sample identification number. After this is completed, the calculator sets up the proper gas chromatographic and mass spectrometric conditions.

If a Volatile Organic Analysis (VOA) has been requested, the calculator will automatically initiate the sampling sequence for this analysis. The sampling sequence consists of a purge cycle and a trap desorb cycle followed by a trap vent cycle.

A stream of helium gas is bubbled through the water sample and then passed through a tube of adsorbent material, Tenax GC. Helium carries the volatile organics out of the water and into the adsorbent trap. The helium flow is then reversed through the trap and shunted into the GC/MS. The trap is heated rapidly to 200 °C to drive the volatile organics into the GC/MS system. Volatile organic compounds pass through a GC column where they are separated prior to entering the mass spectrometer. A mass spectrum for each compound is then collected and stored on a magnetic disc.

To aid in the identification of the unknowns, the mass spectrum representing the top of each GC peak is stored along with an appropriate background spectrum. The background spectrum is then subtracted from the unknown spectrum and the result can be displayed and then searched against a library of known spectra.

The relative retention time of the unknown compound is then compared with the relative retention time of the compound identified by the library search. The correlation of the retention times is combined with the library search correlation to produce a combined correlation factor which accurately defines the quality of the tentative identification of the unknown compound. If the total correlation factor is above 0.75, the identification can be considered positive.

Internal standards are very helpful in establishing accurate retention times. They also are used to accurately define a quantitative report. The total abundance peak heights for each component are compared to the peak height for the internal standard. A response factor table stored in the calculator memory converts these peak height ratios to actual concentrations.

All of the qualtitative and quantitative data is brought together and printed out by the calculator in a Final Report, Figure 1.

The same analysis scheme is carried out when any of the other three types of analysis are requested. However in these cases, the sampling is not controlled by the calculator. The operator must carry out the liquid/liquid extraction and concentration. The sample is then injected into the GC/MS by the operator. The GC/MS analysis and data reduction are then completed by the calculator. The Final Report for these analyses can be combined with the Final Report for the VOA to give a complete profile of the organic constituents of a water sample.

Analysis of several different types of water samples were undertaken to demonstrate the versatility of this method. Figure 2 shows the analysis of water collected from San Francisco Bay. The chromatogram reveals the presence of several compounds in the sample. Ten ppb of internal standard, 2-bromo, 1-chloropropane, has been added to help determine accurate quantitation and relative retention times.

FINAL REPORT

The following compounds were found in sample 256

NAME	SPECT.#	RET.TIME	CORR.	CONC(ppb
CHLOROFORM	33	6.82	0.9958	3.3
1,1,1TRICHLOROETHANE	35	8.50	0.9970	1.0
1,1,2TRICHLOROETHANE	37	12.68	0.9955	1.8
INTERNAL STANDARD	39	13.68	0.9942	1.0
BROMOFORM	41	15.38	0.9652	3.0
TETRACHLOROETHANE	43	17.82	0.8832	2.3
TOLUENE	45	18.12	0.7912	1.0
CHLOROBENZENE	47	20.44	0.7734	1.0

Figure 1. Example of final report format.

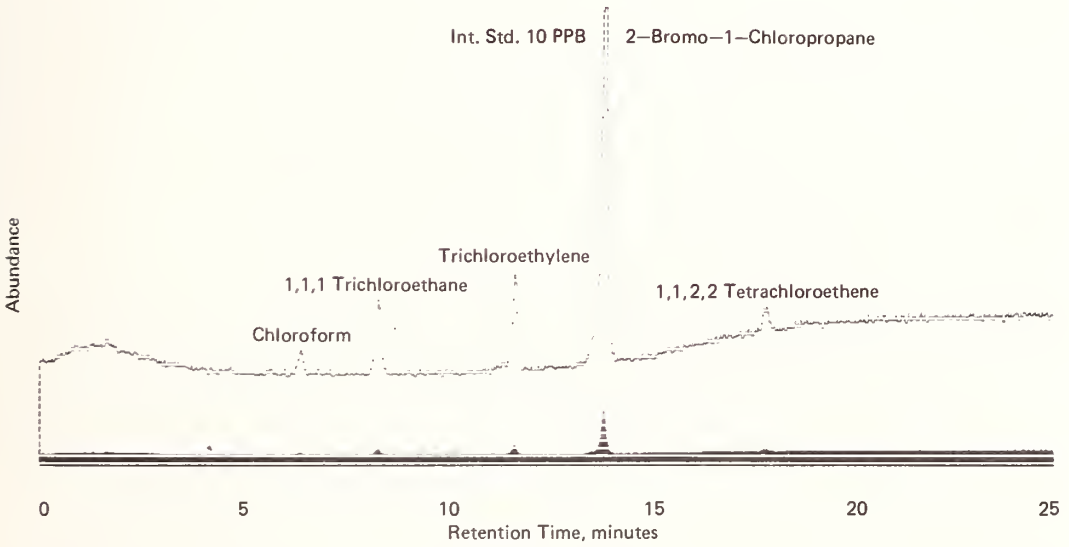

Figure 2. San Francisco Bay water.

Figure 3 is a profile of the volatile organic compounds emanating from an industrial source. A 1 ppb internal standard was added to this sample and the results indicate significant levels of various chlorinated hydrocarbons. A quantitative analysis was made by comparing the total abundance value at the top of each GC peak with the total abundance value for the internal standard.

Quantitative results are shown in Table 1. This example demonstrates the applicability of this method for monitoring the influent and effluent water quality of industrial sites.

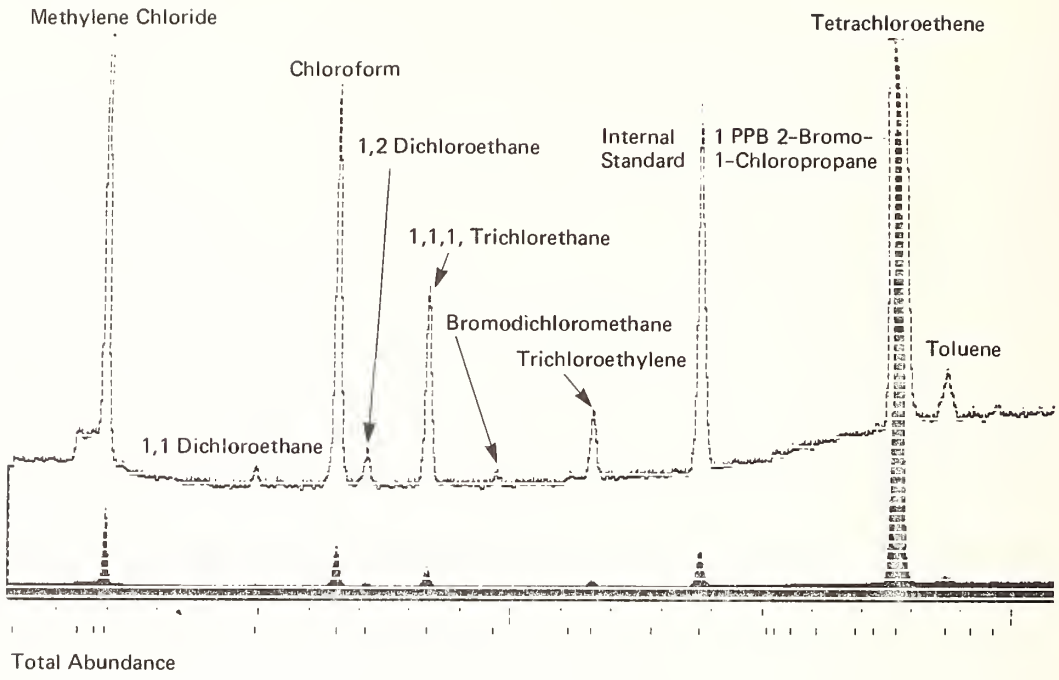

Figure 3. Industrial effluent.

TABLE 1. *Industrial effluent quantitative report*

Compound	Concentration
METHYLENE CHLORIDE	1.9
1,1 DICHLOROTHANE	0.06
CHLOROFORM	1.0
1,2 DICHLOROTHANE	0.1
1,1,1 TRICHLOROTHANE	0.5
BROMODICHLOROMETHANE	0.05
TRICHLOROETHYLENE	0.2
2-BROMO-1 CHLOROPROPANE (INT. STD.)	1.0
1,1,2,2 TETRACHLOROETHANE	17.5
TOLUENE	0.1

If more sensitivity is desired, the mass spectrometer can be operated in the selected ion mode. This mode increases the apparent sensitivity of the instrument by as much as two orders of magnitude and it is possible to detect compounds in the low parts-per-trillion range.

An alternative display method provides qualitative and quantitative results as the compound elutes from the gas chromatograph, Figure 4. Thus the identity and abundance of a GC peak can be printed on top of the peak as it is drawn on the chart paper. This is made possible by a very fast search of a relatively small spectral library. After a successful search is completed the following information is printed on top of the GC peak: compound name, a search correlation factor (how closely the unknown spectrum resembled the library spectrum), the library entry number, the spectrum number of the unknown compound, and the peak height. If the unknown compound is not in the library, "NO MATCH" is displayed. Spectra for all peaks are stored on tape and are available for later off-line display and library searches.

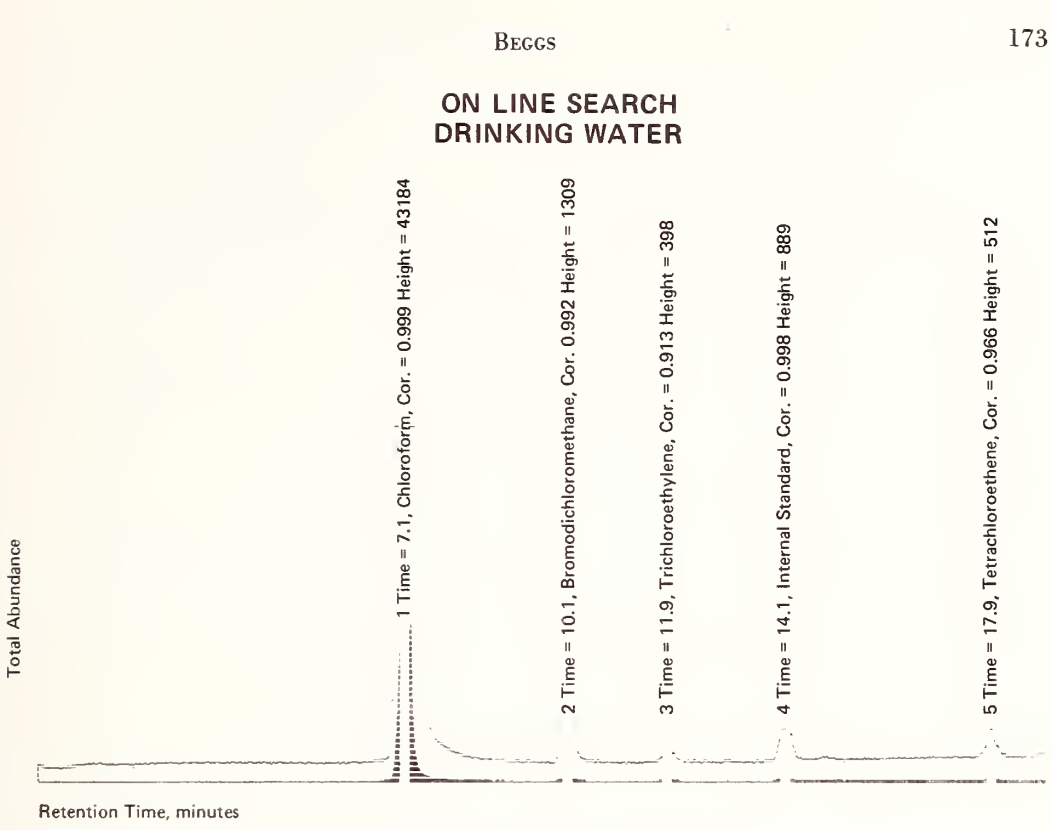

Figure 4. Real time analysis—drinking water.

IV. References

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

A SIMPLE, SENSITIVE METHOD FOR THE QUANTITATIVE ANALYSIS OF CARBON TETRACHLORIDE AND CHLOROFORM IN WATER AT THE PARTS-PER-BILLION LEVEL

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A chromatographic method has been developed and applied to the quantitative analysis of carbon tetrachloride and chloroform in drinking water at the ppb level. The method is simple and rapid with a turnaround time of 20 minutes or less. It is also largely interference free.

Key words: Carbon tetrachloride; chloroform; drinking water analysis; gas chromatography; parts per billion.

I. Introduction

Over the past few years, there has been a great deal of interest shown in identification and quantitation of organic pollutants in water.

Various methods of analysis based on liquid/liquid extractions, head space gas sampling, and adsorption-desorption from traps for sample concentration were evaluated. All these methods proved to be unsatisfactory.

Direct injection of the sample into the chromatograph appears to be the method of choice. Detection limits of this method is 74 ppt CCl_4, and 148 ppt $CHCl_3$. Linearity between the amount injected on the column and G.C. peak area in the range of 740 to 3.7 pg for CCl_4 (Fig. 1), and 1480 to 7.4 pg for $CHCl_3$ (Fig. 3), was achieved. Linearity was also achieved on serial dilution of standards in methanol (Fig. 2).

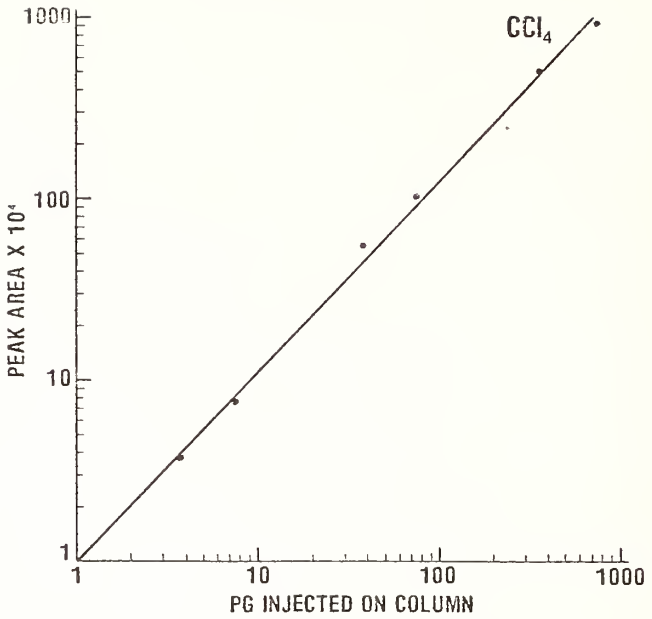
Research has been done on sample storage under various conditions, and the loss of CCl_4 vs. storage time was monitored.

Interferences from various volatile organics such as R-11, R-12, R-22, R-113, CH_2Cl_2, C_2HCl_3 and C_2Cl_4 were investigated. The method proved to be largely interference free. Other volatile organics will be investigated (Table 1).

II. Experimental

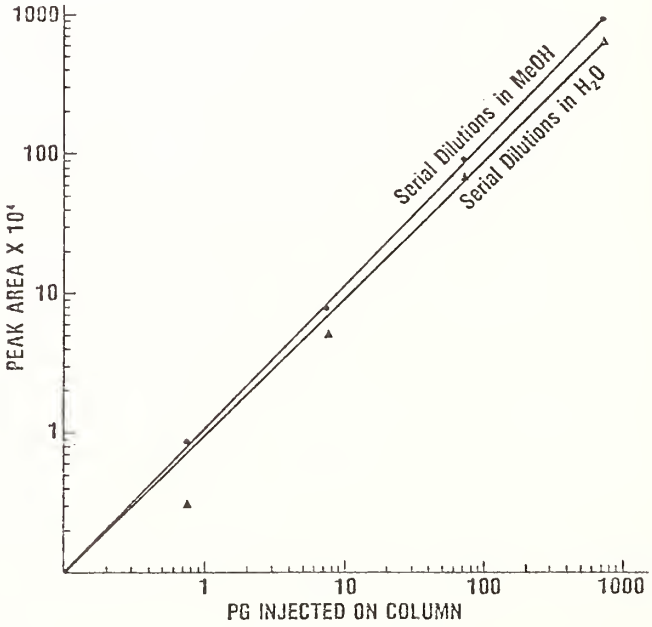
A. GC Analysis Conditions

Instrument	Perkin-Elmer 3920B
Column	4′×4 mm o.d. glass packed with 3% Silar 10C on 80/100 mesh Gas Chrom. Q
Temperatures:	
Column:	Isothermal at 25 °C (kept at this temperature for 7 min by placing a piece of dry ice in the GC oven); then program to 175 °C @ 32°/min and hold for 8 min
EC detector:	300 °C



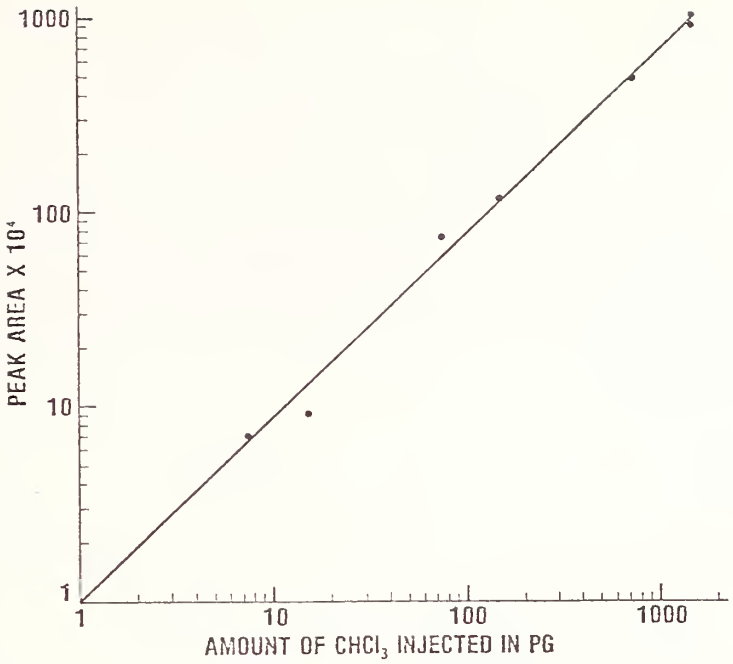
* Using a standard that contains 74 ppb CCl_4, and injecting 10, 5, 1, 0.5, 0.1, and 0.05 ul which amount to 740 to 0.37 pg injected on column, gave nearly a linear plot against peak areas.

Figure 1. Linearity of amounts of CCl_4 injected on column vs. peak areas.*



* Standards that contain from 74 to 0.074 ppb CCl_4 were prepared by serial dilutions in methanol, and water. Plots of amount injected on column vs. peak areas gave nearly a straight line for methanol dilutions but not for water dilutions.

Figure 2. Linearity of dilution plot.*



* As with CCl_4, linearity of amount of $CHCl_3$ injected on column ranging from 1480 to 7.4 pg vs. peak areas, was achieved.

Figure 3. Linearity of amounts of $CHCl_3$ injected on column vs. peak areas.*

TABLE 1.—*Interferences from other low boiling organics at the parts-per-billion level*

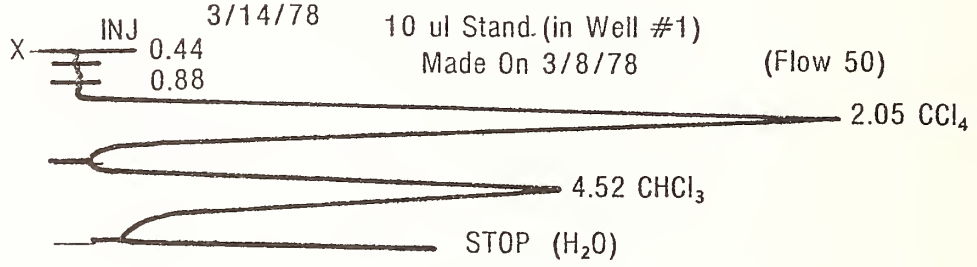
Organic Compound		Interference	
		Yes	No
CCl_2F_2	(R−11)		×
CCl_3F	(R−12)		×
$CHClF_2$	(R−22)		×
$C_2Cl_3F_3$	(R−113)		×
	CH_2Cl_2		×
	C_2H_5Cl		×
	$C_2H_4Cl_2$		×
	$C_2H_3Cl_3$		×
	$C_3H_2Cl_4$	× (slight)	
	C_2Cl_6		×
	C_2HCl_3	×	
	C_2Cl_4		×

Injection port:	150 °C
Flow of carrier gas:	50 mL/min 95% argon-5% methane mixture
Injection size:	10 μl (direct injection)

B. STANDARDS PREPARATION

A standard that contains 74 ppb CCl_4 and 148 ppb $CHCl_3$ is made by adding 5 μL CCl_4 and 10 μL $CHCl_3$ to a 100 mL volumetric flask filled to mark with methanol. Then 1 mL of this methanol solution is pipetted into a 1000 mL volumetric flask of organic-free water (Fig. 4).

Standards containing lower levels of CCl_4 and $CHCl_3$ can be made by serial dilutions of parent standard in methanol.



* As seen above, the column is selected so that CCl_4 and $CHCl_3$ have shorter retention time than water, thus minimizing any interference from water, and maximizing sensitivity to CCl_4 and $CHCl_3$.

Figure 4. G.C. Chromatogram for 10 μL injection of standard containing 74 ppb CCl_4 and 148 ppb $CHCl_3$.*

C. SAMPLES AND STANDARDS STORAGE

Various ways of standards storage were investigated:

1. Storage in volumetric flasks whether at room temperature or 4 °C showed a substantial loss of CCl_4 amounting to as high as 90% in a storage period of 45 days and over 20% in a period of 5 days.
2. Storage in 150 mL serum vials capped with Teflon caps and stored at 4 °C lost 20–25% CCl_4 in 45 days. However, when stored at room temperature, loss was substantial.
3. Storage in 125 mL dark bottles capped with polyethylene caps at 4 °C, lost about 25% CCl_4 in 5 days, but no further loss was monitored after longer periods of time.
4. Storage in 125 mL dark bottles as before with relatively no head space, in which water used was organic free hard water, (11.8 mg $CaCO_3$/liter) yielded no appreciable loss of CCl_4 or $CHCl_3$ over a 1 week period. (Distilled water used to prepare all other standards had only 0.08 mg $CaCO_3$/liter.) It remains to be seen if hardness or oxygen content of water used to prepare standards has anything to do with the loss of CCl_4 and $CHCl_3$. Samples stored in the same way as standards showed no apparent loss of these organics.

III. Conclusion

It is essential for any method, especially one needed for monitoring organic pollutants in drinking water on a regular basis, to be both sensitive and rapid.

This method offers high sensitivity to meet the increasing demand for lower detection limits for organic pollutants in water, and also speeds the analysis of the largest number of samples in the shortest possible time.

A 10 μL injection on column and an electron capture detector yielded sensitivities on the order of 100 parts per trillion. It is important to select a column on which the retention times for CCl_4 and $CHCl_3$ are shorter than that of water. Retention times in this order provide enhanced sensitivity since the response of water can in no way interfere with the responses of the chlorinated compounds. The technique offers an accurate and reliable analysis with a turnaround time of 20 minutes or less.

NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

ANALYSIS OF TIRE CORD EMISSIONS

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Current concern over industrial emissions has led us to study emissions produced by dipped tire cords when they are tensilized. Thermogravimetric analysis and gas chromatographic analysis have been used to study tensilization emissions. A simple device has been designed to produce emissions on a laboratory scale.

Key words: Emissions; gas chromatography; tensilization; thermogravimetric analysis; tire cord; tire cord dip; tire cord finish.

I. Objective

Current concern over industrial emissions has led us to study emissions produced by dipped tire cords when they are tensilized. A survey of the current literature has not shown any work reported concerning these studies. We wish to report a simple method to determine the qualitative and quantitative loss of finish and dip components from tire cord during tensilization. We also wish to report a simple device for producing these emissions on a laboratory scale.

II. Experimental

A. Quantitative Determination

The quantitative loss of finish and dip components from tire cord during tensilization was determined by two methods:

(1) Finish emissions were determined indirectly using a gravimetric/chromatographic analysis. Samples of cord obtained before and after tensilization were extracted with cyclohexane in a Soxhlet apparatus for 16 hours in order to remove finish components. After removal of the solvent, the extracts were weighed and analyzed by gas chromatography and chemical ionization mass spectrometry to identify the chromatographic peaks.

(2) Finish and dip emissions were determined directly using a thermogravimetric analysis. Samples were conditioned at 70 °F and 65% relative humidity at least 48 hours before analysis. The samples were weighed and adjusted to 100% at room temperature to 160 °C and held for 5 minutes; programmed to 270 °C and the run terminated. The weight losses at 160 °C and 238 °C were recorded.

B. Qualitative Analysis

The quantitative analysis of individual finish components in the cord extracts from above also provided an indirect method for identifying the composition of the finish emissions. A method for direct analysis of both finish and dip emissions on a laboratory scale using the apparatus in Figure 1.

Tire cord was placed in the oven, heated to 110 °C, and the emissions were collected for 5 minutes in a dry ice/acetone cold trap. The trap was changed and the oven heated to 238 °C and

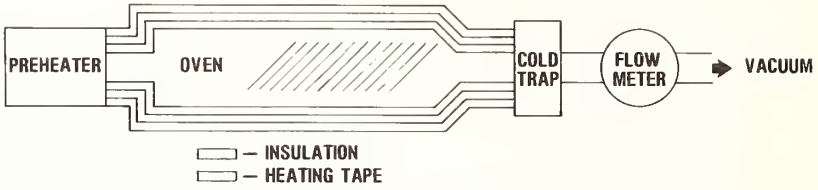

Figure 1. Oven for collection of tensilizations emissions.

again the emissions were collected for 5 minutes. Collected emissions were then analyzed by appropriate techniques.

C. Gas Chromatographic Conditions

Instrument:	Hewlett-Packard 5700A
Column:	6 ft. × 2 mm i.d. glass packed with 3% Dexsil 300
Carrier:	Helium at 20 mL/min.
Oven temperature:	Programmed from 100 °C to 370 °C at 16°/min. and held
Injection temperature:	350 °C
Detection temperature:	400 °C
Detector:	Flame ionization

D. Conditions for Thermogravimetric Aanalysis

Instrument: duPont 951 Thermogravimetric Analyzer

Sample weight:	25 mg
Program Rate:	50 °C/min.
Supression:	90%
Purge gas:	Air
Purge rate:	150 mL/min.
Scale:	90–100% 5 min.

III. Results and Discussion

The thermogravimetric analysis provides a simple, rapid screening method for determining the moisture content and the maximum level of emissions expected from both dipped and undipped tire cord during tensilization. The values obtained are necessarily a maximum due to the higher surface temperature attained by the cord. Below is diagrammed an emissions profile of a commercial polyester tire cord (Fig. 2).

When finish emissions were generated in the laboratory, we also observed numerous degradation products usually consisting of alkyl esters. However, these comprised only a small percentage of the observed emissions. A typical gas chromatogram of the emissions is shown in Figure 3.

The gravimetric/chromatographic analysis not only provides an accurate method of determining the gross loss of finish components from tire cord, but also enables the determination of individual components lost. Tire cord finishes are typically composed of lubricants, such as stearate esters and coconut oil, and various surfactants. We have found that only the more volatile lubricants are lost in significant amounts during tensilization, and that all such lubricants can be quantified by gas chromatography. Below are listed the results from a typical commercial tire cord (Table 1).

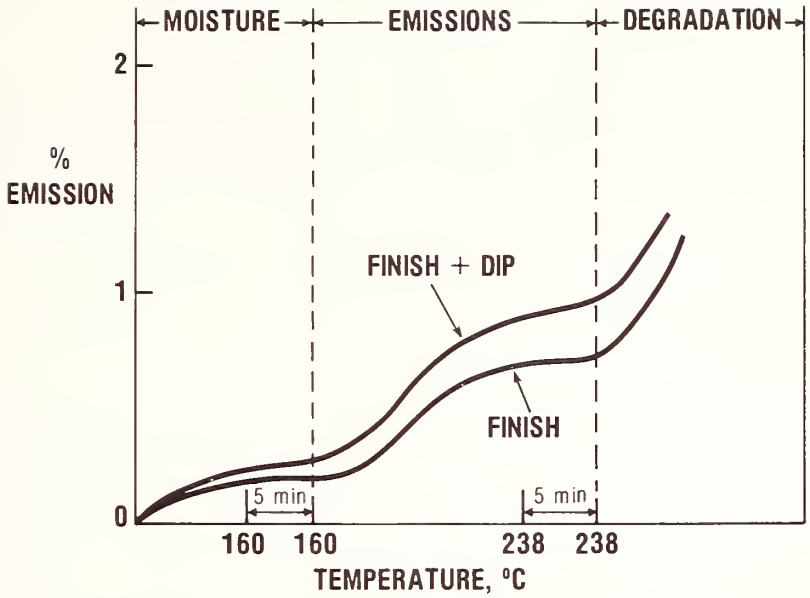

Figure 2. Thermogravimetric analysis in air of commercial polyester tire cord.

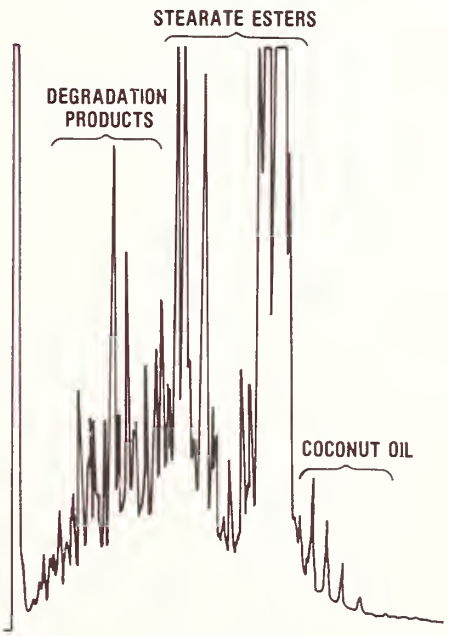

Figure 3. Chromatogram of emissions from a commercial tire cord.

TABLE 1. *Analysis of finish emissions from a commercial polyester tire cord*

Finish level before tensilization	0.75%
Finish level after tensilization	0.37%
% loss of total components	51
% stearate esters lost	61
% coconut oil lost	50

IV. Conclusions

A simple method to determine the amount and composition of finish and dip emissions from tire cord during tensilization has been developed. Using the results, finishes and dips without emissions can be developed.

Thermogravimetric analysis provides an easy method for determining the moisture content of tire cords.

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TRACE ORGANIC ANALYSIS OF WASTEWATER BY LIQUID CHROMATOGRAPHY

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In the "trace enrichment" method, 1–2 liters of the water to be analyzed are pumped through a column containing reverse-phase, octadecyl-silica bonded packing. This packing retains hydrophobic, weakly-polar solutes while allowing highly polar and ionized solutes to pass. After loading the column, distilled water is passed, followed by a linear water-to-methanol gradient. The absorbed solutes are released in decreasing order of their polarity. The ultraviolet absorbance of the effluent is recorded.

The technique has been applied to treated wastewaters, tap waters and natural waters. Characteristic chromatograms are obtained that depend on the pH of the water that is loaded; the lower is the pH, the more is the absorption and subsequent release of humic acids and weak acids of low molecular weight. The effects of various kinds of wastewater treatment are noted; for example, reverse osmosis removes humic acids and ionized compounds, but allows less-polar nonionic compounds to pass; carbon treatment removes humic acids and solutes of low polarity, but not ionic compounds and solutes of intermediate polarity.

Effluent fractions are collected, concentrated, and examined by ultraviolet spectroscopy, as well as liquid chromatography on porous polymer gels and capillary gas chromatography followed by mass spectrometry. Certain compound classes have been identified, such as phthalate esters and phenol ethers of the type $R.C_6H_4.OR'$. A "flush peak" appearing when the column is flushed with distilled water following the loading step indicates the presence of carboxylic acids; its origin will be discussed.

Key words: Liquid chromatography; trace enrichment; wastewater analysis.

I. Introduction

Many publications have appeared concerning the volatile organic compounds present in natural and polluted water. They have been removed and concentrated by various means, including solvent extraction [1], adsorption on a porous solid [2,3], and "sparging" or vaporization in a current of gas [4,5]. Further analysis has been made by gas chromatography, with identification in many cases by mass spectrometry.

The nonvolatile organic impurities, on the other hand, are much more difficult to characterize, and few publications have appeared in this field. Most of the organic carbon in natural waters and wastewaters is in the form of compounds of high molecular weight, known loosely as humic substances. These compounds have the character of colloidal electrolytes. They can form micelles and solubilize small molecules of nonpolar compounds, and they can also form stable complexes with trace metals. Not only are they hard to characterize themselves, but they make the characterization of simpler substances more difficult.

One approach to the analysis of trace organic compounds in water is the "trace enrichment" method [6–9]. A large volume of water is pumped through a small column packed with a chromatographic adsorbent, generally a "reversed-phase" adsorbent like porous silica bonded with octadecyl groups. Trace organic compounds are retained and concentrated at the entrance to the column. Then this same column is used to perform a chromatographic separation. A solvent is passed that removes the adsorbed material in a selective manner, the more polar, more weakly bound compounds being removed first, and the less polar compounds later. It is convenient to use a solvent that is miscible with water and to use it in a gradient mode, increasing the proportion of the extracting solvent continuously with time according to a predetermined program. Methanol,

ethanol and acetonitrile are suitable solvents. As the liquid emerges from the column it passes through a detector, generally one that follows the absorbtion of ultraviolet light at 254 nm. The absorbance is recorded as a function of time and volume.

II. Experimental

A. Apparatus

The apparatus we used is shown schematically in Figure 1. Two liquid-chromatography pumps (Waters Model 6000A) feed into a mixing chamber and then into a stainless-steel column, 50 cm × 1.0 cm internal diameter, which is packed with Waters "Bondapak-C_{18}", a porous silica, particle size 37–75 micrometers, coated with octadecyl groups. One of the pumps carries water, the other methanol. The two pumps are controlled by a solvent programmer, Waters Model 600. The pump carrying water has an inlet valve that allows one to pass either distilled water or the water which is to be analyzed.

A large column packed with coarse adsorbent was used, in preference to the popular microparticulate reversed-phase columns, because, first, we needed the capacity of a large column to process large sample volumes, and second, we found that microparticulate columns became irreversibly plugged after pumping large volumes of secondary sewage effluent. The Bondapak column, by contrast, passed hundreds of liters of sewage effluent with no increase in back pressure and no permanent change except that, after many months of use, approximately 100 mg of white waxy material could be extracted from the column by chloroform.

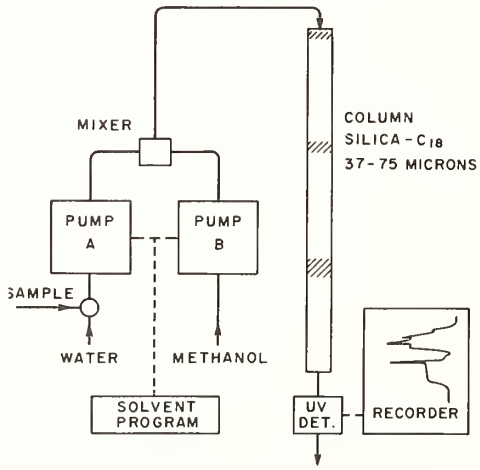

Figure 1. Apparatus for trace enrichment.

B. Sample Preparation

Water samples were degassed and filtered before pumping through the column. They were heated to 80°–90 °C and then filtered under suction, first through a coarse glass fiber filter, then through a fine glass fiber filter (Whatman GF-F, pore size 0.7 micrometers). (At first we used polycarbonate membrane filters, but we stopped using them after we found that they retained naphthalene from aqueous solutions; see paper by D. A. Kurtz in this Symposium.) Dissolved air and carbon dioxide were lost during the suction filtration. The pH of the filtrate was adjusted to 7.8 by adding 0.5 mol/L nitric acid.

C. OPERATION

Starting with the column filled with distilled water, the inlet valve of pump "A" (see Fig. 1) was turned to admit the sample, and a volume of 1–3 liters was pumped through the column at 7 mL/min. Then the inlet valve was turned to admit distilled water, which was passed for 15 minutes at 5 mL/min. The ultraviolet absorption was meanwhile recorded. After the 15-minute water flush, a linear gradient from pure water to pure methanol was begun; the gradient period was 30 minutes, the flow rate 5 mL/min. Finally, pure methanol was passed until the absorbance returned to its base line; this took some 20 minutes.

Fractions of the effluent were collected, each about 30 mL; they were concentrated on a rotary vacuum evaporator to a final volume of 1-2 mL. The concentrated fractions were used for toxicity tests (see below) and for further chromatographic examination.

Blank experiments were run from time to time, and generally showed a very small peak near the end of the water-to-methanol gradient. For low blanks it was essential to use methanol of high quality (Burdick and Jackson "Distilled-in-Glass") and water that had been passed through a large column of washed Amberlite XAD-2 resin and then redistilled.

III. Results

A. CHROMATOGRAPHIC

A typical chromatogram is shown in schematic form in Figure 2. The absorbance starts to rise as soon as the sample solution reaches the detector, then climbs slowly to a limit. Here, highly-polar and ionic compounds are passing through the column with only weak adsorption. When distilled water is passed to flush the column, the absorbance rises very sharply as soon as the water front reaches the detector; then it falls gradually to the base line. Through various tests with known solutions we think that this "flush peak" is due in part to salts of weak aromatic

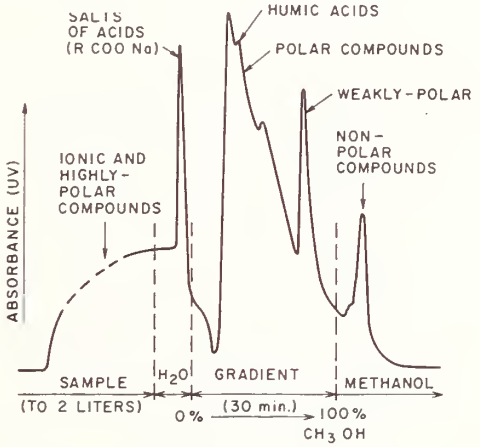

Figure 2. Chromatogram obtained in trace enrichment (schematic).

acids, like sodium benzoate, that are held on the column in the presence of electrolytes. (Solutions of sodium benzoate in pure water or deionized water do not give "flush peaks").

When the first increments of methanol reach the detector the absorbance starts to rise, and rises to a high, sharp peak that indicates humic acids. The solution emerging from the column at this point is a strong yellow-brown color. With about 30% methanol at the column exit the color changes to green and remains blue-green for some 2 minutes. The green compound has a well-defined absorption spectrum in the visible, with a maximum at 630 nm; we have seen it in nearly all wastewaters.

The chromatogram of an actual secondary-treated sewage is shown in Figure 3. The "humic acid" peak, with its associated effluent color, extends over the whole gradient. Its width depends on the pH; the higher the pH, the more colored humic material emerges in the flush peak or before, and the narrower is the peak in the gradient. At lower pH values the gradient peak is broader and shows more secondary maxima like those indicated in Figure 2. Presumably these secondary maxima are caused by weak acids having pK values in the range 5–7.

The ubiquitous nature of humic substances is shown by Figure 4, the chromatogram of water from a spring in a forested valley where there was no human activity. (The small peak at the end of the gradient is the solvent blank.)

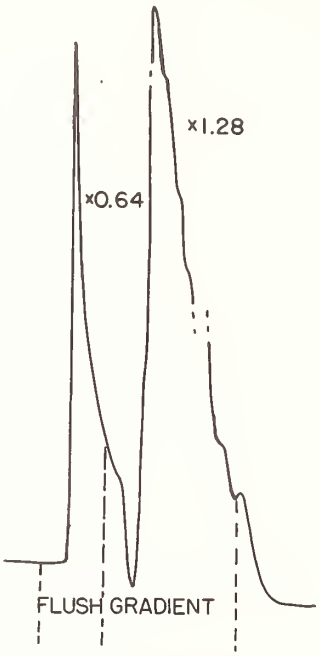

Figure 3. Chromatogram of 1 liter of filtered secondary effluent from Los Angeles County, adjusted to pH 7.8. Ordinates are absorbance at 254 nm.

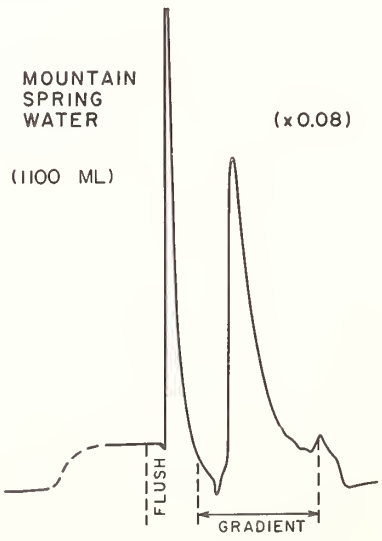

Figure 4. Chromatogram of a "pristine" water. Ordinates, absorbance at 254 nm with full-scale deflection 0.08 unit. The small peak at end of gradient is due to the solvent blank.

B. TOXICITY TESTS

One purpose of this investigation was to identify the fractions of wastewater that were toxic and thus to evaluate, from the health point of view, the effectiveness of various tertiary treatments. Our fractions were tested for toxicity by their effects on human blood cells. Drs. C. Solomons, D. A. Norris and W. L. Weston, of the University of Colorado Medical Center, performed these tests, which will be described elsewhere. They showed that the highest toxicity came near the end of the water-to-methanol gradient, and that the brown humic fractions, which make up most of the mass of the organic compounds retained by the column, are relatively non-toxic.

C. EVALUATION OF TERTIARY TREATMENTS

By comparing the chromatograms of water before and after treatment, one can form an idea of the effectiveness of the treatment. Figure 5 shows the effects of two different kinds of treatment, performed in an experimental way at the pilot plant of the Metropolitan Denver Sewage Treatment plant. Reverse osmosis removes the dissolved salts and suppresses the flush peak; the membrane also filters out, perhaps on a size-exclusion basis, most of the humic acids. A large peak appears before the end of the gradient with minor peaks afterwards, corresponding to substances of low polarity, which *a priori* are likely to be toxic, though we had no opportunity to test their toxicity at the time. Carbon treatment, which is commonly used in tertiary treatment plants today, is more effective at removing compounds of low polarity but does not remove electrolytes. Other tests have shown that carbon lets pass a significant fraction of the humic acids that appear early in the gradient.

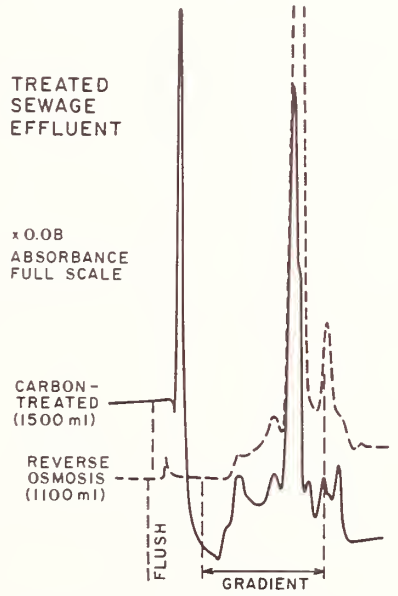

Figure 5. Chromatograms of Denver sewage effluent after treatments as shown.

D. SECONDARY CHROMATOGRAPHY AND CHEMICAL IDENTIFICATION

The Bondapak-C_{18} column achieves only a partial separation of the tens of hundreds of different chemical species that may exist in wastewater, and above all, it does not effectively separate low-molecular-weight from high-molecular-weight compounds. Repeatedly we have injected the concentrated fractions into gas chromatographs with packed or capillary columns, before and after derivatization with dimethyl sulfate, and the only compounds definitely identified

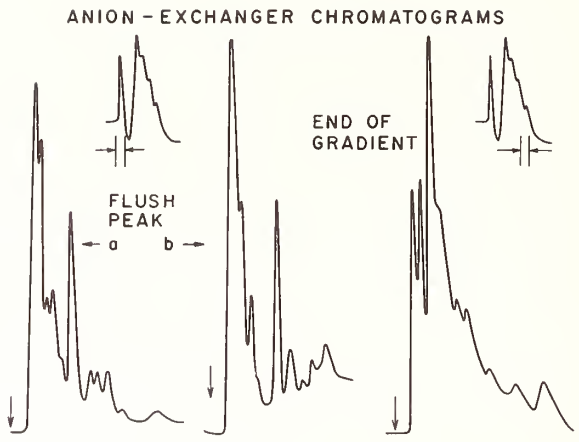

Figure 6. Chromatograms of fractions from the trace enrichment run, after concentration. The column was packed with the anion-exchange resin Aminex A-25, particle size 17 micrometers, a strong-base anion-exchanger. The eluent was 0.1 mol/L ammonium acetate in 25% ethyl alcohol, adjusted to pH 6.5.

have been surfactants and plasticizers (which are found everywhere in industrial environments). It seems that further separations are needed before we can hope to identify and measure specific chemical compounds.

One promising way to achieve this further separation is by liquid chromatography on an anion-exchange resin. The results of preliminary tests on such a resin are shown in Figure 6. An attractive feature of these resin columns is that they retain humic acids very strongly indeed at pH values above 6, effectively filtering out the high-molecular-weight materials. We can get an idea of the acidic or basic nature of the compounds that give the peaks in Figure 6 by using eluents of different pH values and different salt concentrations. It is not only anions that are held by these resins, but neutral molecules as well.

The mass of solute in any one of the peaks in Figure 6 is very small indeed. To have any hope of identification by mass spectrometry or otherwise, it is imperative to process much larger samples. One way to do this is to start with freeze concentration. Another way is to load columns of Bondapak-C_{18} on-site, pumping large volumes of wastewater through them. Most of the highly-polar material will be lost, but we can expect to retain those fractions of low polarity that are potentially the most toxic.

IV. Acknowledgment

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ANALYSIS OF SOIL AND SEDIMENT TO DETERMINE POTENTIAL PESTICIDE CONTAMINATION OF A WATER SUPPLY IMPOUNDMENT

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Analysis of soil, sediment, and water for insecticides, miticides, and fungicides was carried out to assess the potential for contamination of a water supply impoundment. The drainage area which feeds the impoundment is primarily agricultural with land in orchards or row crops. The impoundment is the source of water for a community of approximately 25,000.

An analytical method was developed to allow for the determination of eleven pesticides in a single extract. The compounds were chosen from about 25 compounds known to have been used in the area. The compounds selected were those most extensively used and/or known to be persistent. The compounds determined were the organochlorides: lindane, dieldrin, endrin, DDT and metabolites, and kelthane; the organophosphates: malathion, parathion, and guthion; the carbamates: folpet and captan; and tetradifon.

All of these compounds were determined by gas chromatograph with an electron capture detector after extraction. Using an OV-17/QF-1 column all compounds except captan and folpet could be detected separately, although kelthane and malathion were poorly resolved as were p,pDDE and dieldrin.

The soil and sediment samples were extracted with acetone. The pesticides were flooded out of the acetone and extracted with 10 percent diethyl ether in petroleum ether. The water samples were extracted with 1:1 methylene chloride:hexane mixture. The detection limits using the method were 0.004 μg lindane per gram of soil or sediment; 0.009 μg DDE, DDD, DDT, dieldrin, endrin; 0.04 μg kelthane, malathion, parathion, captan/folpet; 0.08 μg tetradifon; and 4 μg guthion using 50 g of air-dried soil or sediment. For the water samples the sensitivity was about a factor of 100 lower using 500 mL of water and concentrating the final extract to 5 mL.

Soils contained primarily dieldrin and DDE, while sediment contained dieldrin and DDD. None of the pesticides were detected in water. Recoveries were on the order of 75 to 95 percent for all compounds from both clay-like and humus-type soils.

Confirmatory studies were carried out using flame photometric detector, *p*-values, and saponification.

The method proved to be efficient, simple, and straightforward in providing reliable results. The method could be easily run on a routine basis by a technician after minimal training.

Key words: Carbamates; gas chromatography; organochlorides; pesticides; sediment and water.

I. Introduction

The reported work was part of the study of a recent water supply impoundment in southern Illinois. The area surrounding the impoundment and drained by the watershed feeding the impoundment is under intensive agricultural usage. The drainage area is orchard land or row crop fields. There was concern about the potential for contamination of the water with pesticides due to a past history of extensive usage. Review of historical data indicates that about 25 pesticides had been used at various times and in various amounts. Based on this data a series of 11 compounds were chosen for analysis. The compounds were chosen based on expected persistence and/or amount applied. The compounds selected for analysis were lindane, dieldrin p,p′DDT and metabolites p,p′DDD and p,p′DDE, endrin, parathion, malathion, kelthane, captan, folpet, tetradifon, and guthion.

An analysis scheme which could be used to determine all the pesticides listed above was developed to allow for more rapid sample analysis and to eliminate the need for multiple sample

manipulation. This involved a consideration of two separate phases: (1) an extraction and clean-up procedure that resulted in efficient recovery (better than 80%) for all compounds and (2) a gas chromatographic method that resulted in resolution of all compounds.

Samples consisted of soil plugs from fields and sediments from the reservoir bottom. Samples were stored in glass bottles and frozen until analyzed. There were 71 soils and 37 sediments.

The general procedure developed consists of the following steps:

(1) isolation of pesticides from sample matrix;

(2) transfer of pesticides to a less polar solvent (this step also serves as a clean-up step);

(3) pre-separation of pesticides into groups (this was only performed in select cases due to the efficiency of the gas chromatographic method);

(4) separation and quantitation of the individual pesticide present (by electron capture gas chromatography);

(5) identification (usually chemical reaction followed by G.C.).

II. Experimental Work

A. Extraction Method

In order to determine the best method for extracting all compounds of interest from the samples, an evaluation of several solvent systems was made. Review of the literature indicated that procedures for individual compounds [1], multiple organochloride residues [2–9], multiple organophosphorous residues [10,11], and mixed residues [12–14] were available. However, no system was reported which included all the compounds of interest to us. The solvents considered for extraction were based on literature review, standard procedures [15,16], and our experience. The solvent systems considered for evaluation were hexane, 10% ethyl ether in hexane, acetonitrile, 50% methylene chloride in hexane, and acetone.

Our objective was to achieve a balance between maximum extraction efficiency for the compounds of interest with minimum extraction of interfering components from the matrix. Our approach was somewhat reversed of the more conventional method in that we evaluated the amount of interfering substances obtained using the various solvents to extract a soil sample known to have not been under agricultural usage for at least 15 years. Surprisingly all solvent systems showed the same interferents, all of which were primarily in the early part of the chromatogram and would interfere with lindane, kelthane, malathion, and parathion primarily.

All solvents were evaluated using 50 g of air dried soil wet with 50 mL of 95% ethyl alcohol in a 500 mL wide mouth Erlenmeyer flask. One hundred mL of the solvent to be tested was added. The samples were allowed to set for 24 hours with occasional swirling. Samples were vacuum filtered through Whatman #2 paper in a porcelain filter. At this point the various solvents were treated differently. The hexane and hexane mixtures were air evaporated in a sand bath at 30 °C to about 10 mL. Additional 10 mL portions of hexane were added and evaporated several times to completely remove ether and methylene chloride from the mixtures. Samples were extracted with 5 portions of acetonitrile in a 2:1 ratio of acetonitrile to hexane in glass separatory funnels. The acetonitrile extracts from the hexane solvents, the acetonitrile from the soil extract, and the acetone from the soil extract were all treated the same. Four hundred mL of distilled water and 100 mL of saturated sodium chloride were added to the organic solvents in 1 liter separatory funnels. This mixture was extracted with 100 mL of 10% diethyl ether in petroleum ether. The aqueous phase was discarded and the ether mixture washed 4 times by pouring 100 mL portions of distilled water through it. After discarding the water washings, the ether mixture was transferred to a beaker and air evaporated to approximately 5 mL. The ether was dried by adding a small amount of anhydrous crystalline sodium sulfate, then transferred to graduated 25 mL Erlenmeyer and diluted to 10 mL with hexane. A 5 μL portion was injected into the gas

chromatograph. A typical chromatogram is shown in Figure 1; the arrows indicate the position of some of the pesticides of interest.

The extracts were put through a florisil column, approximately 20 g in a 25 mm i.d.×50 cm column with 1.2 cm of anhydrous granular Na_2SO_4. The column was eluted successively with 120 mL of 6%, 15% and 50% diethyl ether in petroleum ether. The 6% fraction after concentration to 10 mL was almost identical to the untreated sample with slight reduction in peak height for some of the early peaks and the longest retention time peak missing. The 15% fraction showed much lower peaks in the early portion of the chromatogram with some interference for those compounds expected in the 15% fraction. No peaks of any significant size were observed in the

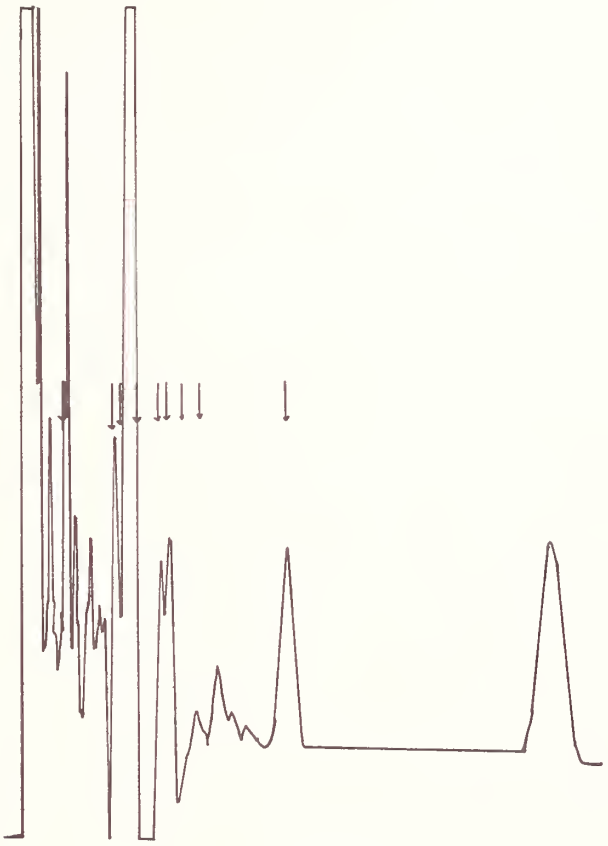

Figure 1. Soil blank.

50% fraction. The results indicated that the conventional florisil method would not be effective for separation of the pesticide from potentially interfering components. Although other absorbents besides florisil, i.e., silica gel [17–19] alumina, celite [18,19], carbon [19,20], etc., have been used for clean-up, it was decided to not pursue this approach because of anticipated problems with complex solvent systems to obtain complete recovery of all pesticides.

We attempted making dilutions of the extract to determine under what conditions the interfering components did not present a problem. At a dilution of 1 to 10 a relatively clean chromatogram was obtained as shown in Figure 2. The major peak shown would obscure the parathion. This was considered acceptable because the interferant did not respond using flame photometric procedure as seen in Figure 3. Since acceptable sensitivity was obtained using a 1/10 dilution of the extract, it was decided to use this approach rather than to attempt to develop a suitable clean-up procedure. The sensitivity expected is shown in Table 1.

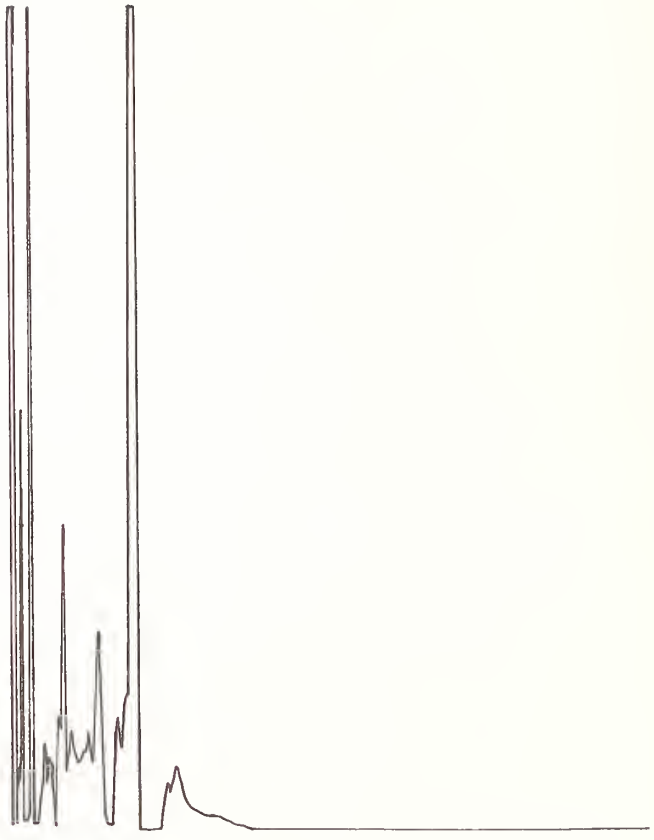

Figure 2. Soil blank 1/10 dilution.

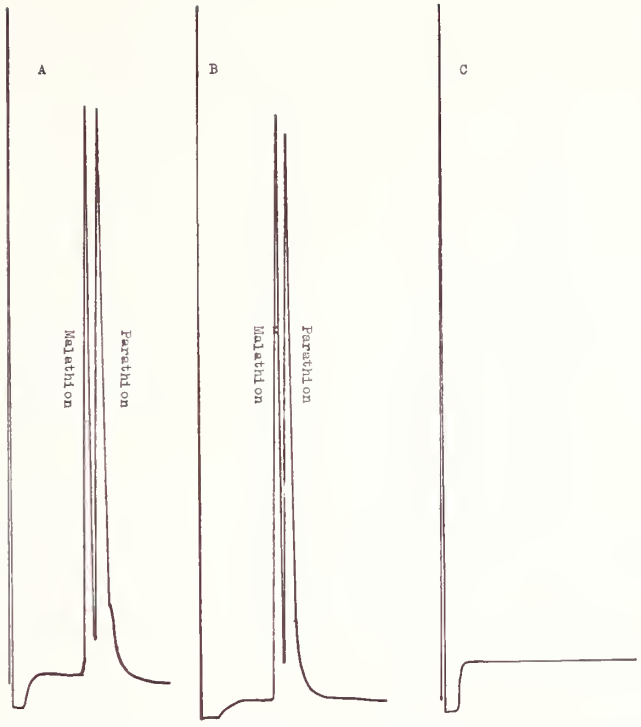

Figure 3. Flame photometric detector. (A) Standard; (B) Spiked soil; (C) Blank.

TABLE 1. *Detection limits*

Compound	Cond. (μg/g)
Lindane	0.004
Dieldrin, endrin	0.009
Kelthane, malathion	
Parathion, captan/folpet	0.04
Tetradifon	0.08
Guthion	4.0/0.4

The two extractive solvents most attractive to us were acetonitrile and acetone because of the potential for direct flood-out of the pesticides after extraction. Work was performed using acetone. The results indicated satisfactory recovery for all compounds of interest except captan and folpet. Recovery data for spiked soils and sediments are given in Table 2. This system was chosen to be used for the samples.

Studies were carried out on the effect of the contact time between sample and solvent. Times of 4, 8, 12 and 24 h were used for contact. No differences in recovery were noted using the various times. For reasons of convenience and operational efficiency, a 24 h contact time was selected.

B. GAS CHROMATOGRAPHIC

A review of pesticides retention times [21–24] indicated that captan and folpet would provide the most difficult separation. A method for separating captan and folpet is available using 3% XE-60 at 195–200 °C [25] and QF-1 or XE-60 [26], however the behavior of other pesticides

TABLE 2. *Recoveries*

Compound	% Recovery from Soil	Sediment
Lindane	80%	83
Kelthane	93	61*
Malathion	98	76*†
Parathion	95	--*†
p,p'DDE	80	73
Dieldrin	82	76
Captan/folpet	30	56
Endrin	97	106
p,p'DDT	80	78
Tetradifon	112	111
Guthion	98	110

* Interfering peak.
† 102 and 97% by flame photometric.

of interest was unknown using XE-60 and incomplete resolution expected using QF-1. Based on the similarity of the two compounds relative to behavior, chemistry, and toxicity, it was decided to report as captan/folpet without concern about determining which was actually present.

Based on reported retention times and experience, a 1.25% OV17/1.95% QF-1 on 100–120 mesh supelcoport was used for all analyses. A typical chromatography containing the compounds of interest is shown in Figure 4. Guthion is not shown since its retention times is about 2 times that for tetradifon or about 60 min. Potential interference between the organophosphorous compounds malathion and parathion and the organocholoride compounds kelthane, heptachlor expoxide, γ-chlordane and α-chlordane is possible. This problem was solved by the use of a flame photometric detector in the phosphorous mode. The operating conditions are shown in Table 3 for the electron capture detector. Similar conditions were used for the flame photometric detector on a Tracor 2200.

A 20 cm column packed with 3% SE-30 was used to decrease the retention time and improve the sensitivity for guthion. Using the short column a retention time of about 10 min was obtained and the sensitivity was improved by a factor of 10 over that observed for the normal operating conditions.

III. Procedure

Based on the results of the experimental studies, a final procedure was developed which was found to be acceptable for the samples. A 50 gram sample of air dried soil or sediment was weighed into a 500 mL wide mouth Erlenmeyer flask. The sample was wet with 50 mL of ethyl alcohol and 100 mL of acetone added. The samples were allowed to set at room temperature for 24 h with occasional swirling. The solvent was vacuum filtered through Whatman #2 paper in a porcelain buchner funnel. The acetone extract was transferred to a 1000 mL separatory funnel. The acetone was diluted with 400 mL of distilled water and 100 mL of saturated NaCl. The acetone-water mixture was extracted with 100 mL of 10% diethyl ether in petroleum ether. The acetone-water mixture was discarded and the ether mixture washed 3 times by passing 100 mL of distilled water through it. After discarding water washings, the ether is transferred to a 250 mL beaker and allowed to air evaporate under a hood on a sand bath maintained at 30 °C. When the sample evaporated to approximately 5 mL, anhydrous sodium sulfate was added to remove moisture, and the solvent transferred with hexane washings to a graduated 25 mL Erlenmeyer flask. The sample was diluted to 10 mL and a 1/10 dilution made. A 5 μL portion of the 1/10 dilution was injected into the gas chromatograph. Appropriate dilutions were made where

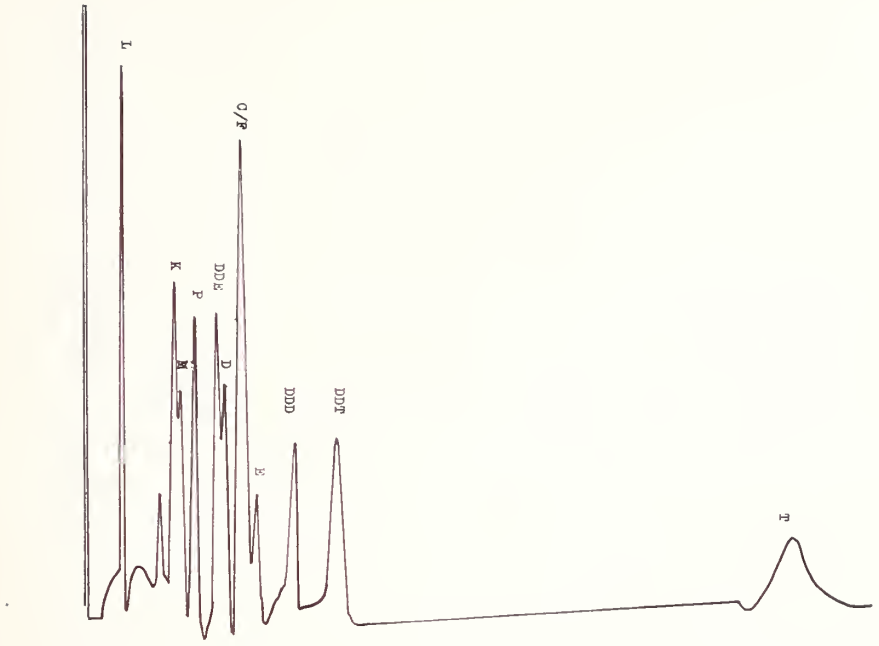

Figure 4. Standards: L–Lindane 0.01 ng/μL; K–Kalthane 0.10 ng/μL; M–Malathion 0.10 ng/μL; P–Parathion 0.10 ng/μL; DDE–p,p'DDe 0.02 ng/μL; D–Dieldrin 0.02 ng/μL; C–Captain 0.10 ng/μL; F–Folpet 0.10 ng/μL; E–Endrin 0.02 ng/μL; DDD–p,p'DDD 0.02 ng/μL; DDT–p,p'DDT 0.02 ng/μL; T–Tetradifon 0.10 ng/μL.

TABLE 3. *Instrument operating conditions*

Instrument	Bendix 2500
Detector	Ni^{63} electron capture
Column	1.5 m×4 mm (i.d.) glass
Packing	1.25% OV17/2.0% QF-1 on 60–80 mesh supelcoport
Carrier gas	N_2–30–40 mL/min
Temperatures	Injector–200 °C
	Oven–190 °C
	Transfer–220 °C
	Detector–275 °C

necessary. Typical chromatograms for a soil are shown in Figure 5 and for a sediment in Figure 6.

Samples containing high levels of p,p'DDE or showing evidence of a shoulder on the DDE peak were run through a florisil column. The column was eluted with 120 mL of 6% diethyl ether in petroleum ether followed by 120 mL of 15% diethyl ether in petroleum ether to separate the p,p'DDE and dieldrin.

The eluants were air evaporated to approximately 5 mL and treated as the original extracts relative to dilution and G.C. Typical chromatograms of a 6% fraction and a 15% fraction are shown in Figure 7 and of the sample before florisil in Figure 8.

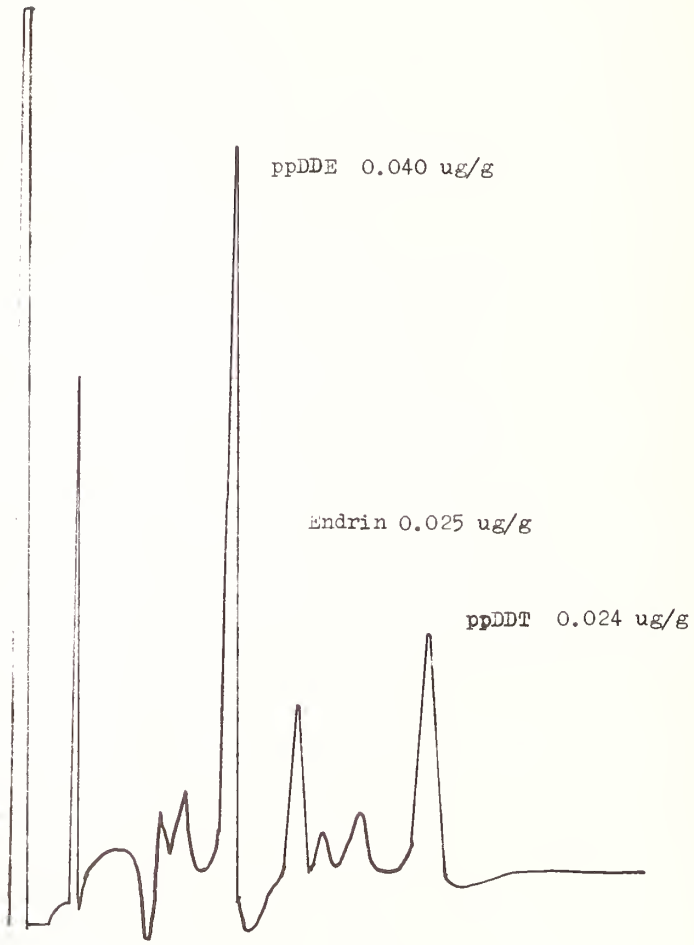

Figure 5. Soil extract.

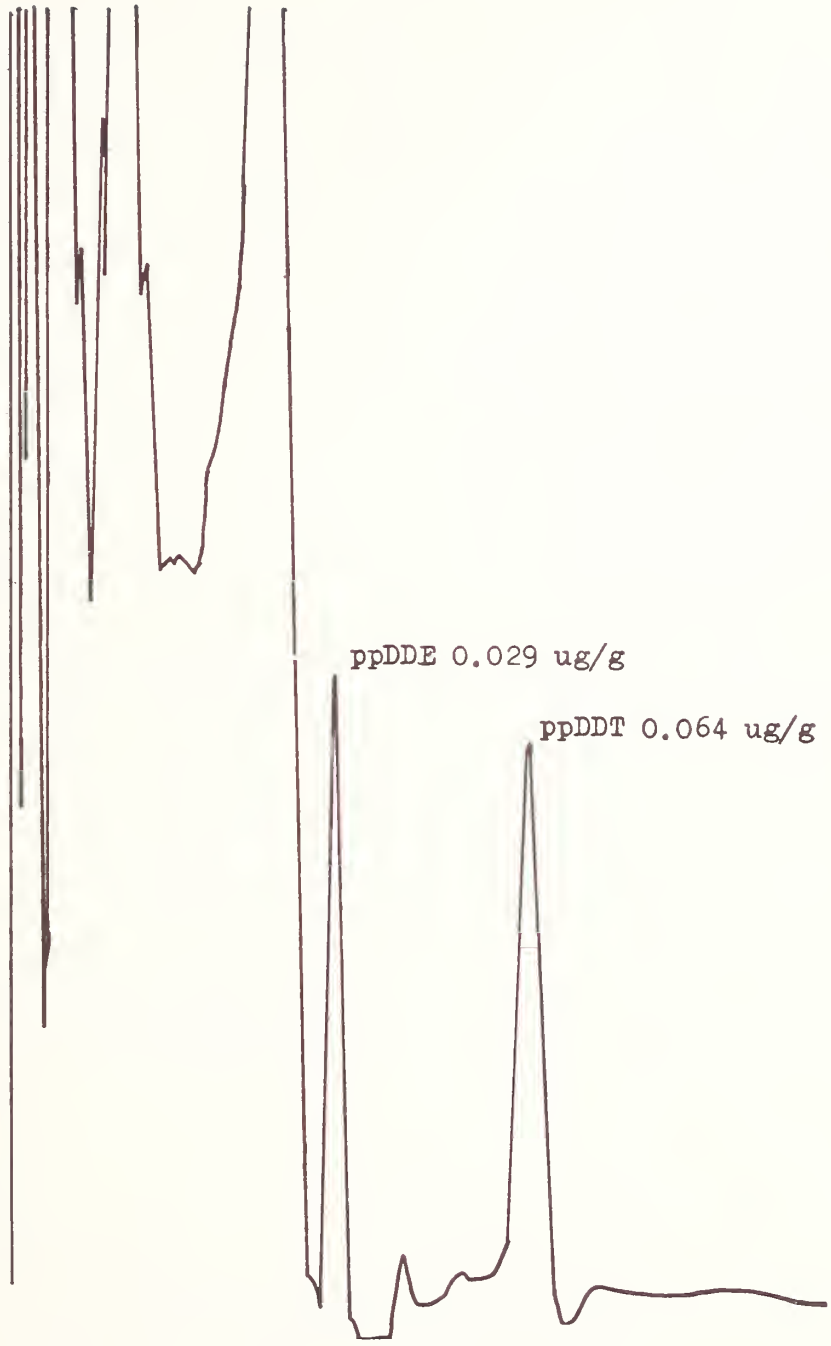

Figure 6. Sediment extract.

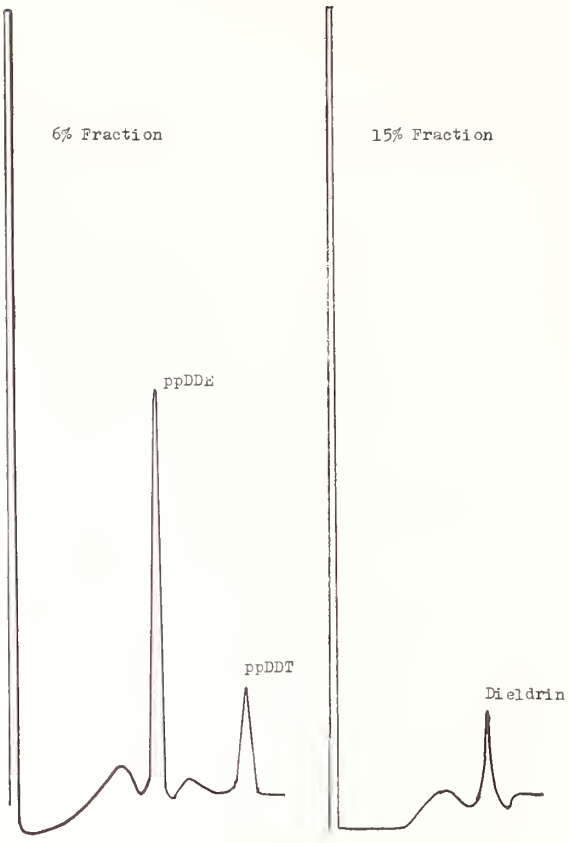

Figure 7. Florisil fractions.

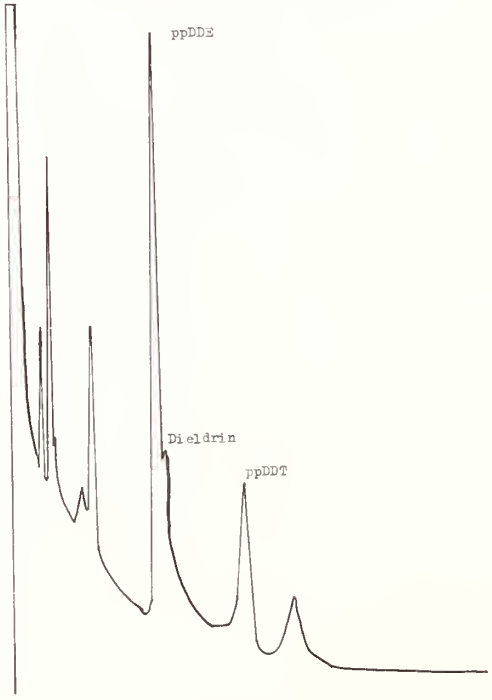

Figure 8. Sample before florisil.

IV. Results and Discussion

The method as outlined was used to identify pesticides and determine levels present in some 71 core soil samples and 37 sediments. The sediments were found to contain p,p′DDD, p,p′DDE, and p,p′DDT with p,p′DDD being present in the greatest amount in most samples. Levels of p,p′DDE ranged from none detected to 0.26 μg/g with a mean of 0.049. Levels of p,p′DD ranged from none detected to 0.45 μg/g with a mean of 0.06. Levels of p,p′DDT ranged from none detected to 0.02 with a mean of 0.0055. The soils contained primarily p,p′DDE, p,p′DDD, and p,p′DDT with p,p′DDE being present in the greatest amounts. The p,p′DDE ranged from none detected to 2.4 μg/g with a mean of 0.27 μg/g. The p,p′DDT ranged from none detected to 2.3 μg/g with a mean of 0.25 μg/g. Only traces of DDD were detected in a few samples. Five samples contained dieldrin ranging from 0.02 and 0.4 μg/g. Two samples contained endrin at levels of 0.02 and 0.4 μg/g. These two samples were the lower and upper 15 cm portion of a 30 cm core sample.

Sixteen soil samples were run in duplicate or triplicate to determine method reproducibility. Samples were run on different days. Five sediment samples were rerun on different days. Typical results are shown in Table 4.

The sediment samples showed interference in the early part of the chromatogram. The effect was thought to be due to sulfur. Recently a method was reported for removing interference due to sulfur by treatment with tetrabutylammonium sulfite [27]. Our work samples were treated with mercury as reported by Geoerlety and Law [28]. A typical chromatogram of a sediment with and without Hg treatment is shown in Figure 9. The sulfur present did not interfere in any of the sediment samples analyzed since p,p′DDE was the earliest eluting peak and was observed on the tail of the sulfur peak.

Twelve soil samples and nine sediment samples were put through the florisil column. This was used to confirm p,p′DDE, p,p′DDD, p,p′DDT, dieldrin, and endrin and also to determine if dieldrin was present in samples containing large amounts of p,p′DDE. Some typical results are shown in Table 5.

Fifty-one soils and eight sediment extracts were run using a flame photometric detector in the phosphorous mode. No phosphorous compounds were detected. The sediments required clean-up with Hg since the high levels of sulfur interfered with the photometric detector. In addition, 12 samples from areas known to have used guthion, were run using the short column to allow for lower detection limit. No guthion was detected indicating complete breakdown or removal of the compound.

Identification of the pesticides were made based on retention times, florisil column fraction, flame photometric detector, *p*-values [29] and chemical reactions [30].

The *p*-value for the acetonitrile-hexane system was used to confirm the samples containing endrin. A *p*-value of 0.436 was determined for endrin in the sample and a value of 0.441 for the sample.

TABLE 4. *Repeat analyses* (μg/g)

Sample	Soils DDE	DDT
A	0.38, 0.48, 0.37	0.36, 0.49, 0.39
B	0.04, 0.05, 0.05	0.05, 0.06, 0.06

Sample	Sediments DDE	DDD	DDT
A	0.01, 0.01	0.009, 0.001	
B	0.13, 0.08	0.06, 0.08	0.02, 0.03

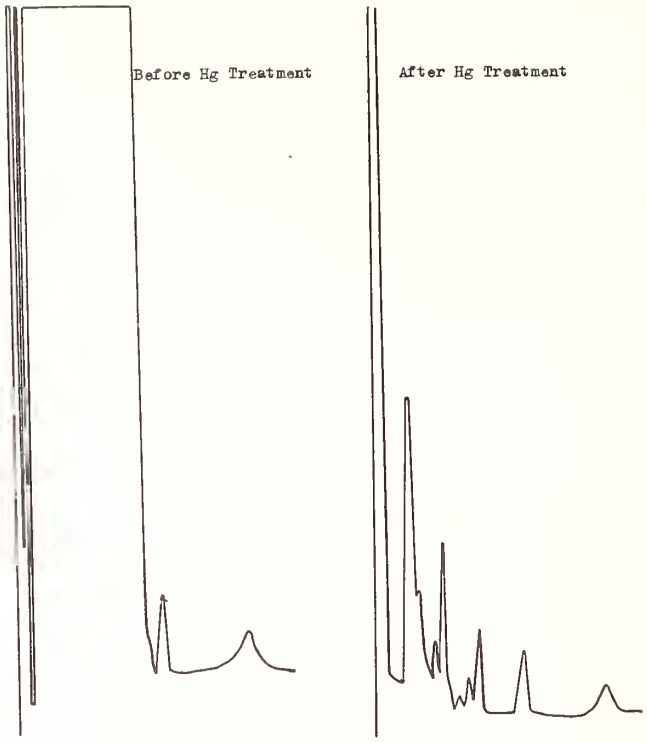

Figure 9. Effect of Hg treatment.

TABLE 5. *Florisil treatment*

	Compound					
	DDE		DDT		Dieldrin	
Sample	With	Without	With	Without	With	Without
44	0.48	0.53	0.58	0.62	0.51	0.42
38	0.27	0.32	0.20	0.17	0.02	0.02
65	0.31	0.38	0.38	0.36	0.06	--
40	0.26	0.31	0.23	0.21	0.12	--
45	0.38	0.40	0.38	0.32	0.33	0.28

Results of saponification are shown for soils and sediments in Table 6. Results are in good agreement for DDE after saponification and DDE+DDT before treatment.

Treatment of samples with fuming sulfuric acid resulted in the loss of dieldrin and endrin in the samples containing these compounds as expected. Standards containing the compounds of interest were treated at the same time as the samples for comparative purposes.

The method developed was found to satisfy the need of a single procedure for determining all the compounds of interest. Adequate recovery, reproducibility, and sensitivity were obtained without the need for multiple sample treatment.

TABLE 6. *Saponification data*

Sample	DDE (after sapon.)	DDE+DDT
40	0.61	0.52
37	0.29	0.20
18	4.83	5.15
67	0.15	0.15

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

SAMPLING AND ANALYSIS OF BETA-CHLOROETHERS IN THE ENVIRONMENT

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Under contract with the U.S. Environmental Protection Agency, Office of Toxic Substances, Monsanto Research Corporation has developed and successfully utilized methods for the sampling and analysis of trace levels of β-chloroethers in the environment. The six compounds analyzed in this study were: 2-chloroethylethyl ether, 2-chloroethylvinyl ether, bis(2-chloroethoxy) methane, and 1,2 bis(2-chloroethoxy) ethane. The methods for the sampling, workup, and analysis of air, water, soil, and sediment samples collected in the vicinity of potential producers and users are discussed in this paper.

Key words: Air; β-chloroethers; environment; gas chromatography/mass spectrometry; sampling; sediment; trace analysis; water.

I. Introduction and Background

Monsanto Research Corporation (MRC), under a contract with the U.S. Environmental Protection Agency, Office of Toxic Substances, was requested to develop sampling and analysis methods for trace levels of β-chloroethers in the environment. The methods developed were used to sample air, water, soil, and sediment in the vicinity of eight potential producers or users of β-chloroethers. The six compounds analyzed in this study were: 2-chloroethylethyl ether, 2-chloroethylvinyl ether, bis(2-chloroethyl) ether, bis(2-chloroisopropyl) ether, bis(2-chloroethoxy) methane and 1,2-bis(2-chloroethoxy) ethane. The structures of these six compounds are shown in Figure 1.

Three main sources of potential emission of β-chloroethers into the environment are 1) as byproducts, e.g., propylene oxide and ethylene oxide production via the chlorohydrin process; 2) as intermediates, e.g., production of polysulfide rubbers; and 3) as solvents, e.g., degreasing or dewaxing operations. Figure 2 shows the locations in the United States of sites where β-chloroethers have been detected since they were first reported in extracts of water samples by Rosen, et al. in 1963 [1]. β-Chloroethers had been found exclusively in water samples until Bursey, et al. [2] reported the presence of bis(2-chloroisopropyl) ether in ambient air samples collected at Freeport, Texas.

The following sections describe the sampling, workup, and analysis methods used, and the results of our application of these methods to the environmental samples collected around potential emission sources.

II. Sampling Methods

Air samples were collected on Tenax-GC in Pyrex glass sampling tubes. The sampling tube is shown schematically in Figure 3. The air sample was drawn through the tube with a portable

$ClCH_2CH_2OCH_2{=}CH_2$

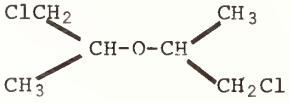
chloroethylvinyl ether (CEVE)

$ClCH_2CH_2OCH_2CH_3$

chloroethylethyl ether (CEEE)

$ClCH_2CH_2OCH_2CH_2Cl$

bis(2-chloroethyl) ether (BCEE)



bis(2-chloroisopropyl) ether (BCIPE)

$ClCH_2CH_2OCH_2OCH_2CH_2Cl$

bis(2-chloroethoxy) methane (BCEXM)

$ClCH_2CH_2OCH_2CH_2OCH_2CH_2Cl$

bis(2-chloroethoxy) ethane (BCEXE)

Figure 1. Structures of the six β-chloroethers analyzed in this work.

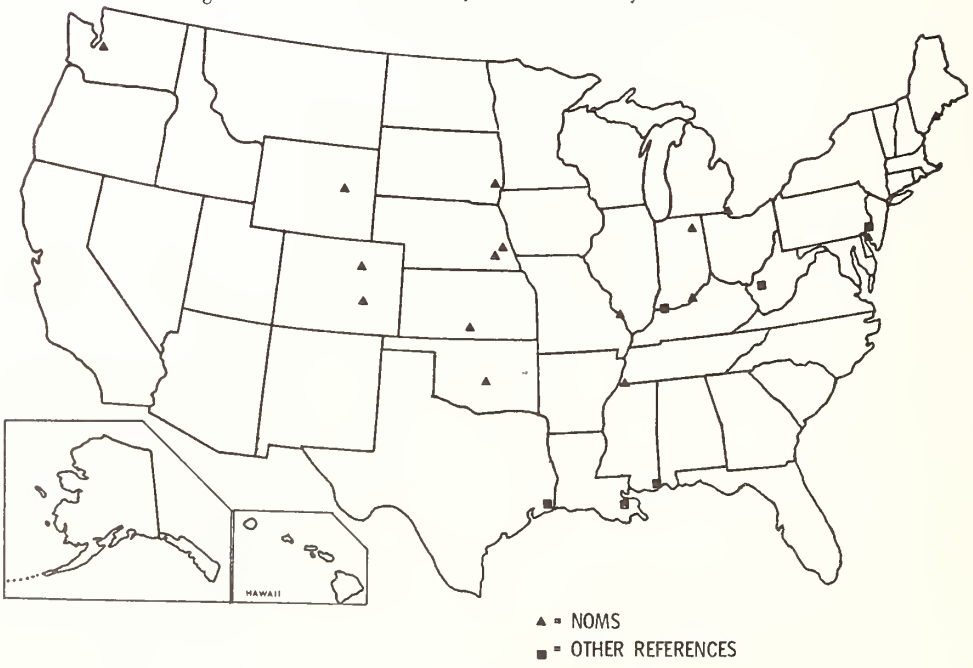


Figure 2. Sites where β-chloroethers have been found.

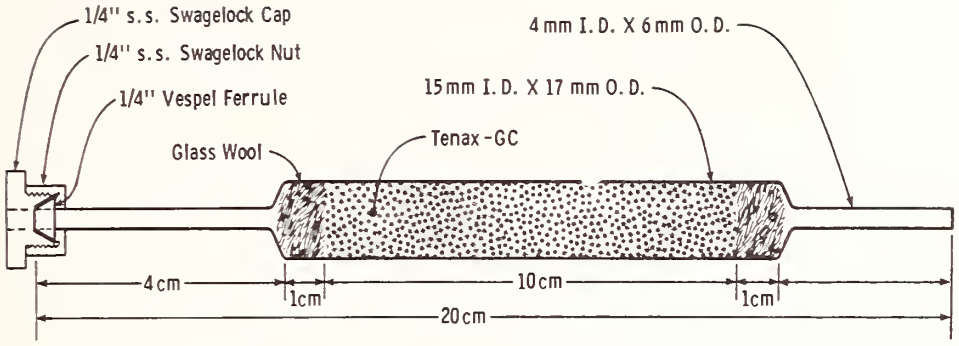


Figure 3. Pyrex sampling tube packed with Tenax-GC.

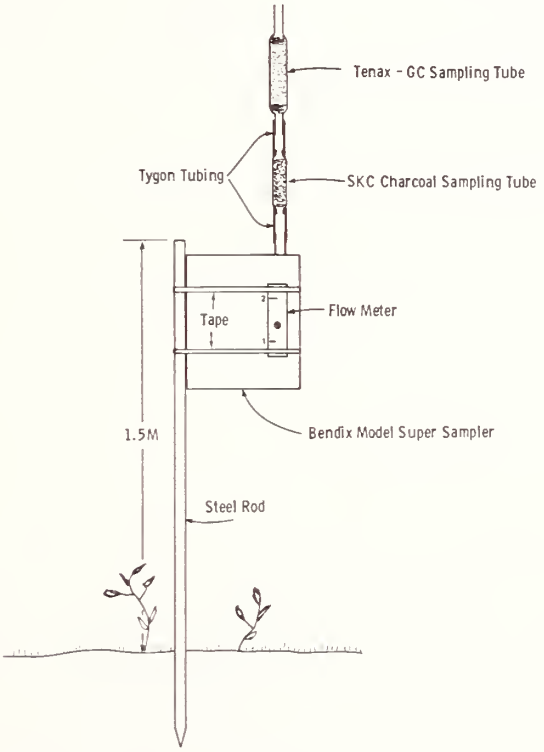


Figure 4. Air sampler arrangement.

personnel sampler. The samplers are the type utilized to sample air in workplace environments. A typical air sampler arrangement is depicted in Figure 4. Flow rate through the tube was 1.0 L/min and sampling time was 8 hours. Sample tubes were capped during shipment with polyethylene slip-on caps.

Twenty-four hour integrated water samples were collected using a peristalic pump. The water sampler arrangement is shown in Figure 5. Samples were collected at two downstream and one upstream location on the waterway receiving the water discharge from the plant. Samples were stored in amber glass bottles with Teflon-lined caps.

Soil samples were collected around the perimeter of the plant using a tulip bulb planter, and stored for shipment in glass canning jars. Sediment samples were scooped from the bottom of the waterway receiving the plant discharge and stored in glass canning jars.

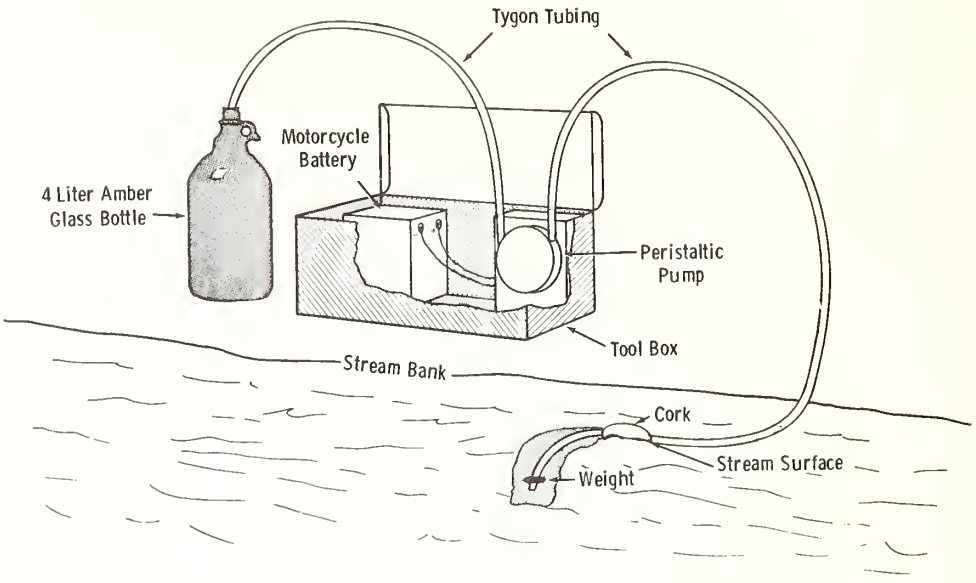


Figure 5. Water sampler arrangement.

III. Sample Workup and Analysis Methods

The Tenax tubes were solvent-desorbed with 4 milliliters of methanol using a method developed at MRC [3]. The apparatus for desorbing the tubes is shown in Figure 6. The percentage of the spike recovered from a tube spiked with the six β-chloroethers for each 1 milliliter increment is shown in Table 1. The average recovery of the β-chloroethers spiked on Tenax-GC tubes was 93.0% in the laboratory and 74.8% for tubes spiked in the field.

One liter of each water sample was extracted 3 times with 100 mL of methylene chloride. These three extracts were combined and concentrated in a Kuderna-Danish evaporator to a final volume of 2 to 3 milliliters. The average recovery of the β-chloroethers spiked in distilled water in the laboratory was 83.9%. When the same concentration of β-chloroethers was spiked in the field in 1 liter upstream water samples from six field sites, the average recovery was 77.3%.

Fifty-gram samples of soil and sediment were Soxhlet-extracted for 16 hours with methylene chloride. Each extract was concentrated to 2 to 3 milliliters with a Kuderna-Danish evaporator. The average recovery of the six β-chloroethers from soil samples spiked in the laboratory was 56.1%.

All of the concentrates were analyzed with a Hewlett-Packard model 5983 gas chromatograph/mass spectrometer. The mass spectrometer was operated in the selected ion monitoring (SIM) mode. The characteristic ions for each β-chloroether were monitored. SIM chromatograms for the six β-chloroethers are shown in Figures 7 and 8. The gas chromatographic conditions for the analysis of the β-chloroethers are shown in Table 2. Average detection limits for the β-chloroethers using GC/MS/SIM for each type of sample are shown in Table 3.

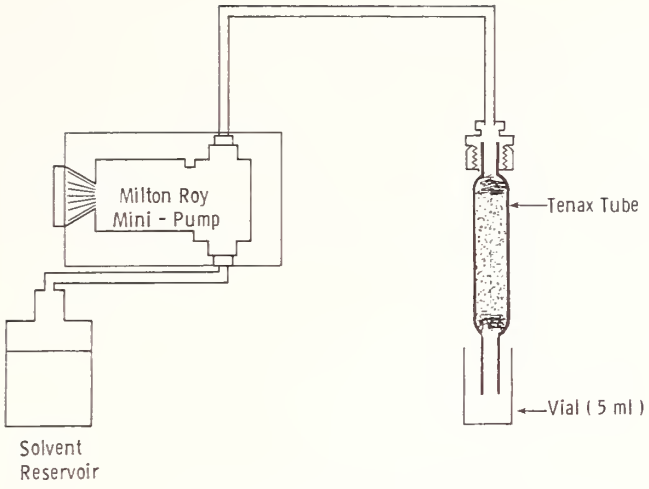


Figure 6. Solvent desorption apparatus.

TABLE 1. *Recovery of β-chloroethers from Tenax-GC. 100 mL of ~2.5×10⁻⁴ g/cm³ of each β-chloroether added*

	% Recovery				
cm^3	CEVE/CEEE	BCEE	BCIPE	BCEXM	BCEXE
0–1	68	75	67	71	67
1–2	14	18	16	11	15
2–3	N.D.	N.D.	N.D.	N.D.	N.D.
3–4	N.D.	N.D.	N.D.	N.D.	N.D.
Total	82	93	83	82	82

TABLE 2. *Gas chromatograph/mass spectrometer conditions for β-chloroether analysis*

Instrument: Hewlett-Packard Model 5983 GC/MS/DS

Column: 1.82 m×0.21 mm (i.d.) glass packed with Tenax-GC (60/80 mesh)

Injection Port Temperature: 280 °C

Transfer Line Temperature: 280 °C

Column Temperature Programming: From 140 °C to 260 °C at 8 °C/min. Hold at 260 °C for 5 minutes

Helium Flow Rate: 30 mL/min

Ion Source Temperature: 150 °C

TABLE 3. *Average detection limits for β-chloroethers in four types of environmental samples*

Type of sample	Detection limit
Air	7.0×10^{-7} g/m³ (0.12 ppb)
Water	2.0×10^{-7} g/L (0.2 ppb)
Soil/Sediment	4.1×10^{-9} g/g (4.0 ppb)

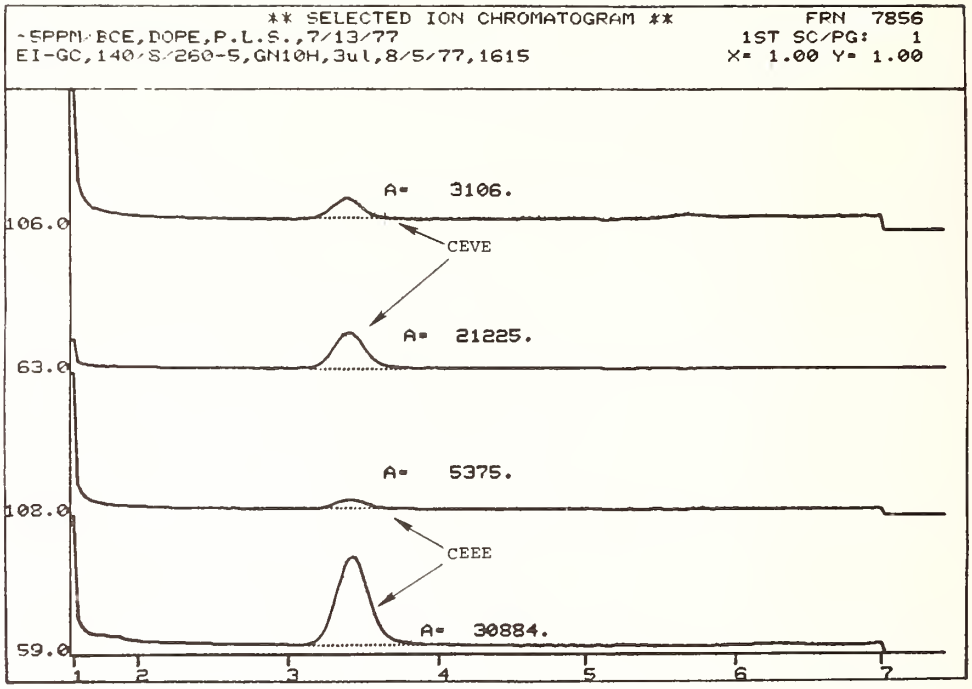


Figure 7. SIM chromatograms for β-chloroethers (1.5–7.5 min).

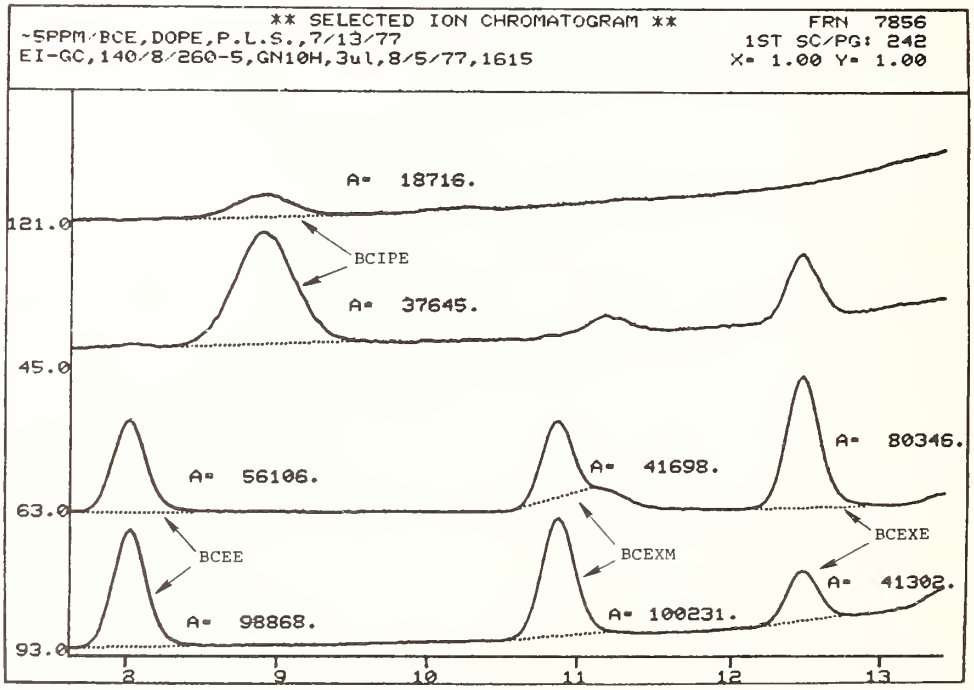


Figure 8. SIM chromatograms for β-chloroethers (7.5–13.5 min).

IV. Results of Sampling

The geographic locations of the eight sampling sites are shown in Figure 9. The total number of samples of each type collected and the number of each in which β-chloroethers were found are listed in Table 4. Only bis(2-chloroethyl) ether, bis(2-chloroisopropyl) ether, and bis(2-chloroethoxy) methane were found in the samples collected.

Three raw water samples were also collected at Sioux Falls, South Dakota in August and September 1977. None of these three samples contained β-chloroethers. Detection limits for bis(2-chloroethyl) ether and bis(2-chloroisopropyl) ether in these samples was 3×10^{-8} g/L.

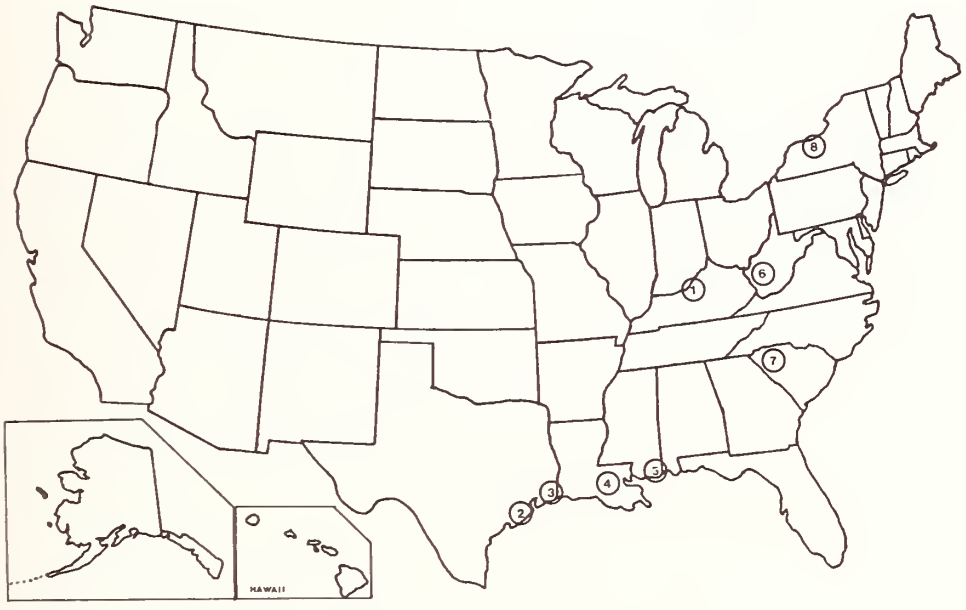


Figure 9. β-chloroethers sampling sites.

TABLE 4. *Number of samples collected and number of samples containing β-chloroethers for each type sample*

Sample type	Number of samples collected	Number of samples containing β-chloroethers
Air	72	2
Water	35	8
Soil	54	1
Sediment	12	2

V. Acknowledgment

This project was funded under EPA Contract 68-01-1980.

VI. References

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

A STATISTICAL STUDY OF ATMOSPHERIC SAMPLING FOR AN ENCLOSED INHABITED ENVIRONMENT

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Maintaining the quality of an enclosed inhabited environment, such as in the nuclear-powered submarine, requires monitoring of the atmosphere by various sampling procedures. One type of sampling procedure is grab sampling, whereby atmosphere samples are obtained essentially instantaneously in evacuated flasks. However, in many instances these samples cannot be analyzed for days or months. An extensive statistically based program was conducted on selected contaminants to determine the effect of time (shelf life) on the samples. In addition, a parallel study was conducted in the grab sampling technique to determine if there was a difference in flask preparation.

Although this study did not reveal any differences in flask preparation, it did reveal that the samples do have a shelf life. For all contaminants evaluated except the refrigerants and methane, a statistically significant exponential time decay is present in the shelf life of a contaminant.

Key words: Aging; analysis; atmosphere; sampling.

I. Introduction

Closed inhabited environmental systems such as the nuclear-powered submarine and diving chambers are susceptible to the build-up of toxic contaminants. These contaminants may be produced by a wide variety of factors including human effluents, out-gassing of instruments, refrigerant leakage, cleaning solvents, etc. Although these factors are normally insignificant, atmospheric recycling over extended time periods may result in significant increases in the concentration levels of these contaminants.

On board the nuclear-powered submarine there are atmospheric analyzers. At times, however, an unusual contaminant is detected which is beyond the scope of training of the operator and/or analyzer. By means of a small oil-free compressor a "grab sample" of the atmosphere is pumped into an evacuated 1.64 liter volume type 304 stainless steel bottle to a pressure of about 50 psig. When convenient the sample is forwarded to NRL for analysis.

Because of the delay in time from sampling to that of analysis, which might be 70 days or more, a statistical evaluation was made on several previously detected contaminants for their stability or shelf life in the stainless steel bottles.

The study described in this report was a joint effort. The grab sampling and associated chemical analyses were conducted by the Chemistry Division, Naval Research Laboratory. The statistical analysis was conducted by Desmatics, Incorporated.

II. Experimental Procedure

A. General

A number of factors can affect the results obtained in a sampling method such as this. For purposes of this investigation, the following contributing factors were considered:

(1) Concentration level
(2) Sampling method
(3) Flask
(4) Measurement (analysis)
(5) Time to analysis (delay time).

In addition, the interaction effects of these sources were also investigated.

For purposes of this study, four grab sampling methods were of interest. The methods were defined by differences in flask preparation (vacuum, bake, and combination of the two) and filling of the flask (single or purge fill). Hence, the gas samples were collected under the following sampling methods:

#1 : Vacuum-bake, Single fill
#2 : Vacuum only, Single fill
#3 : Vacuum-bake, Purge fill
#4 : Vacuum only, Purge fill

A gas mixture containing 11 contaminants in air, listed in Table 1, were investigated in this study. Each contaminant was studied at a high (60–110 ppm) and a low (5–12 ppm) concentration. The use of more than one concentration permitted the estimation of any differential effects which concentration may have had on the sampling method or shelf life.

TABLE 1. *Contaminants of interest and value of the estimated parameter* α *in time* $e^{\alpha t}$

Contaminant	Estimated Value of α	Predicted change after 70 days
1. Methane	-	-
2. Carbon monoxide	–0.001614	–10.7%
3. Dichlorodifluoromethane (R-12)	+0.000404	+ 2.9%
4. Dichlorotetrafluoroethane (R-114)	+0.000896	+ 6.5%
5. Vinylidene chloride	–0.000425	– 2.9%
6. Trichlorotrifluoroethane (R-113)	-	-
7. Hexane	–0.000424	– 2.9%
8. Methyl chloroform	–0.000546	– 3.7%
9. Benzene	–0.000682	– 4.7%
10. Trichloroethylene	–0.000697	– 4.8%
11. Toluene	–0.000825	– 5.6%

B. Obtaining Grab Samples

The sampling bottles, Figure 1, used in the evaluation were 1.64 liter volume type 304 stainless steel obtained from Alloy Products Corporation, Waukesha, Wisconsin. The bottles were equipped with brass Whitey shut-off valves, part number B-14DKM4,. obtained from Potomac Valve and Fitting, Incorporated, Rockville, Maryland. The test gases were obtained from Union Carbide Corporation, Linde Division, Keasby, New Jersey.

Figure 1. Gas sampling bottle.

Forty-eight bottles were used in this study to build the statistical base. One-half of the bottles in three groups of eight were tested with the low concentration and half, also in three groups of eight, with the high concentration test gases. Two evacuated bottles for the four sampling methods described above, making a total of the eight bottles in each group, were randomly attached to a heated (60 °C) manifold. One end of the manifold was connected via Teflon tubing to a test gas bottle. After purging the manifold, the opened end was closed and pressurized to 40 psig.

The sample bottles were opened and filled with the test gas. Those bottles designated for single fill were closed. Those bottles designated for purge fill were left open while the manifold pressure was released to atmospheric and then repressurized. This step was repeated three times to obtain the purge fill method.

To study the shelf life of these bottles they were analyzed at day 0 and every 10 days thereafter to day 70.

C. Gas Chromatographic Analysis

A Beckman GC-5 gas chromatograph with dual hydrogen flame ionization detectors was used in the evaluation of the gas sampling bottles. A schematic of the gas handling and analyzing system is shown in Figure 2. Subsystem I was designed to detect all the organic atmospheric contaminants as shown in Figure 3. Methane and carbon monoxide were detected on subsystem II as shown in Figure 4.

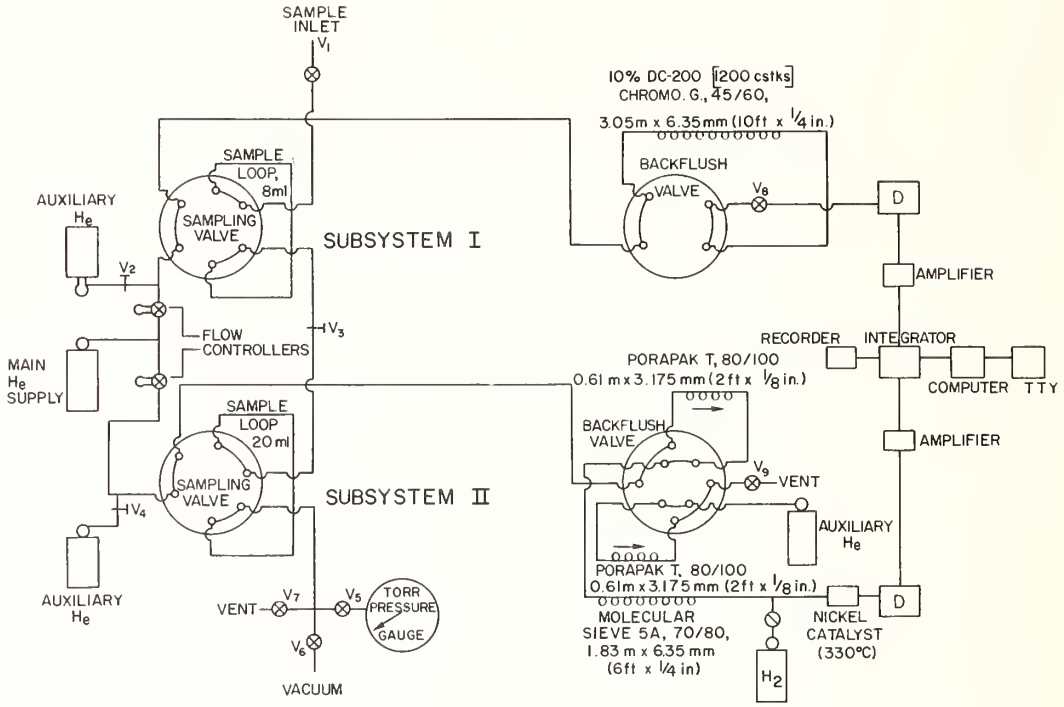

Figure 2. Gas handling and analyzing system.

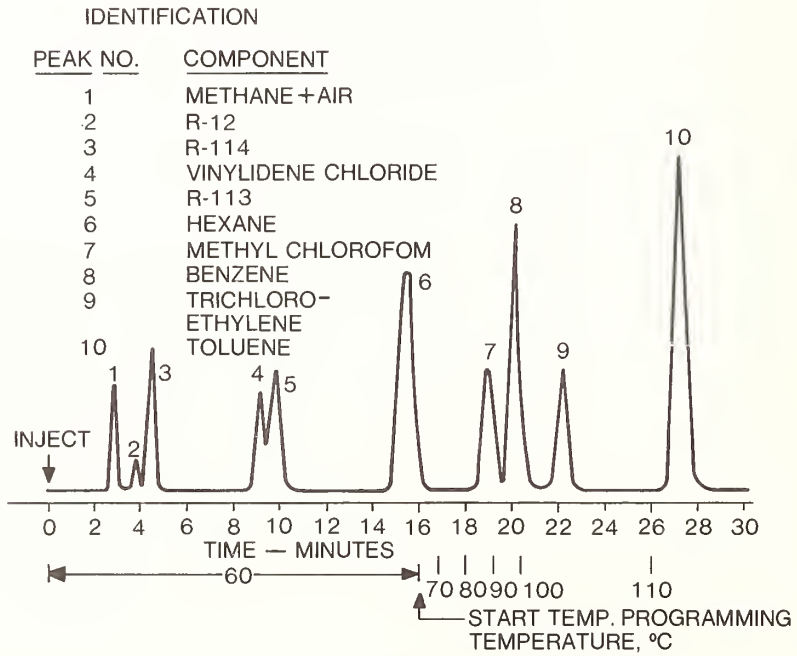

Figure 3. Separation of contaminants on Subsystem I.

IDENTIFICATION

PEAK NO.	COMPONENT
1	OXYGEN
2	METHANE
3	CARBON MONOXIDE

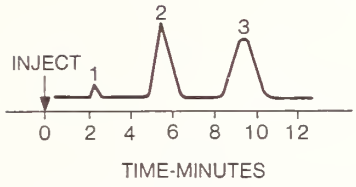

Figure 4. Separation of contaminants on Subsystem II at 60 °C.

D. Statistical Experimental Design

The usual model assumed in an experimental situation is one in which a given observation y is a composite of a number of additive effects:

$$Y_{ijkmnp} = M + C_i + G_{j(i)} + S_k + GS_{jk(i)}\ F_{m(ijk)} + A_{n(ijkm)} + T_p + CT_{ip} + ST_{kp} + CST_{ikp} + GT_{jp(i)} + GST_{jkp(i)} + FT_{mp(ijk)} + \epsilon_{np(ijkm)} \quad (1)$$

where the capital letters refer to the sources of the effects. That is,

M = an overall mean effect
C = the concentration level effect
G = the group effect
S = the sampling method effect
F = the flask effect
A = the analysis effect
T = the time effect
ϵ = a random error effect.

The two- and three-letter combinations in the model indicate the interactive effects between the various factors, while the subscripts denote the specific level of any factor or factor combination. A subscript expression containing parentheses indicates that the particular factor levels outside the parentheses are nested in a hierarchical relationship within the factor levels inside the parentheses.

The analysis of variance was used to break out the total variation in the experiment and ascribe it to each of the components in the additive model (eq. (1)).

III. Results

By employing the appropriate tests using the analysis of variance, the data indicated that there was little statistical evidence that any of the sampling methods gave different results for any of the contaminants investigated. The data did show that the observed concentration of the contaminants with time was proportional to $e^{\alpha t}$ where:

t = number of days elapsed from sampling to analysis time
α = constant decay parameter

Table 1 shows the estimated parameter α for the nine contaminants for which a statistically significant time trend was found. There was no statistically significant time effect for contaminants methane and trichlorotrifluoroethane. The table also indicates the predicted change in concentration over a 70-day period.

Figure 5 presents a graph of the significant time trend for carbon monoxide as well as the observed value at each time. The observed values here are averages (on a logarithmic scale) over all samples and groups for each particular time. Also included are the upper and lower 95% confidence bounds on the estimated time trend.

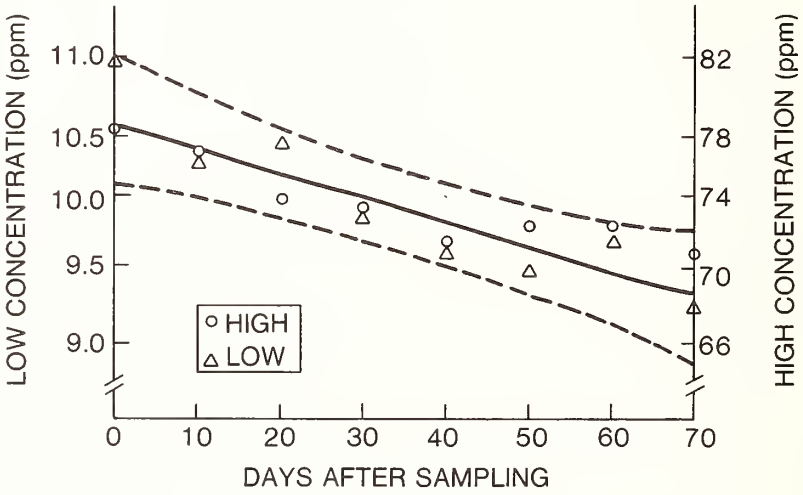

Figure 5. Time trend for contaminant #2 (carbon monoxide) with 95% confidence bounds.

IV. Summary and Conclusion

The two main topics addressed in this investigation were (1) whether difference existed in the performance of the four grab sampling methods used, and (2) whether the grab samples have a shelf life. The statistical analysis of the experimental data indicates that each of the sampling methods perform equally well.

The data did indicate that the samples do have a shelf life. For all contaminants except the refrigerants and methane, a statistically significant exponential time decay is present in the shelf life of a sample. Although decay parameter values are not the same for each contaminant, the parameter value is independent of the concentrations evaluated. For two of the refrigerants (R-12 and R-114) an unexplained statistically significant exponential time growth was found in the shelf life, again independent of concentration.

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DEVELOPMENT OF AN AQUEOUS POLYNUCLEAR AROMATIC HYDROCARBON STANDARD REFERENCE MATERIAL

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The National Bureau of Standards currently issues over 900 Standard Reference Materials (SRM's), with various groups being represented, such as: clinical laboratory standards, trace element standards, nuclear materials, glass viscosity standards, rubber materials, color standards, and coating thickness standards. We are now endeavoring to add to this list an additional group, namely trace organic chemical Standard Reference Materials.

The first SRM from this new group of materials will be an aqueous polynuclear aromatic hydrocarbon (PAH) Standard Reference Material. There are several problems associated with the preparation, storage and handling of aqueous solutions of PAH that previously prevented the development of this SRM. Now, through the use of a dynamic coupled-column liquid chromatographic method developed in this laboratory, we have been able to circumvent these problems. The use of this technique for the preparation and certification of an aqueous PAH SRM will be discussed.

Key words: Dynamic coupled-column liquid chromatography; extractor column; generator column; polynuclear aromatic hydrocarbon; Standard Reference Material.

I. Introduction

In the fall of 1975, the National Bureau of Standards (NBS) and the Environmental Protection Agency (EPA) jointly sponsored a series of workshops entitled "Standards and Reference Materials for Environmental Analysis Associated with Energy Development." The objective of these workshops was to obtain input to NBS on the methodology and certified standards needed for the accurate analysis of environmental samples associated with the production of alternate fuels.

At the conclusion of these workshops, a number of Standard Reference Materials (SRM's) were recommended by the participants for NBS consideration. One of the SRM's recommended was a polynuclear aromatic hydrocarbon (PAH) in a water matrix. Although many PAH have demonstrated mutagenic properties, this SRM was given a low priority because of the presumed difficulties associated with the preparation and stabilization of such a material.

II. Problems Associated with the Preparation and Stabilization of a PAH/Water SRM

Preparation of aqueous PAH solutions of known concentration by gravimetric procedures is difficult because of the extremely low aqueous solubilities of PAH. As shown in Table 1 many PAH have aqueous solubilities of less than 500 μg/kg (ppb). Preparation of aqueous solutions of known concentration by serial dilutions of a more concentrated organic solution is both hazardous and wasteful. After small aliquots are taken, large volumes of organic solvent containing toxic and expensive chemicals remain to be disposed of.

Preservation of stable aqueous solutions of PAH is hampered by absorptive losses of the PAH to the surfaces of containers and transfer tools. The magnitude of the adsorptive effect is variable and is a function of the manner in which the solutions are handled. The adsorptive

TABLE 1. *The aqueous solubilities of some aromatic hydrocarbons as determined by several investigators*

Compound	MacKay and Shiu[a]	May et al.[b]	Schwarz[c]
Anthracene	73	45	41
Phenanthrene	1290	1002	1151
2-Methylanthracene	39	21	
1-Methylphenanthrene		269	
Fluoranthene	260	206	
Pyrene	135	132	129
1,2-Benzanthracene	14	9.4	
Chrysene	2	1.8	
Naphthacene	0.6		
Triphenylene	43	6.6	
3,4-Benzpyrene	0.4		

[a] Solubilities determined by method described in ref. 1.
[b] Solubilities determined by DCCLC method described in ref. 2.
[c] Solubilities determined by fluorimetric method described in ref. 3.

TABLE 2. *Surface adsorption characteristics of phenanthrene, chrysene, and benz(a)pyrene in aqueous solutions*[a]

	Percent loss from solution					
	Phenanthrene		Chrysene		Benz(a)pyrene	
	1 h	13 h	1 h	13 h	1 h	13 h
Glass	8		46	71	53	82
Silanized glass	5	73	64	81	73	93
Platinum	35	87	66	85	57	93
Aluminum	5	76	50	89	67	95

[a] The concentration of each PAH was 1 μg/kg. Surface to volume ratios are approximately 1 cm^2/cm^3 for all materials studied.

properties of three PAH on four different surfaces are shown in Table 2. These results show that losses of PAH from static solutions to surfaces occur in short periods of time. Stirring such solutions only slightly reduces such losses.

III. Preparation and Quantitation of Dilute PAH Solutions by Dynamic Coupled-Column Liquid Chromatography

Recently, we have developed a dynamic coupled-column liquid chromatographic (DCCLC) method [2] for investigating the aqueous solubility behavior of PAH. In the DCCLC method, saturated aqueous solutions of PAH are generated by pumping water through a column packed with glass beads that have been coated with the compound of interest ("generator column"). The concentration of the desired compound in the effluent of the "generator column" is measured by a modification of the coupled-column liquid chromatographic process that has been previously described by May et al. [4]. A flow diagram of this system is shown in Figure 1.

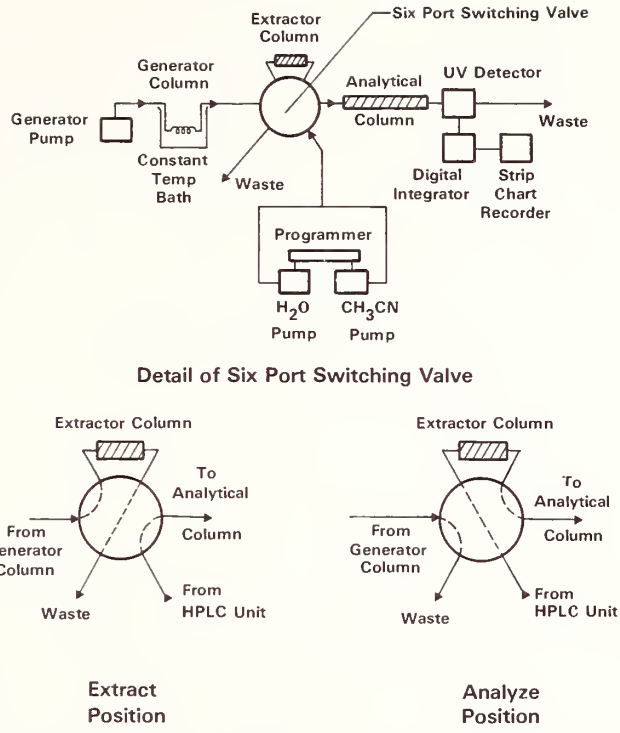

Figure 1. Flow diagram of dynamic coupled-column liquid chromatographic system.

A. Preparation of Standard Aqueous PAH Solutions Using "Generator Columns"

The aqueous solubility of a compound is a well-defined thermodynamic quantity. Therefore, saturated aqueous solutions produced by "generator columns" may also be defined as standard solutions at a given temperature. "Generator columns" are prepared by packing 60×0.6 cm stainless steel tubes with 60–80 mesh glass beads coated with 1 percent (wt/wt) of the compound of interest. The beads are coated by adding 20 grams of the beads to 200 mL of 0.1 percent methylene chloride solution of the PAH of interest and removing the solvent with a rotary evaporator.

Stable saturated aqueous solutions are eluted from "generator columns" after an initial aqueous purge volume of between 100 mL and 200 mL. After this initial conditioning, equilibrium is obtained and the PAH concentration at constant temperature has been shown to be independent of flow rate between 0.1 and 5 mL/min (see Table 3). Equilibrium can be re-established after a change in temperature by passing 10 mL of water through the column under the new conditions. A purge volume of approximately 100 mL was necessary to re-equilibrate a "generator column" after a shelf storage period as long as 3 months.

B. Quantitative Analysis of Generated Aqueous PAH Solutions by DCCLC

Extraction of the generated PAH solutions is accomplished by passing a measured volume of that solution through an "extractor column" (see Fig. 1). This 60×0.6 cm column is packed with a superficially porous bonded C_{18} stationary phase (Bondapak C_{18}, Waters Associates, Milford, MA) and provides better than 99 percent extraction efficiency for less than 25 mL volumes of aqueous PAH solutions.

After extraction, a water-acetonitrile solvent blend is passed through the "extractor column" to elute the adsorbed PAH. This elute is then passed through a microparticulate analytical column

TABLE 3. *Phenanthrene concentration dependence on the aqueous flow rate through a "generator column"*

Flow rate (mL/min.)	Concentration[a] (ppb)
5.0	865±7
0.4	868±4
0.1	866±3
Temperature	22.0 °C

[a] The concentrations reported represent the averages of five measurements at each temperature. The uncertainties represent the standard deviation of the mean at each respective temperature.

(μBondapak C_{18}) for separation of the PAH from non-analyte interferences. Individual response factors are calculated by replacing the "extractor column" with a calibrated sample loop, and injecting known amounts of the PAH of interest dissolved in acetonitrile.

IV. Preparation of Dilute Organic PAH Solutions Using "Generator Columns"

Small volumes of dilute organic solutions may also be accurately prepared by extracting the generated aqueous solutions with the desired volume of an immiscible organic solvent, such as hexane. Since PAH hexane/water distribution coefficients are very large, the concentration of these dilute solutions can be calculated if the volume ratios are known. For example, 5 mL of a 10 μg/kg (ppb) solution of chrysene in hexane can not be prepared gravimetrically. Such a solution would have to be prepared by serial dilutions of a more concentrated solution. This process is hazardous and wasteful and can be imprecise. However, such a solution can be prepared by extracting 25 mL of a saturated aqueous solution (~2 ppb) of chrysene with 5 mL of hexane. This is only a hypothetical example, but it does demonstrate the utility of using "generator columns" to prepare organic PAH solutions of known concentrations indirectly.

V. Evaluation of DCCLC as a Method for the Preparation and Certification of an Aqueous PAH SRM

There are several factors that make the DCCLC approach ideal for the preparation and analysis of very dilute aqueous solutions of individual PAHs:

A. Saturated solutions are prepared by an equilibrium process. The reversible nature of this process was demonstrated by the following experiment. Distilled water was pumped through two independently thermostated anthracene generator columns that were connected in series. The temperature of the first column, A, was maintained at 24.3 °C. Concentration measurements were made on the effluent from the second column (B) in the series, at temperatures of 25.3°, 12.8°, and 6.6 °C. The concentration of the solution that eluted from column B at each temperature, was identical, within experimental error, to the concentration that had been obtained from column B alone. The data from this experiment are presented in Table 4.

Normally, saturated PAH solutions were generated by pumping distilled water through "generator columns." Solutions of the same concentration (saturated solutions) were also obtained when the PAH concentration in the feed solution was greater than that in the effluent. This could only happen through an equilibrium process. It has also been shown that PAH concentration is independent of the rate of flow through a "generator column" between 0.1 and 5.0 mL/min.

TABLE 4. *A demonstration of the equilibrium-reversible nature of the solution generation process for a "generator column"*

Generator column "A"		Generator column "B"		Generator column "A"+"B" in series	
Temp. (°C)	Conc. (μg/kg)	Temp. (°C)	Conc. (μg/kg)	Temp. (°C)	Conc. (μg/kg)
24.3	42.7	25.3	45.5±0.2	25.3	45.4±0.1
		12.8	21.3±0.2	12.8	20.7±0.1
		6.6	14.0±0.1	6.6	14.2±0.2

Flow rate through anthracene generator columns was 5.0 mL/min.

TABLE 5. *Anthracene solubility* $= 0.0013t^3 - 0.0097t^2 + 8.886t + 8.21$ *(μg/kg)**

t (°C)	Concentration measured (mol/liter×10^{-7})	Concentration measured (μg/kg)	Concentration calculated (μg/kg)
28.7	3.13	55.7±0.7	55.8
24.6	2.44	43.4±0.1	43.1
22.4	2.09	37.2±1.1	37.5
18.3	1.63	29.1±0.6	29.0
14.1	1.25	22.2±0.1	22.3
10.0	0.98	17.5±0.3	17.4
5.2	0.71	12.7±0.4	12.7

* t=temperature in °C.

B. The use of "generator columns" to produce aqueous PAH solutions circumvents the problems that are usually associated with storing such solutions, since the solutions need not be generated until they are needed.

C. The concentration of the generated aqueous solutions is a function of temperature, and may be expressed in terms of least squares fits of the concentration as a function of temperature to within ±2% of the experimentally determined values between 5° and 30 °C (see Table 5).

D. Both the short and long term precision with which aqueous PAH solutions can be generated appear to be ⩽2% (see Table 6).

E. Shelf storage of "generator columns" does not seem to present a problem. They have been shown to be stable for longer than 1-1/2 years and through more than 100 liters of aqueous purge.

F. DCCLC is a rapid and accurate method for analyzing the generated dilute aqueous PAH solutions. Analytical errors due to adsorption are minimized in DCCLC because the solution is extracted and concentrated, on line, in less than 500 ms after generation. It has been estimated that this analytical method has an uncertainty of less than 2 percent [2].

TABLE 6. *Precision with which anthracene solutions may be generated and measured at 25.4 °C*

Volume of distilled water eluted through column (mL)	Concentration measured (μg/kg)
1	45.2
190	47.0
230	46.6
500	46.1
775	46.9
910	44.6
1175	45.5
Average measured concentration (6-21-77)	46.0±0.9
Concentration calculated from calibration curve (12-20-76)	45.7±0.2
Concentration measured by solvent extraction followed by GC analysis (6-21-77)	45.8±1.0

VI. Conclusion

The DCCLC technique has been shown to be an ideal method for the preparation and certification of an aqueous PAH SRM. Tentative plans are to issue individually certified anthracene, 1,2 benzanthracene, and 3,4 benzpyrene "generator columns" by early 1979. Investigations are in progress to determine the feasibility of preparing multi-PAH "generator columns."

VII. References

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[2] May, W. E., Wasik, S. P., and Freeman, D. H., *Anal. Chem.* **50,** 175 (1978).
[3] Schwarz, F., *J. Chem. Eng. Data* **22,** 273 (1977).
[4] May, W. E., Chesler, S. N., Cram, S. P., Gump, B. H., Hertz, H. S., Enagonio, D. P., and Dyszel, S. M., *J. Chromatogr. Sci.* **13,** 535 (1975).

NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

NEW COLUMN TECHNOLOGY IN THE ANALYSIS OF ORGANICS IN WATER BY GAS CHROMATOGRAPHY

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Recently EPA issued a protocol on the analysis of 114 organic pollutants in water. The proposed method of analysis is by gas chromatography using selective columns and detectors. The organic pollutants are concentrated from water samples by solvent extraction or purge and trap techniques. The volatile pollutants, which are purged and trapped, are analyzed on a 80/100 Carbopack C/0.2% Carbowax 1500 column. Base-neutrals and pesticides are extracted from water with methylene chloride and analyzed on a 1% SP-2250 column. The pesticides and Aroclors are analyzed with selective detectors such as electron capture or Hall conductivity. The analysis of phenols has been improved with the use of a new deactivated phase, SP-1240 DA, instead of the EPA recommendation of Tenax.

Key words: Aqueous organics; phenols; volatile organics, Carbopack C/0.2% Carbowax 1500.

I. Introduction

In 1974, Congress enacted into law the Safe Drinking Water Act and thereby required the Environmental Protection Agency to establish the control and purity requirements of the water we drink and use daily. As a result of this law, EPA has issued a protocol entitled "Sampling and Analysis Procedure for the Survey of Industrial Effluents for Priority Pollutants," IFB No. WA-77-B133 [1]. This procedure established the guidelines for the screening and analysis of industrial waste water for some 129 priority pollutants. Encompassed in this list are 114 organic and 15 inorganic compounds. The proposed method of analysis for the organics is by gas chromatography using sensitive, selective detectors with mass spectrometry confirmation.

The 114 organic pollutants are normally present at sub ppb concentrations in water. Samples, therefore, must be concentrated by either solvent extraction or purge and trap techniques. Four classifications of organic pollutants have been created, based on the method of concentration: volatiles, pesticides & PCBs, base neutrals, and acidic compounds.

II. Volatile Organics

In the analysis of volatile pollutants, the water sample is purged with helium gas and the volatiles are collected on an adsorbent trap of Tenax-Silica Gel 15. The proposed analysis of the 30 volatiles is to be conducted on a 6′×1/8″ SS column with Carbopack C modified with 0.2% Carbowax 1500 80/100 mesh and is shown in Figure 1. The analysis shown in Figure 4 was conducted on a Hall conductivity detector at 1 ng/mL concentration. Those aromatic compounds listed as volatiles are not shown here due to the lack of response on the Hall detector. Acrolein and acrylonitrile are also considered as volatiles, but are not efficiently recovered in the purge and trap technique. Therefore, they are analyzed by direct aqueous injection on the Carbopack C/0.2% Carbowax 1500 column. The lowest detectable amount of acrolein or acrylonitrile is 0.1 ng/mL by direct aqueous injection with flame ionization detection.

Due to the upper temperature limit of 175 °C of Carbowax 1500 an alternate column packing, 0.3% Carbowax 20M on Carbopack C 80/100, was proposed. Figure 2 shows the analysis of the volatile pollutants with this packing, as well as, the dichloro benzenes which were

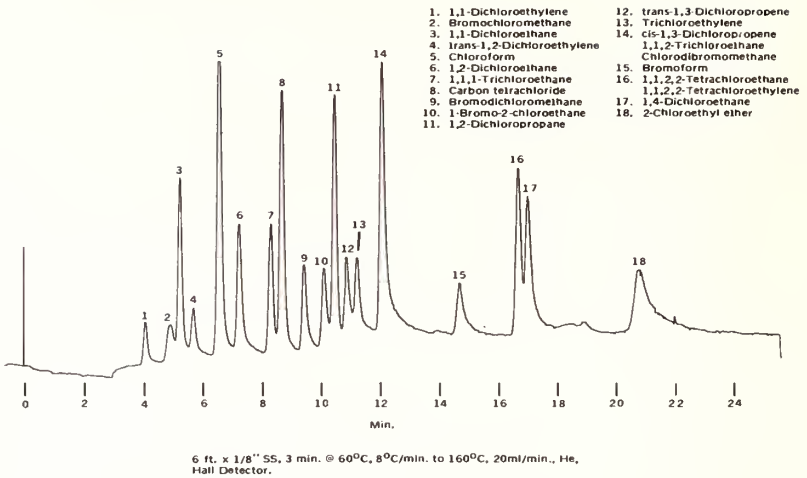


Figure 1. Carbowax 1500/Carbopack C—Hall detector analysis of volatiles.

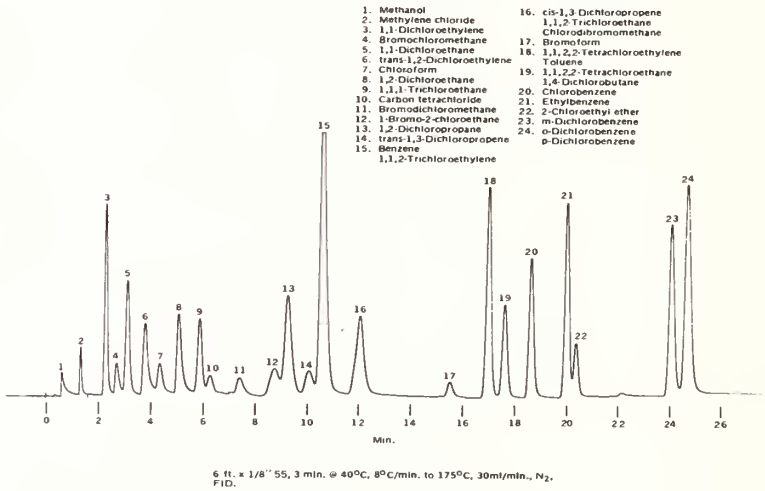
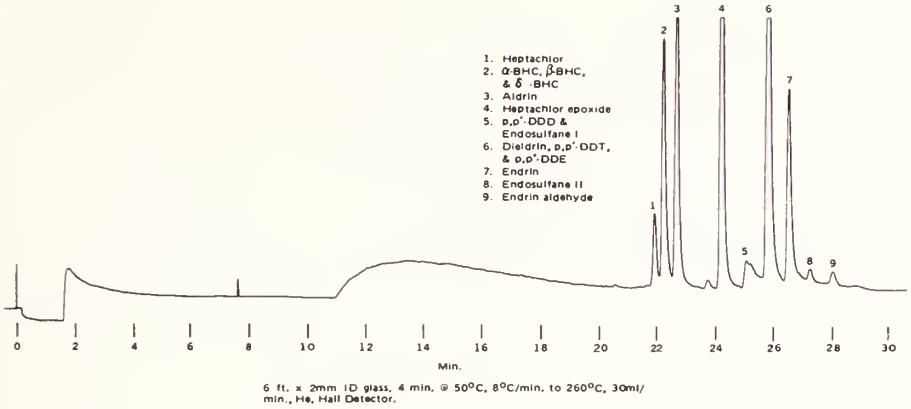


Figure 2. Carbowax 20M/Carbopack C.

not eluted on the Carbowax 1500/Carbopack C column. The analysis of the volatiles shown in both Figures 1 and 2 was by direct syringe injection of dilute solutions of water standards now available from Supelco.

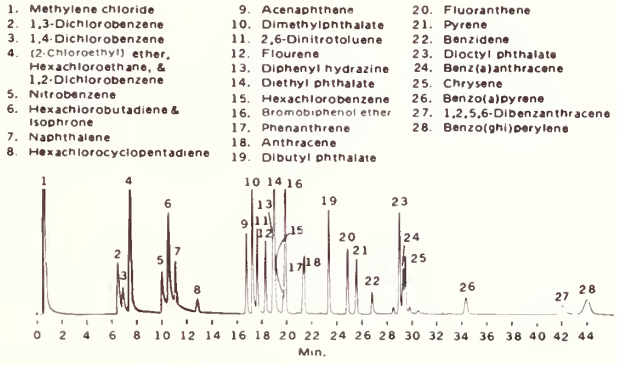
III. Pesticides & PCBs

For the analysis of pesticides, PCBs, and base-neutrals a single multipurpose column was chosen, 1% SP-2250 on 100/120 Supelcoport. SP-2250 is a methyl phenyl silicone with a temperature range of 50 °C to 360 °C, which allows for the complete analysis of the aforementioned compounds. The pesticides and PCBs are extracted from water with 15% methylene chloride in hexane, concentrated, and taken up in hexane for a final concentration to 5 mL or less. Figure 3 shows the analysis of the pesticides listed in the protocol using a 6′×2 mm ID glass column containing 1% SP-2250 on 100/120 Supelcoport. Though the resolution of the pesticides is limited with this column, the pesticides are differentiated from the multiple peak PCBs, chlordane and toxaphene which elute after the pesticides.



6 ft. x 2mm ID glass, 4 min. @ 50°C, 8°C/min. to 260°C, 30ml/min., He, Hall Detector.

Figure 3. Pesticides on 1% SP-2250.



6 ft. x 2mm ID glass, 4 min. @ 50°C, 8°C/min. to 260°C, 20ml/min., N_2, FID.

Figure 4. Base-neutrals on 1% SP-2250.

IV. Base-Neutrals

In the analysis of the base-neutrals, the water sample is treated with 6N NaOH to an adjusted pH of 11 and extracted with methylene chloride. The extract is analyzed on the 1% SP-2250 on 100/120 Supelcoport column, as shown in Figure 4. The analysis shown here was conducted with a temperature program of 50 °C to 260 °C @ 8 °C/min. An alternative to the use of 1% SP-2250 would be the 1% SP-2250 DB, specially deactivated for basic compounds.

V. Acidic Compounds (Phenols)

The acidic compounds consist of 11 substituted phenols. Prior to extraction of the water sample with methylene chloride, the water sample is acidified with 6N HCl to a pH of 2. The protocol proposes the use of a Tenax column, which is marginal at best as shown in Figure 5. Note, the loss of many of the nitrophenols and tailing seen with many of the other chloro substituted phenols. Supelco working in conjunction with EPA has developed a new chromatographic packing, SP-1240 DA. Specially deactivated for the analysis of acidic compounds, SP-1240 DA gives complete resolution of the 11 phenols with good peak shape as shown in Figure 6. With a temperature limit of 200 °C, the bleed of SP-1240 DA is noticeable,

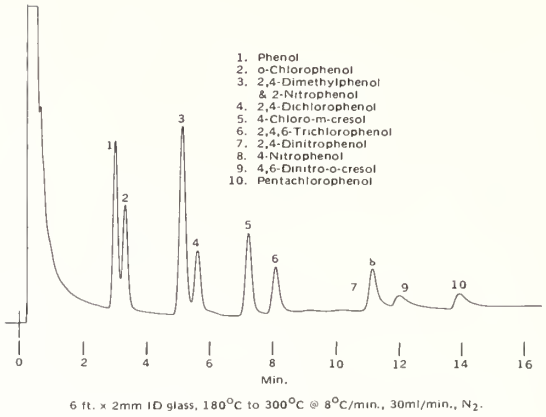


Figure 5. Phenols on Tenax.

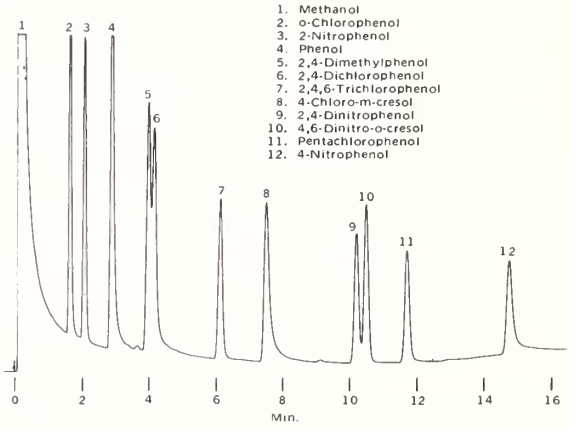


Figure 6. Phenols on SP-1240 DA.

though it does not interfere in the mass spectrometry conformation analysis. During evalution of the SP-1240 DA, it was necessary to periodically replace the phosphoric acid-treated glass wool at the inlet to the column to prevent adsorption of phenols due to accumulated oils and other contaminants. The SP-1240 DA column was used continuously for a period of over 2 months with only periodic changing of the glass wool at the inlet to the column.

VI. Conclusions

The analysis of volatiles in water has been shown with the use of 80/100 mesh Carbopack C/0.2% Carbowax 1500 for direct aqueous injection or purge-trap concentration. A 1% SP-2250 column has been shown to have the versatility and upper temperature limit for use as a general column in the analysis of the base-neutrals, pesticides, and PCBs. The development and extensive evaluation of SP-1240 DA provides a welcomed and proven column packing for the analysis of phenols in water.

VII. Acknowledgments

The authors would like to acknowledge Analytical Services Section, Survalence and Analytical Division, Region 4, Environmental Protection Agency, Athens, Ga., Tracor, Inc. for the

use of a Hall conductivity detector used in the evaluation of columns for the analysis of volatile organics and pesticides, and Hewlett-Packard for the use of an HP-5840 A equipped with a new pulse frequency electron capture detector, for the evaluation of columns in the analysis of volatile organics and pesticides.

VIII. Reference

[1] Requests for the "Sampling and Analysis Procedures for Survey of Industrial Effluents for Priority Pollutants" should be directed to U.S. EPA Effluent Guidelines Division, Washington, DC, 20460, Attention: Mr. Telliard.

Section II. FOOD ANALYSIS

NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

SAMPLING METHODS FOR TRACE ORGANIC ANALYSIS IN FOODS

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The operation of sampling permits a small, conveniently handled portion to provide information as to the condition or composition of a large lot. How well the small portion represents the large portion is a function of the nature of the sampling operation, number of units in the sample, and the homogeneity of the parent lot. Many of the problems in analytical food chemistry at μg and mg per kg levels involve a very heterogeneous distribution of toxic compounds, causing the sampling error to be very much greater than the analytical error. In such a situation it does little good to improve the precision of the method of analysis, because the improvement is simply "swamped out" by the magnitude of the sampling error. More reliable results can only be obtained by taking and analyzing more and/or larger samples.

Key words: Analysis; analytical food chemistry; errors; sampling.

Analytical chemists should have an annual ritual of rereading the article entitled "The Chemical Analysis of Things as They Are" presented over 40 years ago by Dr. G. E. F. Lundell at the 85th meeting of the American Chemical Society and republished in the 1972 NBS Special Publication 351. In this article, Dr. Lundell stated that he had chosen the subject because so many talks and articles dealt with "The Chemical Analysis of Things as They are NOT." He stressed that this situation had been caused by a constant drift toward specialization. As a consequence, there was a tendency to devote more time to the determinative step and less time to chemical analysis itself. In other words, chemical analysis was considered as dealing with one or two variables instead of the dozen or more variables which might be involved in everyday analysis. Dr. Lundell's viewpoint of analytical chemistry is as applicable today as it was then. The editors of analytical journals should send a reprint of this article to authors who report a precision for their newly developed methods of the order of magnitude of 1% or less.

We will discuss briefly the objectives and important areas of analytical food chemistry in order to focus on sampling and its impact on analysis of food for organic compounds at trace levels. We will use as examples the programs and methods of the Food and Drug Administration (FDA) with which we are most familiar, such as pesticide residues, aflatoxin, and nitrosamines. Furthermore, we intend to treat the subject from a practical, and not from a theoretical or academic point of view.

I. Objectives of Food Analysis

Analyses of food are conducted for many different reasons. The most frequent practical objectives are: (1) to determine the safety of the product; (2) to ascertain if the product meets legal, nutritional, or commercial specifications; and (3) to specify the quality of the product and the resultant economic value. Industrial and academic laboratories have additional technological and research objectives. Because food may be easily debased, defiled, decomposed, or contaminated, legal tolerances, standards, and guidelines have been established by the responsible government agencies to ensure safety, integrity, and nutritional content. These specifications involve additives or contaminants (safety), conformity to standards (integrity), and economic

attributes (quality). Some of the important areas of analytical food chemistry, with current examples, are summarized in Table 1. You will recognize immediately that many of these problems involve analyzing for trace levels of organic compounds, in the μg and mg per kg range. In conducting such analyses, we must cope with: (1) a large number of commodities of varied chemical and physical composition and stability; (2) potential presence of a large number of compounds and their alteration products, with a wide range of chemical properties resulting from natural occurrence, direct addition or application, translocation from the environment, ingestion by animals, migration from packaging materials, etc.; and (3) different orders of magnitude of quantitation required for different chemicals, based on technological, legal, or toxicological consideration.

From these areas we will pick a number of important topics that illustrate the significance of sampling in food control problems. There are many other collateral aspects of handling the sample, which are important for reliable analytical results, which we will not cover, such as: containers, preservation, transportation, identification, and record keeping. These items are discussed from the point of view of food analysis in a recent book on quality assurance [1].

Throughout this discussion, keep in mind the significance of the sample—it is only a means to an end. You are usually only incidentally interested in the sample per se; most of the time you are really interested in the composition of the lot that the sample represents. To paraphrase a profound advertisement by the Eli Lilly Company depicting a drug dosage form with the caption "The one you take is never tested," "the sample you analyze is never eaten." The capital and operating costs of analysis for trace constituents in food are extremely high. Very often the decisions made as a result of these analyses have an important significance on human health as well as social, economic, and political implications. Analysis of samples from which a valid conclusion cannot be made is not only a waste of resources but a potential cause of considerable social and political mischief.

TABLE 1. *Same important areas and problems of analytical food chemistry (1978), and examples*

I. FOOD SAFETY
- A. Additives and their metabolites
  1. Pesticide residues (organochlorine, organophosphorus, organonitrogen)
  2. Drug residues in animal tissues (growth promotants, antibiotics, arsenicals)
  3. Food additives (preservatives, antioxidants, artificial sweeteners, nitrites, vinyl chloride, acrylonitrile)
  4. Color additives
- B. Contaminants
  1. Natural poisons
     a. Mycotoxins (aflatoxins, ochratoxin)
     b. Marine toxins (paralytic shellfish poison)
     c. Plant toxins (solanine, cyanogenic glycosides)
  2. Toxic elements (lead, cadmium, mercury, arsenic)
  3. Industrial chemicals
     a. Organohalogen compounds (chlorodioxins, polychlorinated biphenyls (PCB), polybrominated biphenyls (PBB))
     b. Polynuclear hydrocarbons (benzo(a)pyrene)
     c. Asbestos
- C. Biological vectors (sanitation)

II. FOOD COMPOSITION
- A. Decomposition (volatile acids and bases, histamine)
- B. Commodity standards, specifications, and identity
- C. Nutrient labeling
  1. Proximate analysis (moisture, ash, protein, carbohydrates)
  2. Minerals (calcium, phosphorus, iodine, iron)
  3. Vitamins

III. FOOD QUALITY
- A. Value
- B. Grade

II. Pesticide Residues

Food chemists have always recognized the importance of sampling because of the heterogeneous nature of many of their commodities. Initially, problems with trace levels of organic compounds involved such matters as phase distribution and solubility, in the cause of preservatives, or the distribution of solid vitamin and mineral additives in cereal products.

The passage of the Pesticide Amendment to the Federal Food, Drug, and Cosmetic Act in 1954 resulted in full appreciation of the contribution of sampling to the variability of an analysis for a constituent present in a substrate in the mg/kg range. Initially, pesticides are applied to growing crops in experimental plots to obtain information on the maximum potential residue to be expected at harvest when utilizing good agricultural practices. This residue data, decay rates, and safety considerations result in the establishment of a regulation permitting a maximum level (tolerance) of the pesticide residue on the crop as it is shipped in interstate commerce. After the issuance of a regulation which permits use of a pesticide, the commodities may be sampled by an FDA inspector and analyzed to determine if any residue found is above the tolerance. The sampling procedures to develop residue data required for establishing a regulation are different from those necessary to enforce a regulation. They are also different from programs designed to monitor trends in residue levels in foods, as in FDA's "total diet" program. Data from the total diet program are used in making important decisions on continued pesticide usage. This leads to one of the important principles of proper sampling.

Define the Purpose of Your Sample

If the purpose of the work is to determine the concentration of residue reasonably expected as a result of the application of a particular pesticide to a specific crop, consider the following partial list of factors which may contribute to the variability of the final answer:

Geographical location, involving soil, topography, and climate;

Size of test plots as they affect the sufficiency of material for replications and controls and as they minimize nonuniformity from drift, skips, and edge effects in application of the pesticide;

Placement factors of dosage levels, formulations, and application techniques (aerial, spray, dust, soil treatments, encapsulation, etc.);

Nature and maturity of the crop and time of application for proper pest control;

Time of sampling to permit a determination of the decay pattern.

With these factors in mind, a number of gross samples must be taken from the field in such a manner that a reasonable representation of the crop is obtained. Fortunately, the design and analysis of experiments in agronomy formed the basis of modern statistical analysis. It has its basis in the concept of randomness, which requires that every portion of the material involved have an equal chance of being incorporated into the sample. In general, the larger the sample, the greater the probability that it truly represents the entire experiment. It is not at all uncommon for several hundred pounds of material to be taken from the experimental plot to represent the pesticide residue pattern at a given time. This sample must be accompanied by a sizable control, grown under similar conditions but without the pesticide treatment, in order to have a crop blank and untreated material for recovery studies to verify analyst and method performance.

It is obviously very difficult to generalize in this area. The general situation for most field crops requires several hundred pounds of primary sample. However, it may have to be greater for watermelons and smaller for mustard seed. The commodity may not even be a fruit or vegetable crop but rather an animal product such as meat, milk, or eggs. A single quart of milk may adequately represent 10,000 pounds of homogenized milk but a single liver would not represent an entire herd of animals even if they all consumed the same feed.

Several hundred pounds of anything is too much to handle in the laboratory so the primary sample must be subdivided into a more manageable portion, still keeping in mind the requirement

of representativeness. The primary sample must therefore be cut, chopped, or ground into smaller pieces, remixed, and resampled. The process is continued until about a kg of ground sample is obtained for the laboratory. It is useful to obtain replicates of these kg subsamples since analyses of these separately will provide an estimate of the subsampling error.

The question is often asked if the process can be shortened. The answer is yes, it can—but only at the expense of reliability. Six apples removed from a field sample of 1000 can be chopped and analyzed and provide a result but it will be a matter of chance if the next set of six apples from the remaining 994 apples come anywhere near the first value. The extent of agreement between the first and subsequent samples and to what extent we can extrapolate to the parent population is the subject of samping statistics.

The accepted manner of obtaining the laboratory sample [2] is by mixing and quartering the field sample. Any of the 4 quarters is expected to represent the field sample if it contains a sufficiently large number of subunits and is well mixed. If this is so, 3 quarters can be discarded. The remaining quarter is processed to remove ordinarily inedible portions such as crowns, stalks, shells, hulls, stems, leaves, and similar inedible waste material. However, the pesticide regulations [3] must be consulted as to what legally constitutes the agricultural commodity. The regulations contain some surprises, such as: corn consists of the kernels plus cob with husks removed; sugar beets include both the roots and tops; and peas sometimes include the pods and sometimes not. The quartered sample is now of a size that can be cut, ground, chopped, or otherwise reduced into finer particles which can be further mixed and quartered down to a manageable laboratory sample. The standard compendia on food analysis such as the AOAC book of methods [4] should be consulted for suitable methods of reducing various foods.

The Pesticide Analytical Manual of FDA [5] provides valuable information specific to preparation of samples for pesticide residue analysis. If all else fails, freezing with Dry Ice or liquid nitrogen will usually permit grinding otherwise intractable commodities, but they must be protected from moisture condensation during the process.

Field sampling as described above is usually conducted in a research atmosphere by a pesticide manufacturer to produce data in support of a petition to a government agency to establish a legal tolerance for the pesticide residue. Once the tolerance has been granted, the problem becomes one of practical compliance sampling to see that the tolerance is not exceeded. The Food and Drug Administration has this responsibility for pesticide residues. Such sampling usually is performed not by the analytical laboratory, but by a separate inspectional organization over which the laboratory ordinarily has little control. Although the sampling purpose is again the same as before—to obtain a representation of the lot—the situation is quite different. We are no longer confronted with agronomic and application variables, but rather with marketing and distribution variables. An inspector may be faced with a carload of wheat, a truckload of watermelons, or a stack of cartons of lettuce. Large lots in transit or in storage bins are the most common sampling problem faced by a regulatory agency—not the removal of several cans of processed foods from a supermarket shelf.

When food is stored in bulk, those parts of the mass with the greatest exposure to the environment usually undergo greater change in composition than those parts in the sheltered interior. But an inspector faced with two doors of a railroad car or a stock of 10,000 cases of canned goods in a warehouse does not have time to calculate the surface to volume ratio and sample accordingly. An entire day might be required to shift a stack of canned goods to obtain a random sample. The practical solution, when the time of sampling is unimportant, is to perform the sampling operation when the lot is being moved. Then all parts of the lot are accessible. When sampling cannot be delayed, particularly with perishable commodities, compromises must be made. However, compromises invariably result in increased sampling errors.

About the best we can ask of the inspector is to try to preserve the identity of individual lots, if marked. The greatest variability within a single shipment is likely to be the lot-to-lot variation. For packaged products, a lot is defined as a collection of primary containers or units of

the same size, type, and style, produced under conditions as uniform as possible, designated by a common container code or marking, or in the absence of any code or marking, a day's production [6]. A shipment of food that has been sampled as a single lot, when it actually consists of a number of lots, will exhibit greater between-sample variability than if each lot were sampled and analyzed individually. But sampling each identifiable lot individually will require the collection and analysis of more samples than sampling the entire shipment as a single lot. There can be occasions when time and cost factors are more important than increased sampling error.

The definition given for processed foods in identifiable containers is not satisfactory for bulk commodities, such as a shipload of grain or a truckload of potatoes. Here, even if primary identifiable containers are available, such as compartments or sacks, repeated loading and unloading of the material undoubtedly accomplishes a certain degree of dispersion of any homogeneity factors traceable to origin or processing. Removal of samples from bulk lots is best accomplished by hand during the loading or unloading process for large commodities, or with a continuous sampler or by cup samples at relatively constant intervals, for small sized commodities. In a static situation, long compartmented probes, triers, or thiefs are used to attempt to reach representative portions of the lot in a random fashion.

Fish present an interesting special case from many points of view. Fish are the ultimate bioaccumulators of fat-soluble pesticides and industrial chemicals. They are sampled because they can concentrate unmeasurable amounts of contaminants in the water into measurable amounts in their bodies. But what constitutes a suitable lot sample and analytical sample? Larger fish of a given specie contain higher concentrations of fat-soluble compounds than smaller fish because of a longer exposure time. Some fish have their fat storage depots under the skin while others have the fat more evenly distributed. Fish are not covered by pesticide tolerances so there is no legal indication of the portion to be analyzed. For environmental purposes, the whole fish—scales, bones, skin, and viscera as well as the flesh—would constitute the proper sample. For application of the Food, Drug, and Cosmetic Act, the edible part is the proper sample. It is important not to compare analytical values obtained on whole raw fish with values obtained on the edible raw portion or with values obtained on the edible cooked portion.

Different values obtained from separate samples taken from the same shipment or lot, unfortunately, are often ascribed to poor analytical work or to unsatisfactory methods of analysis rather than to the generally more likely cause of improper sampling and/or inherent variability of the lot or of the shipment. In any case, the importance of proper sampling cannot be over-emphasized. If replicates from the same laboratory-prepared samples produce closely agreeing values, but the averages of the individual laboratory-prepared samples from the same lot do not agree with each other, improper sampling or sample preparation, or extreme variability of the lot are likely causes. Quality assurance of the sampling operation is necessarily a more difficult and expensive operation than quality assurance of the laboratory operations because proper sampling ordinarily can only be checked by procedures which include the laboratory component.

The importance of sampling errors for analytical chemists in the field of trace analysis lies in its significance for research in improved analytical methods. If we take the academic viewpoint that an "improved" method of analysis is one with better accuracy and better precision, and possibly a lower limit of detection, then sampling and not method will often be the limiting factor in practical trace analysis. As pointed out by Youden [7], if the analytical error is already less than one-third of the sampling error, further reduction of the analytical error is of no importance.

The overall precision of methods of analysis used in food and drug analysis in general may be more a function of concentration of analyte than of the method of analysis [1]. The extremes thus far reviewed in detail indicate overall precisions (between-laboratories), expressed as coefficients of variation, of approximately 32% for aflatoxin analyses [8] at levels of μg/kg to 2–4% at levels of 0.1 gram to kg/kg (0.1–100%) in pharmaceutical analyses [9]. For pesticide residue analysis [10] at the mg/kg level, the interlaboratory coefficient of variation is about 16%. As a first approximation, the intralaboratory coefficient of variation is about one-half to two-thirds

the interlaboratory coefficient of variation [8]. Therefore the within-laboratory (i.e., analyst) coefficient of variation in pesticide analysis would be about 10%.

There are very few data on sampling error in pesticide residue analysis since each investigation usually involved a single pesticide on a single crop under experimental conditions. The Food and Drug Administration conducted an investigation of the variabilities involved when sampling six categories of crops as follows:

(1) Large hard fruits (e.g., apples, pears, peaches)
(2) Small soft fruits (e.g., berries, grapes, currants, cranberries)
(3) Leaf and stem vegetables (e.g., spinach, celery, lettuce, cabbage)
(4) Vine and ear vegetables (e.g., cucumbers, squash, corn, tomatoes, peas)
(5) Root vegetables (e.g., potatoes, carrots, yams, turnips)
(6) Forage (e.g., hay, silage)

The primary purpose of the investigation was to determine if analysis of a composite sample from 10 subdivisions could reflect the pesticide content of a lot as accurately as analyses of the 10 individual subdivisions. Although our analysis and interpretation of the data are not yet complete, they show [11], as predicted by statistical theory, a 3 to 1 ratio of the coefficients of variation of the two types of samples: Individual subdivisions (10 analyses), Coefficient of Variation (CV)= 60%; composites (of 10), CV = 20%.

Now comparing the 20% relative sampling error of composites with the 10% relative within-laboratory analytical error, it can be seen that we are approaching Youden's break-even point of three to one. There is some room for improvement in pesticide residue methods, say from a CV of 10% to a CV of 7%. In practice this might improve the 95% confidence limits (the region within which we would expect to find the "true" value 95 times in 100) for 1.00 mg/kg residue from 0.80–1.20 to 0.86–1.14. However, this improvement is in the face of the ever-present sampling variability with its corresponding confidence limits (assuming use of composite samples of 10 subdivisions) of 0.65–1.35. The confidence limits reflecting the two effects (analysis and sampling) are obtained by pooling variances. For the 10% analytical CV, the confidence limits for the combined errors would be 0.60–1.40; for the 7% analytical CV, 0.63–1.37. So you can see that even if a substantial improvement can be obtained in the reliability of the analysis, it will achieve very little in the face of the overwhelming effect of the sampling variability. If improved reliability is required, we must concentrate on reducing the sampling error. Sampling error can be reduced only by increasing the size and number of units in the lot sample and analytical subsample.

III. Aflatoxins

In case you think we have taken a very pessimistic and unreasonable estimate of sampling error in pesticide residue analysis, let us examine the only throughly documented case of sources of variability in the analysis of a very low level contaminant—aflatoxin.

Aflatoxins are a group of toxic compounds produced by the mold *Aspergillus flavus,* from which the name was derived. They contain the difuranocoumarin moiety with the lactone ring oxygen conjugated with a double bond. Aflatoxin B_1, the major member of the group, is a very potent carcinogen. Here we are concerned with contamination in the low μg/kg range. Among the commodities which are unavoidably contaminated with aflatoxin are peanuts.

The problem of sampling shelled peanuts for aflatoxin has been investigated extensively, both theoretically and experimentally, in a remarkable series of papers by Whitaker and his colleagues [12–16]. First, they point out that the distribution of aflatoxin-contaminated units in shelled peanuts resembles the characteristics of the incidence of contagious diseases, which have been studied successfully using the negative binomial distribution. The negative binomial probability function describes a situation with high probability of zero counts and low probabilities of very high counts. This has been demonstrated for peanuts, cottonseed, and corn [8]. Levels of

the order of magnitude of 1,000,000 μg aflatoxin/kg (0.1%) in a few individual kernels have been reported. The general applicability of this distribution was tested against data collected under the peanut marketing program of the U.S. Department of Agriculture in its examination of over 30,000 lots of peanuts for aflatoxin [13,14]. The actual data were found to fit the negative binomial model fairly well [15]. With this information, efficient sampling procedures (sample size and number of samples and analyses per lot) can be devised to determine, within acceptable confidence limits, if the true average level of aflatoxin in a lot is above or below a given acceptance level.

The relative distribution of analytical and sampling errors was the specific objective of one of Whitaker's studies [16]. Each of 29 lots of 120 pounds of shelled peanuts (about 106,000 kernels) was divided by a riffle into ten 12-pound subsamples. Each 12-pound subsample (10,600 kernels) was comminuted in a mill, from which a 280-gram (10 ounces, 550 kernels) subsample was removed and extracted. All aflatoxin assays were conducted in a single laboratory by solvent extraction, thin layer chromotography, and densitometric measurement of spot intensity. Figure 1, taken from this paper, shows the design of the experiment. Replication of various points in the design provided the data for estimating the variance of the individual steps.

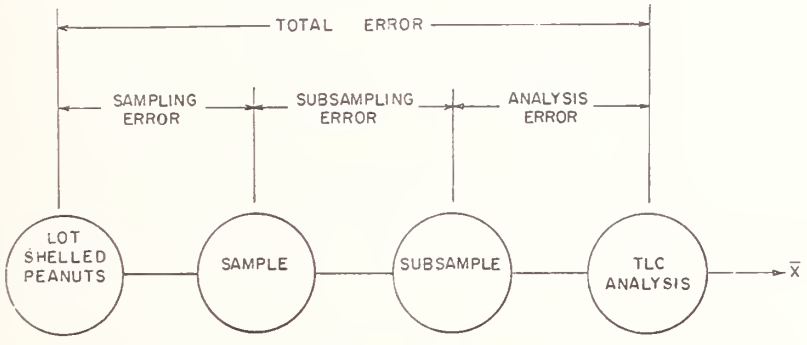

Figure 1. Design of experiment to determine error components in aflatoxin analysis. TLC=thin-layer chromatography. (From Whitaker, Dickens, and Monroe [16]).

Figure 2 shows the coefficient of variation as a function of total aflatoxin concentration in μg/kg (ppb) for each of the three important error components of this experiment. At the critical 20 ppb guideline level, the relative contribution of each component to variability, expressed in terms useful to the chemist (relative standard deviation or coefficient of variation) and to the statistician (variance), are shown in Table 2. The substantial difference between the two columns is a mathematical artifact. The standard deviation is in the same units as the measurement; the variance is the standard deviation squared, an operation which exaggerates the effect of a large deviation. The statistician manipulates and interprets his results in terms of the variance because variances are additive. Standard deviations do not add directly. Note that at the 20 ppb level, almost 90% of the total error results from sampling and subsampling, and not from the analysis. Therefore it will do little good to improve the precision of our methods of analysis for aflatoxin as long as nature can permit a single moldy peanut to contaminate a large lot of peanuts with measureable amounts of aflatoxin after grinding and mixing.

One thing we can do in this situation is to take and handle larger samples. Another approach is that used in the current USDA peanut marketing order. It uses a sequential analysis plan with three 48 pound (22 kg) samples submitted to the laboratory. Only the first is ground until the results of duplicate analyses are known. Results below or above statistically defined limits permit an unqualified acceptance or rejection of the lot. If the result is equivocal, the second 48 pound portion is ground and analyzed and a decision is based upon the average of the four

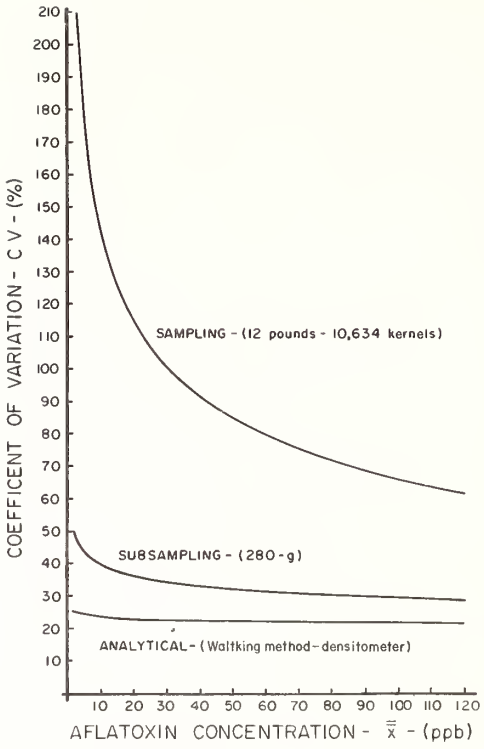

Figure 2. Coefficient of variation of the error components as a function of aflatoxin concentration (from Whitaker, Dickens, and Monroe [16]).

TABLE 2. *Relative errors at aflatoxin concentration of 20 μg/kg expressed in terms of standard deviation and variance*

Error Source	% of total as function of: Std Dev.	Total Variance
Sampling	67	89
Subsampling	20	8
Analysis	13	3
Total	100	100

results, using narrower confidence limits derived from the larger sample and greater number of analyses. If the value is still equivocal, the third portion is ground and analyzed and a decision is made for acceptance or rejection based upon the average of the six analyses from the three samples.

Commercial firms utilizing peanuts for peanut butter, confectionery, and other food purposes also have elaborate sampling and inspection programs to avoid the introduction of aflatoxin contamination into their products. Although analysis is the critical final step in the control process, at least an equal amount of care and effort is required in the sampling procedure to obtain the samples for analysis. As long as the sampling error constituted as much as 90% of the total error in the analysis for aflatoxins, there was little incentive to spend much time in "improving" the thin layer chromatographic methods currently in use to control aflatoxin contamination of foods.

Edible seed products provide an example of a case of contamination when it is inadvisable to seek the contaminant by representative sampling. In certain types of adulteration, a food is considered adulterated if it consists *in whole or in part* of impure material. Experienced public health inspectors deliberately attempt to locate those portions of a lot that show the adulteration "in part." Damaged, moldy, or discolored seeds are considered to be adulterated *per se.* If pockets of imperfect seeds or nuts are discovered, collection of a representative sample of the bulk material will result only in the dilution of the adulterant, often to a point well below the limit of detection. Selective sampling is ordinarily performed when it is suspected that a deliberate attempt has been made to hide damaged goods by mixing it with sound material. This example again emphasizes the importance of defining the purpose of the sample.

IV. *N*-nitrosamines

The analysis for trace quantities of *N*-nitrosamines in foods has also received intensive attention in our laboratories during the last several years as well as in other laboratories all over the world. These contaminants, the majority of which have been demonstrated to be carcinogenic, may be formed by the action of added nitrites on secondary and tertiary amines which are normally present in many foods. In addition, it has also been found that nitrosamines can be formed through the nitrosation of primary amines and quaternary ammonium compounds. Nitrite additives have been widely used for preservation (control of *Clostridium botulinum)* and in some cases for color fixation in meat and fish.

Numerous surveys, using gas chromatographic—mass spectrometric determinative procedures developed in our laboratories, have been performed on those foods which have the potential for nitrosamine formation. In general, these compounds, including *N*-nitrosodimethylamine, *N*-nitrosopiperidine, and *N*-nitrosopyrrolidine, have been found sporadically in some meat and fish products at low μg/kg levels. Some of these occurrences have been traced to use of nitrite-spice curing premixes, which, it is hoped, are no longer a problem, since FDA has amended the regulations regarding the use of nitrites and/or nitrates in cure mixtures. However, as has been pointed out, it is still possible that amines in the spices may react with nitrite in the finished meat product during storage or cooking. It is also important to note that the presence of *N*-nitrosamines has been verified in raw fish with no prior history of nitrite treatment. Obviously, the very randomness of the occurrence of these compounds presents a problem in how and what to sample. However, the food product that has received the most publicity in this area is bacon. This food presents some unique problems in sampling and sample preparation.

Unlike the random occurrence of *N*-nitrosamines in meat and fish products, *N*-nitrosopyrrolidine has been shown to occur rather consistently in the μg/kg range in *cooked* bacon. The compound has not been found in raw bacon, although some investigators have reported low levels of *N*-nitrosodimethylamine in the uncooked product, unconfirmed by mass spectrometry. The mechanism of formation of the nitrosopyrrolidine in the cooked bacon has not been resolved, although available evidence indicates that the nitrosation of proline, with subsequent decarboxylation on heating, is involved. During the past few years, the meat industry has been able to significantly reduce the nitrosamine content by increased addition of ascorbic acid (which inhibits nitrosamine formation) and by reduction of the nitrite level. Considerable activity is also being devoted to finding a substitute for nitrite.

While these studies are being conducted in an attempt to control or eliminate the problem, the sampling and analysis of bacon must be continued to provide up-to-date data on the product in the marketplace. Sampling of bacon is of particular interest since it is one of the few products which is obtained directly from the retail market. Retail sampling is necessary because the industry has been unable to provide bacon, either experimentally or from the processing line,

which shows the levels of *N*-nitrosopyrrolidine encountered in the retail samples. Apparently, other factors such as abuse or quality control in distribution channels are involved.

The preparation of the bacon sample is also unique in that the product must be fried to produce the nitrosopyrrolidine contaminant. The bacon is purchased in retail packages and individual strips are fried in a calibrated electric frying pan under standardized conditions of 340 °F for 3 minutes on each side to a "golden crispness." The fried strips are then blotted with paper towels to remove the excess rendered fat as usually done by the housewife. The strips are then ground in a chopper and composited, and replicate portions are taken for analysis. It is important to note that the nitrosopyrrolidine is volatile and fat soluble so that appreciable quantities of the compound are found in the vapor above the frying bacon and higher concentrations are found in the rendered fat than in the cooked bacon.

You need not be an experienced bacon eater to realize that the circumstances described cannot be called controlled conditions. Assume a frying pan having a constant surface temperature of 340 °F is used at the start. After exactly 6 minutes of exposure of bacon strips of variable thickness, with irregular ratios of fat to lean, and a variable tendency to form an undulating pattern, superimposed on a poorly designed steam distillation and a simultaneous rendering operation, the results is a magnificant example of uncontrolled variables introduced into the analytical process.

V. Other Systems

We have gone into some detail on these three important areas of interest to FDA as illustrations of the problems which exist in bringing an analytical sample to the chemist. The remaining time will be used to outline a few other sampling problems that have had to be resolved.

Whenever there is a major oil spill, our primary concern is to ensure the wholesomeness of fish and seafood from the affected area which may be offered in commercial channels. But there appears to be a background of "normal" endogenous or absorbed polynuclear hydrocarbons in seafood. Perhaps they are the residue of a long history of exposure to natural or accidental oil spills. In any case it is essential to have a blank or control of the identical species living under similar, if not identical conditions. Such a control sample is critical to an indisputable conclusion of contamination. The control sample has been taken from as far away as 150 miles from the spill to obtain suitable uncontaminated samples for background and recovery studies.

Many polymers are produced by different manufacturers for use in the further formulation of containers for food, as films or rigid surfaces which contact food, or as adhesives and cements which are components of articles which may contact food. Many of these polymers and their adjuvants are produced in experimental lots prior to commercial production. It is almost impossible to produce a polymer without trace amounts of unreacted monomer remaining. The amount of unreacted monomer in the resin will vary from batch to batch and from manufacturer to manufacturer, depending upon further variations in processing procedures. Interest in the monomers lies in the fact that some of them have been shown to be carcinogenic. The sampling problem here is one of complete market coverage and the difficulty in acquiring a true control sample totally free of the component under investigation.

Sometimes systems will not remain constant with the passage of time. Antioxidants will disappear from foods so that samples taken at different times will not be reproducible. This is an expected phenomenon since antioxidants are expected to decompose as they perform their function of reacting with oxygen before the oxygen reacts with the food.

One final example from the area of animal drug residues might be termed secondary sampling. Drugs are frequently fed to animals for therapeutic, prophylactic, and growth promoting purposes. The drug is distributed throughout the body; part is excreted, part is deposited, and part is metabolized. It would be extremely difficult to sample a whole cow or even a whole chicken. In

a *Federal Register* document which has not yet received from analytical chemists the full attention that it deserves, the FDA announced that a "marker residue" in a "target tissue" would constitute the appropriate sample for examination for residues of carcinogenic concern [17]. The target tissue, often liver, is that tissue in which the residue is most likely to occur at the highest level, thereby providing a more "reliable" measure of the total residue in all edible tissues; the marker residue is that residue (the compound or a metabolite, or any combination) whose level in the target tissue is a reliable measure of the total residue of carcinogenic concern in all edible tissue. Considerable analytical work is required to establish which tissue in what organ is the suitable sample for regulatory analysis, but once established it constitutes the legal sample for examination for the drug residue of concern.

VI. Conclusion

We have discussed a number of aspects of sampling foods as the prerequisite to the analysis of constituents present in the μg and mg/kg levels. Through a detailed discussion of three important examples—pesticide residues, aflatoxins, and nitrosamines—we have emphasized a number of important points regarding sampling:

The results of your laboratory sample are really not as important as what it implies regarding the composition of the parent lot it represents.

The purpose of the work determines to a great extent the sampling procedure. Establishing a regulation, enforcing a regulation, or determining the concentration level in the general food supply require different sampling procedures.

In many contaminant problems, the compound of interest is distributed in a heterogeneous random fashion throughout the lot. In such cases, the sampling error is so great that it overwhelms the analytical error; therefore, it does little good to improve the analytical procedure. Greater reliability is achieved only through examination of more and/or larger samples.

Although we have emphasized the vagaries of sampling, we should not overlook the intermediate steps from the lot sample to the analytical portion—containers, storage, mixing, size reduction, subsampling, protection from contamination, and above all, proper record keeping.

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THE ROLE OF SAMPLE PREPARATION IN NUTRITIONAL LABELING ANALYSIS

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The broad spectrum of food products requiring analysis for nutritional labeling and the ubiquitous nature of many of the substances being determined has created many diverse problems for the food scientist. Among these is the need to obtain from rather large representative lots, products prepared in such a manner so as to guarantee uniformity of the sample and the integrity of their nutrients which are by their very nature highly reactive and most labile. The problem is compounded by the fact that in the final actual analysis minute quantities of the original sample must be analyzed by using semi-micro and even micro methods with the same precision and accuracy demanded by macro techniques. This paper will discuss some of the diversified products encountered by the food analyst in his daily routine and the special techniques and equipment which must be utilized to handle such samples. Also stressed will be the importance of sample preparation and how good sample handling techniques can be used to minimize the loss of nutrients and sampling errors which, unless corrected, are greatly amplified as a consequence of nutritional labeling.

Key words: Composite sampling; nutritional analysis; nutritional labeling; sample preparation.

The advent of nutritional labeling has placed an ever-increasing demand upon the food scientist, and in particular, upon the industrial food analyst. Paramount among these demands is the responsibility to develop information regarding the nutritional composition of foods which represents to the consumer that which is being manufactured by the food processor. This is not the easiest of tasks, particularly when one considers the ubiquitous nature of the nutrients; the trace amounts at which they are found; their susceptibility to further reaction and change; and the wide variety of food types and matrices requiring analysis under such a program. With this in mind, it is the purpose of this presentation to provide an overview of the problem areas encountered by the analyst in the area of sample preparation, and to show how these problems relate to the effective management of a program of developing nutritional labeling information for the consumer. To provide a proper framework for this discussion, however, it is necessary to consider the present federal regulations pertaining to nutritional labeling, summarized in Table 1.

In considering these regulations two portions are directly related. The first establishes the incremental level that each nutrient can be labeled. As seen, these increments increase in units of 2% up to 10% RDA, by units of 5% through 50% RDA, and then increase in increments of 10% through 100% RDA.

The second pertains to the enforcement portion of the regulation which dictates that 12-unit composites of a single day's production, when analyzed by official AOAC methodology, must be within 80% of the declared label value. When one considers the implication of these regulations, it is readily seen that both play a critical part in the manner in which food products are prepared for nutritional analysis. As a case in point, let us consider the problem confronting the food scientist concerned with establishing a label value. Obviously, before any product can be labeled, sufficient analytical data must be developed to establish that the label value is representative of the product being manufactured. In an ideal situation, a particular nutrient varies only slightly within the product; and under these conditions, reliable label values can be established from very small samplings. It is well recognized, however, that there can be wide fluctuations in the nutrient

TABLE 1.

Format		Increments	Enforcement
Serving size			*Naturally occurring* 12-unit composites
No. of servings			When analyzed Within 80% of label value
Calories/serving		5	(120% calories & fat)
Protein, Carbohydrates, Fat	g/serving	1 g	*Fortified food* 12-unit composites When analyzed Within 100% of label value
Protein	% RDA/ Serving	2% up to 10%	
Vitamin A		5% up to 50%	
Vitamin C, Thiamine, Riboflavin		10% up to 100%	
Niacin, Calcium, Iron		over 150%	

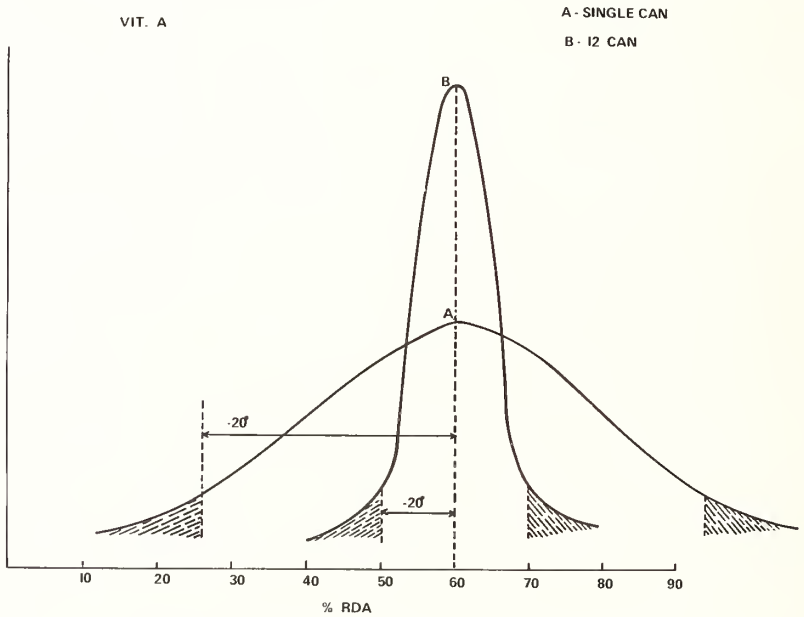

Figure 1. Normal distribution curves for Vitamin A Content.

content. These fluctuations can occur depending upon such factors as seasonal variability, plant location, processing conditions, and even analytical variability. Under such conditions, it is not easy to arrive at a truly representative value, and often times, this poses some difficulty. On one hand, it would be unfair to the product manufacturer to penalize the product by grossly understating its nutritional content to allow for this variability. On the other hand, as shown above, label compliance generally requires that each nutrient in every unit of the product be within 80% of the label value. Perhaps this can best be illustrated more graphically in Figure 1 which shows two normal distribution curves representing the vitamin A content of a product

containing betacarotene as the primary source of this vitamin. In curve A, vitamin A values were determined from the analysis of individual cans. Curve B shows the same data obtained when 12-unit composites were analyzed and the data plotted to give a normal distribution curve similar to the single can analysis. Referring to Curve A, that is, the data obtained from single can analysis, it is seen that the product has a mean of 60% RDA with a standard deviation of approximately ±20%. Recommended government guidelines, as well as most corporate guidelines, dictate that the label value be set to assure 95% compliance, i.e., at a point two standard deviations from the mean. With such a wide variability, a label value of 24% RDA would ensure 95% compliance. However, as has been indicated previously, government regulations stipulate rounding off in units of 5% above 10% RDA. To move the label value closer to the mean under these conditions would increase the risk of non-compliance. The actual label value must, therefore, be set at 20% RDA, a value considerably removed from the mean value of 60% RDA.

Again, referring to Figure 1, Curve B shows the same data obtained when 12-unit composites are analyzed and the data plotted to give a normal distribution curve similar to the single can analysis. The central limit theorem of statistics states this relationship mathematically as follows:

$$(\text{Stan. Dev.})_n = \frac{(\text{Stan. Dev.})}{\sqrt{n}}$$

In referring to Figure 1, it can be seen that the mean percent RDA for this product (12-unit composites) is exactly equal to the mean found for single can analysis. The effect of 12-unit compositing, however, is to reduce the variability by the $\sqrt{12}$; i.e., by a factor of 3.64. A comparison of the two curves shown in Figure 1 demonstrates quite clearly the effect that compositing has in reducing such variability. This figure also shows that the actual label value (45% RDA) can be set considerably closer to the mean value of 60% RDA with the same degree of confidence that 95% of the product will meet this value. It can be seen from this that compositing of samples solves the problem of assuring label compliance while, at the same time, presenting a much more realistic representation of the true nutritional quality.

From a practical standpoint, however, it is easy to imagine the difficulty associated in preparing for nutritional analysis uniform and completely homogeneous 12-unit composites of such diverse samples as turkeys, pies, lunchmeats, breads, frozen dinners, or institutional sized canned products, etc. Compounding this problem is the fact that often the methods utilized in the determination of many of the nutrients involved are so sensitive that often only a minute quantity of the original composite may be subjected to the actual analysis itself.

Perhaps this point is best demonstrated from Table 2 which shows some of the representative levels of the typical nutrients and the amounts of sample composite that is finally required in the actual analytical determination.

TABLE 2. *Relationship of total composite to % sample actually analyzed*

Nutrient	Total sample composite weight, g*	Final analytical determination % original composite	Final analytical determination Amount of nutrient (micrograms)
Thiamine	2900	4×10^{-5}	5
Riboflavin	2900	2×10^{-6}	0.2
Niacin	2900	7×10^{-6}	1
Vitamin A	2900	9×10^{-6}	0.8
Calcium	2900	9×10^{-6}	5
Iron	2900	8×10^{-6}	25
Sodium	2900	7×10^{-8}	10

*Sample weight 12 units at 8.5 oz. (28.4 g/O_3).

When one considers that the individual composite in some instances must be prepared from as high as several thousand grams, it is easy to see the importance that sample preparation and sample homogeneity have in implementing an effective program of analysis from trace quantities of nutrients.

It is with these points in mind that attention can now be given to the manner in which samples are prepared for nutritional analysis. However, as indicated previously, the principal concern of this paper is to provide an overview of the general approach to this problem and more specific details regarding the handling of individual food types will not be considered here. Aulik [1] in his excellent review has amply covered this subject.

Generally speaking, it is well recognized that many of the normal nutritive constituents of food products are susceptible to oxidation and thermal and photochemical decomposition. For these reasons, the area in which blending or sample preparation is to be conducted is usually chosen to be one with no outside windows or with, at the least, subdued lighting. Ideal conditions are those in which a series of amber or red fluorescent lights are mounted overhead and used during the blending operation. For the most part, products are received and held in the frozen state prior to analysis. The samples should be equilibrated overnight at 4 °C. and then brought to room temperature prior to blending. Experience over the past 5 years covering an extensive program of nutritional labeling has shown that three main tools for blending will usually suffice for handling practically all types of food materials requiring compositing and homogenization. These tools include a large industrial size blender, a heavy-duty meat grinder, and some sort of a high-speed chopper such as a Hobart or Fitzmill unit. Another interesting blending technique which we have had occasion to use in our laboratory is that of Cryogenic Blending. As the name implies, samples are hard-frozen and blended usually in a Waring Blendor following the addition of liquid nitrogen. As the sample returns to room temperature, the nitrogen gas volatilizes leaving a fairly homogeneous and representative product. This technique is particularly useful where it is practically impossible to prepare a homogeneous sample by other ordinary techniques.

Table 3 summarizes the broad spectrum of foods normally encountered in the day-to-day operation of an analytical laboratory whose primary function is the analysis of these nutrients. Also shown in this table are the various types of blending units which can be employed for compositing and homogenizing such samples.

Following compositing of the 12-unit sample in a suitable blending unit, the samples are homogenized until uniform. Here consideration should be given to not extending the homogenization cycle to a point where excessive heat is built-up or to any other condition which can be detrimental to labile nutrients such as riboflavin, vitamin A and vitamin C.

Following blending, the homogenized samples may be placed in a large suitable container and again gently mixed by hand until uniform. In our laboratory we routinely use three 6-ounce jars to collect the sample. Since vitamin C is the most labile nutrient, in normal operating procedure the contents of one of the sample jars is immediately taken to the laboratory where ascorbic acid is determined on the sample in as short a time as possible to provide continuity between blending and the actual analytical step itself. The remaining samples are set aside for the determination of B vitamins and for proximate and mineral analysis.

Any considerations of the effect of sample preparation on trace nutritive analysts for nutritional labeling would not be completed without some consideration being given to the influence of serving size. The federal regulations regarding nutritional labeling clearly stipulate (see Table 1) that all nutrients must be expressed on the basis of the amount in one complete serving. Table 4 illustrates the manner in which the variability associated with such factors as non-uniformity of sample preparation, sampling error, the loss of nutrients, or even analytical precision can, unless minimized, be amplified as a consequence of expressing the nutritive content on a per serving size basis.

Table 4 shows, for example, the increase in label value (expressed as % RDA) as the serving size increases from 142 grams in the case of a soup to a serving size of 1 pound in the case of a

frozen dinner. From a nutritional standpoint, the difference shown is negligible but without the proper precautionary controls associated with blending, sampling or analysis, this effect can be appreciable.

TABLE 3. *Representative food types and equipment used in sample preparation for nutritional analysis*

Product	Sample size (grams) (×12)	Meatgrinder	Fitzmill	Blender
Frozen dinners	7,500		x	
Muffins	4,100	x		
Bread	5,500	x	x	
Crackers	5,500	x		
Canned vegetables and fruits	4,800			x
Canned meats	1,620	x	x	
Meats— Raw and cooked	11,000	x	x	
Baby food	1,350			x
Frozen soups	3,400			x
Frozen institutional foods	38,000	x	x	x
Pies	3,800			x
Pie fillings	4,100			x
Frozen cakes	5,400	x	x	
Canned soups	3,600			x
Canned institutional soups	18,000		x	x
Grains	2,100		x	x
Spice mixes	2,100			x
Main dish	3,700			x

TABLE 4. *Effect of serving size on label value (% RDA for thiamine)*

Content/100 g (variability ±0.01)	Content/serving (0.05 mg ±0.01 mg)	% RDA/serving
	Cond. soup (142 g)	
	0.057 → 0.068 mg	3.8 → 4.5% RDA
	0.057 → 0.045 mg	3.8 → 3.0% RDA
	Main dish (270 g)	
0.05 → 0.06 mg	0.14 → 0.16 mg	9.0 → 10.8% RDA
0.05 → 0.04 mg	0.14 → 0.11 mg	9.0 → 7.2% RDA
	Frozen dinner (450 g)	
	0.19 → 0.27 mg	15.0 → 18.0% RDA
	0.19 → 0.10 mg	15.0 → 12.0% RDA

Summary

The broad spectrum of food products requiring analysis for nutritional labeling and the ubiquitous nature of many of the substances being determined creates many diverse problems for the food scientist. Among these is the need to obtain from rather large representative lots, products prepared in such a manner so as to guarantee uniformity of the sample and the integrity of their nutrients which are by their very nature highly reactive and most labile. The problem is compounded by the fact that in the final actual analysis minute quantities of the original sample must be analyzed by using semi-micro and even micro methods with the same precision and accuracy demanded by macro techniques.

Acknowledgment

The author wishes to express his appreciation to Mr. Edward Mangan, for his technical assistance in preparation of this paper.

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NUTRIENT ANALYSES OF FOODS: A REEXAMINATION

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One of the key areas in the rapidly expanding field of human nutrition is the quantitative analysis of nutrients in foods. Contrary to popular belief, the nutrient analysis of foods is *not* routine, and much research is needed to obtain accurate and precise data on the nutrient composition of foods. This paper presents an overview of the analytical aspects of the chemical analysis of food nutrients, emphasizing the organic chemical nutrients (trace and otherwise), and evaluates the state of current nutrient data, the public health problems associated with certain nutrients, and the analytical methodology. Rapid, inexpensive, specific analytical procedures for trace compounds in complex matrices and certified standards and standard reference materials are needed to acquire the required nutrient data.

Key words: Nutrient analysis; nutrition; standard reference materials.

I. Introduction

Public interest in human nutrition and nutrition related health problems has grown enormously in recent years as has interest in, and commitment to, research in these areas by the federal government, particularly the Department of Agriculture and the Department of Health, Education and Welfare.

To provide nutritious diets to people it is necessary to have a complete understanding of the nutritional requirements of humans and a detailed knowledge of the nutrient contents of foods. Mertz has recently reviewed the status of our knowledge of human nutrient requirements [1]. This paper is concerned with the status of our knowledge of nutrient composition of foods.

There is a considerable difference in the different nutrients and their suspected contribution to existing U.S. public health problems. It is suspected that large numbers of Americans have an inadequate consumption of some nutrients such as vitamin A and iron [2]. On the other hand, a considerable number of persons apparently have an excess intake of some nutrients such as cholesterol. My assessment of the inadequate and/or excessive nutrient intakes and their contribution to existing U.S. public health problems is given in Table 1.

The purpose of this paper is to reexamine the current state of our knowledge regarding the nutrient composition of foods, the underlying factors that bring us to this state, and possible fruitful avenues that could be used to improve our knowledge.

A. Current Knowledge of Nutrient Composition of Foods

Many chemists have analyzed foods for their nutrient content, and these data have been compiled by the U.S. Department of Agriculture, which has a long and respected history of publishing tables of nutrient composition. The latest edition of the fundamental compendium of the nutrient content of foods. Agricultural Handbook No. 8, "Composition of Food—Raw, Processed, and Prepared" [3], was published in 1963, and is currently being extensively revised. Two revised sections on dairy and egg products [4], and spices and herbs [5], have recently been published and revised sections on baby foods, fats and oils, and soups, sauces, and gravies are

TABLE 1. *Inadequate and/or excessive nutrient intakes and their contribution to existing U.S. public health problems*

	No known contribution to existing problems	Suspected to be contributing to existing problems	Accepted as contributing to existing problems
Carbohydrates	Starch	Sugars	Fiber
Lipids		Fatty acids Other sterols Trans-fatty acids	Cholesterol Total fat
Minerals and trace elements	Cobalt Molybdenum Nickel Vanadium Tin	Arsenic Chromium Copper Magnesium Manganese Selenium Silicon Sulfur	Calcium Fluorine Iodine Iron Phosphorus Potassium Sodium Zinc
Proteins and amino acids		Amino acids Total protein	
Vitamins	Biotin Cholin Pantothenic acid	Niacin Vitamin E Vitamin K	Folacin Riboflavin Thiamin Vitamin A Vitamin B_6 Vitamin B_{12} Vitamin C Vitamin D
Other			Total calories

planned for release in 1978. Most of the nutrient analyses included in the 1963 version of Handbook 8 were completed before the late 1950's, much of the data in the recently released revisions was obtained in the 1960's and 1970's.

Many people feel that more data is needed on the nutrient composition of foods particularly for processed and prepared foodstuffs and for those nutrients whose importance has only recently been established. The U.S. Department of Agriculture is aware of these needs [6] and intensive efforts are underway to provide accurate up-to-date data on the nutrient composition of foods "as eaten." In a recent assessment of the state of knowledge of the nutrient composition of foods [7], several critical problems were discussed: a) Almost all the data compiled to date have been on food items to be prepared and eaten in the home; b) most of the data are for commodities and few for prepared foods; and c) little is known about the nutrient content of food served in or by institutions, restaurants, and fast food outlets. Although processing and distribution of the foods for the institutional market differs from that for home consumption, it is not known whether these differences affect the nutrient composition of the foods served.

For some nutrients, almost no adequate data are available for any food item. Of the organic chemical nutrients, there is very little information on the levels of the individual simple sugars; starch; nutrient fiber; plant sterols; trans-unsaturated fatty acids; vitamins A, B_6, B_{12}, D, and E, biotin; choline; and pantothenic acid. There are several reasons for the lack of data.

B. Reasons for Present Lack of Data

Historically, emphasis was first placed on those nutrients whose deficiencies in the diet led to symptoms of poor health. The importance of nutrients such as thiamin, niacin, vitamin C, and calcium prompted extensive study of their occurrence in foods. Other nutrients, even though

known to be essential, received little attention because symptoms of deficiency were unrecognized, or marginal deficiencies existed without showing gross symptoms. The importance of some nutrients has been recognized only recently. The analytical values for classes of compounds such as total fat or total carbohydrates were originally considered adequate, but nutritionists now realize that composition data is required for each of the individual sugars and fatty acids. Since 1950, studies have established that trace amounts of elements such as zinc, copper, chromium, and selenium are essential for good health [1]. Only scant analytical data are available on concentrations of these nutrients in food, for only a few scientists have worked on the detailed methodology and only a few foods have been analyzed.

Acquisition of composition data for some nutrients has had low priority because deficiencies or requirements have not been demonstrated. For example, choline deficiency has not been demonstrated in man and it is difficult to establish biotin deficiencies in man. Although composition data eventually will be acquired for such nutrients, it is understandable why there is little data at present.

Our foods have changed since the early analyses. New varieties of traditional food plants, including the cereal grains, are used and processed foods, are consumed in greater amounts, and in forms that did not exist in the 1950's. The introduction and proliferation of new foods came at a time when there was little interest in performing routine analyses for nutrients. Data on the effects of processing on the nutrient contents are particularly needed. Most of the existing data on processed foods have not come from the same laboratories as the data on raw foods. Thus, changes produced by processing can be only inferred but not proven, because the observed differences are confounded with between-laboratory differences, sample differences, methods differences, or even geographical, seasonal, and varietal differences.

C. Problems in Methodology

Underlying the gaps in our basic knowledge of nutrient composition are the lack of appropriate, reliable methods for assaying all the nutrients. A recent estimate of the state of the analytical methodology for the analysis of nutritionally important forms of nutrients is shown in Table 2. New or improved analytical methods for many nutrients are needed, especially for those nutrients whose nutritional significance has only recently been established, such as the trace elements, individual carbohydrates and some of the lipids.

The complexity of foods, the multiplicity of chemical forms of the nutrients, and the sensitivity required for quantification place very special demands upon the analytical system and the analyst. The assay systems will probably require quantitative extraction of the nutrient from the food, complete separation of the isomeric forms of the nutrient from each other and from the biological antagonistic and inactive forms, and sensitive quantitative detection of each compound of interest. Although difficult, the problems are not insoluble.

Enormous advances have been made in analytical chemistry in the past 10 years and the evolution of analytical chemistry promises to continue at a rapid rate. Little of this technology has been applied to nutrient analysis of foods. I believe the transfer of the new analytical technology to food analysis is appropriate, timely, and under the proper stimulation, could rapidly permit solution of many of the nagging problems of nutrient analysis.

D. Problems Peculiar to the Field of Nutrient Analysis

There are several aspects of nutrient analyses that deserve special mention. Perhaps the most important is that nutrients are usually defined as a biological activity not as a chemical structure. Often several closely related, but different, chemical compounds give the same qualitative biological response; although, the quantitative biological responses may differ. For example, α-, β-, and γ-tocopherols all elicit the biological response defined as vitamin E activity. However, the dose response in humans for each of these isomers of tocopherol is different [8].

TABLE 2. *State of development of methodology for the analysis of nutrients in foods* [7]

	Sufficient	Substantial	Conflicting	Fragmentary	Little to none
Carbohydrates			Fiber Starch	Individual sugars	
Lipids		Cholesterol Fatty acids	Other sterols Total fat	Trans-fatty acids	
Minerals & trace elements	Calcium Copper Magnesium Phosphorus Potassium Sodium Sulfur Zinc	Iron Selenium	Chromium Fluorine Iodine Manganese	Molybdenum	Cobalt Silicon Tin Vanadium
Protein		Most amino acids	Total protein Some amino acids		
Vitamins		Vitamin C Niacin Riboflavin Thiamin	Vitamins A, B_6, B_{12}, D, E Folacin Pantothenic acid	Biotin Choline Vitamin K	

KEY

Factors	Sufficient	Substantial	Conflicting	Fragmentary	Little to none
Probability of correct value	excellent	good-excellent	fair	poor	zero to poor
Speed of analysis	fast	slow	slow-fast	slow	very slow
Cost of analysis	low	high	low-high	very high	very high

Although some chemical isomers elicit the same biological response, seemingly quite similar isomers often elicit no response or may even inhibit the normal response. Often when one optical isomer of a nutrient elicits a positive response, its antipode elicits either no response or inhibition [9]. There is often a species specificity about the biological reactions to various isomers so that extrapolation of assays from one species to another should only be done with extreme caution and after careful study. In Figure 1, the activity of serum vitamin B_{12} activity determined by a radioimmunoassay, is plotted versus the activity determined by a microbiological growth assay. If the two assays were responding to the same compounds, the points should fall on the straight line. However, these two assay systems do not respond in the same manner. It is difficult to determine *a priori* which assay is accurate for vitamin B_{12} levels in serum. The availability of a standard reference serum for vitamin B_{12} would help resolve this dilemma.

E. INTERLABORATORY REPRODUCIBILITY PROBLEMS

Another problem with nutrient analyses is the difficulty of reproducing results in interlaboratory collaborative studies. In Table 3 are listed the results of a series of interlaboratory collaborative studies of nutrient contents of various foods, in which each independent laboratory used the same method and samples of the same food for the assay of a given nutrient. A range of $\pm 20\%$ around the mean would give a ratio of 1.50, and for most nutrient analyses this would be the limit of acceptability. By this standard, the assays for vitamins A, B_6 and niacin are not acceptable for any of the foods assayed. The assays for vitamin C and riboflavin are acceptable for

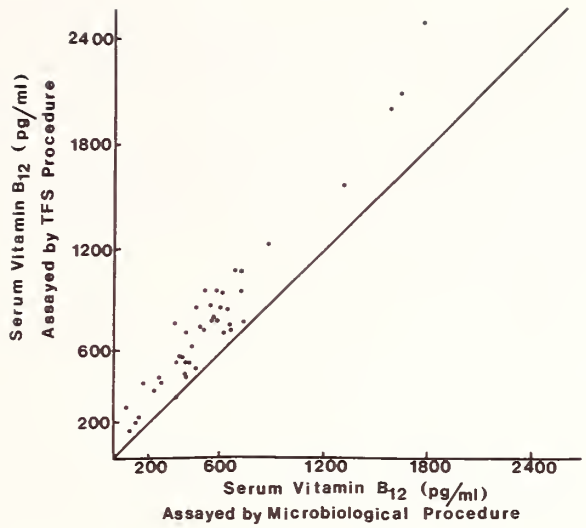

Figure 1. Same serum extracts assayed for vitamin B_{12} by radio immunoassay using toad fish serum and microbiologic assay using *Lactobacillus leichmannii*. The line of identity is indicated. Reprinted from Buchanan et al. [10] in the *Journal of Nuclear Medicine* by permission.

TABLE 3. *Maximum/minimum ratios of the range of nutrient values in different foods found by different laboratories in collaborative studies*

Cholesterol	Vitamin A	Vitamin B_6	Vitamin C	Niacin	Riboflavin
1.41	2.45	2.88	1.15	2.99	1.26
1.52	1.71	1.83	1.02	5.50	1.58
1.57	2.22	3.81	1.11	1.86	1.23
1.23	2.00	5.60		6.78	
2.91	3.70	1.66			
	8.80				

The maximum/minimum ratio equals the maximum range value divided by the minimum range value. Each column lists the values calculated from one collaborative study. Each number represents the ratio for an individual food. The data for the cholesterol study was calculated from the data of Punwar [11], that of vitamin A from the data of Parrish [12], that of vitamin B_6 from the data of Edwards et al. [13], that of vitamin C from the data of Deutsch [14], of niacin from the data of Grass [15] and riboflavin from the data of DeRitter [16]. A range of ±20% around the mean would give a ratio of 1.50.

all the foods assayed and that for cholesterol is acceptable for four of the five foods assayed. Standard reference materials should be useful in reducing the interlaboratory variation found in such studies.

F. Numbers of Required Analyses

The task of assaying nutrient content in foods is not only complex but a great number of analyses are required. Estimates of the total foods and food products available in the U.S. vary, but there seem to be between forty and sixty thousand food items on the market. Nutrient composition varies even within one brand name item in different ways for different nutrients. Conservative estimates indicate that a minimum of 20 to 30 samples will be required per nutrient per food for adequate statistical estimates of the population means for most nutrients and foods.

Thus, the methods of analyses should be rapid and inexpensive as well as accurate and precise. Considerable effort will be required to develop and verify the automation required for

these analyses. Standard reference materials will be needed in the development of these methods and for the maintenance of quality control in the automated analyses.

G. Standards and Standard Reference Materials

Appropriate certified standards and Standard Reference Materials (SRM) are needed to develop and verify these new specific, accurate, precise measurement systems [17,18], and to assure the long term quality control of the field methods necessary to generate the large amounts of needed data. There are two kinds of standards needed.

First are needed highly purified, individual nutrient compounds with sufficient data to accurately identify and certify these compounds for their specific chemical, physical, and spectral properties. These certified standards permit the verification of procedures and calibration of equipment and development of specific nutrient separation and analysis methods.

Well-characterized samples of inorganic nutrients, are readily obtained. However, this is rarely the case with the organic nutrients. Standards are available for a few carbohydrates such as sucrose, glucose, and fructose. There is a cholesterol standard (NBS-SRM) and several of the individual fatty acids are commercially available as pure compounds. Very few of the vitamins are available in sufficient purity and the existence of isomers complicates the problem.

Secondly, selected food samples certified for specific nutrient levels are needed. Almost all measurement systems for accurate determination of a specific nutrient depend upon a quantitative procedure for separating the nutrient from a very complex food matrix. To develop these procedures and to monitor quality control upon their long term use requires certified reference materials of the various matrices which might be analyzed, i.e., different types of foods. There are presently available several biological SRMs from the U.S. National Bureau of Standards which are certified for some of the trace elements of nutritional interest and several of these are potentially useful as SRMs for food analysis. However, additional SRMs in several additional classes of foods such as dairy products and meat are needed. Standard reference materials for other classes of nutrients including the vitamins, individual fatty acids, carbohydrates, and amino acids need to be developed. The establishment and availability of appropriate SRMs in this area will allow the needed development, validation and application of necessary rapid, automated analytical methods for the determination of these nutrients in the food supply.

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MEASUREMENT OF THE TRACE AMOUNTS OF VITAMIN B_{12} PRESENT IN VARIOUS FOODS BY A NEW RADIOMETRIC MICROBIOLOGIC TECHNIQUE

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A simple, sensitive and reliable radiometric microbiological assay was developed for the assay of vitamin B_{12} content in foods. The evolution of $^{14}CO_2$ by *L. leichmannii* from L(guanido ^{14}C) arginine was shown to be directly proportional to the amount of cyanocobalamin added to the usual assay medium. The assay requires less technical time than the standard turbidimetric assay and is semi-automated. Comparison of the vitamin B_{12} content of a variety of foods by this radiometric assay and the standard microbiological assay gave an *r* value of 0.96. Recovery experiments using cyanocobalamin gave good results with foods of animal origin. Recovery from foods of vegetable or grain origin were significantly lower. Reproducibility on a variety of foods and replicate samples of the same foods by the radiometric technique was satisfactory. This simplified radiometric microbiological assay combines the biologic specificity of the microorganism with the precision and automation of measuring a physical event as the end point (quantification of $^{14}CO_2$ evolved from a label substrate).

Key words: Foods; radiometric microbiological assay; vitamin B_{12}.

The need for a simple, sensitive and reliable assay for the determination of vitamin B_{12} content in biological fluids and foods led to our development of a radiometric microbiological assay for this vitamin. Although a number of microorganisms were shown to be dependent on vitamin B_{12} for growth, *Lactobacillus leichmannii (L. leichm.)* has been most widely used for bioassay in recent years. There are advantages in the relative ease of handling of this organism and no clearly established superiority of any of the other more fastidious organisms. This organism forms the basis for the official A.O.A.C. vitamin B_{12} assay [1]. This method, however, is cumbersome and fails to take into account that the standard solutions must be handled in a manner identical to the tissue extracts [2] if accurate test results are to be obtained.

In recent years most laboratories have abandoned the tedious microbiological assay in favor of the competitive protein binding radioassays. Considerable experience has now been gained by direct comparison of the *L. leichm.* microbiological and these radioassays. The competitive binding radioisotopic methods for serum vitamin B_{12} have been found to give higher values than the microbiological method when used for measurement of serum vitamin B_{12} [3–8]. Several investigators have found that the microbiological assay for vitamin B_{12} results better reflect the clinical status of the patient [9–12]. These observations suggest that these competitive protein binders are recognizing chemically similar compounds which are devoid of biologic activity, both for the test microorganism and the B_{12} deficient subject.

In previous work, we identified a high affinity vitamin B_{12} binder present in the serum of the common Chesapeake Bay toadfish (Opsannus tau). This binder was found to be both more stable than intrinsic factor and human serum binders used previously by other investigators and also to follow the principles of isotope dilution (in that it bound vitamin B_{12} in a stoichiometric fashion, in contrast to the other binders mentioned above) [13]. Despite the use of this "ideal" vitamin B_{12} binder and the use of identical sera extracts, we again observed systematic higher results with the assay of human sera using the competitive protein binding assay compared to one of the conventional turbidimetric microbiological assays using *L. leichm.* Accordingly, we elected to

begin exploration of the possibility of developing a radiometric microbiological assay, feeling that if such were technically feasible it would combine the specificity of the organisms for the biologically active forms of vitamin B_{12} and other essential nutrients with the precision of physical (i.e., radioactive decay) measurements.

The detection of microbial metabolism using radionuclides was first described by Levin et al. [14] and has since been applied to many clinical microbial procedures [15–19]. Certain general principles apply to each of the radiometric microbiological assays that we have developed and performed standard quality control tests on to date:

1. A microorganism (species of *Lactobacillus)* was chosen whose growth was known to be proportional to the amount of each specific nutrient to be assayed. These organisms had already been well-characterized and were readily available from the American Type Culture Society.
2. Assay media were commercially available which had been characterized as ideal for the growth of that particular strain except for the selected absence of the specific nutrients.
3. It was then necessary for us to undertake a lengthy search in each instance to identify the appropriate ^{14}C labeled substrate which would be metabolized during growth of the organism to $^{14}CO_2$, with direct proportionality to the amount of the essential nutrient added.
4. To establish that the $^{14}CO_2$ could be measured quantitatively using an ionization chamber and automated sampling and measuring techniques and that each individual method was sensitive, specific and reproducible.

In brief, after testing a number of potential ^{14}C labeled substrates (see Table 1) we found that in the presence of ^{14}C-arginine and cyanocobalamin, *L. leichm.* produced significant amounts of $^{14}CO_2$ within 16–20 hours. The amount of $^{14}CO_2$ evolved was optimized with the use of guanido labeled arginine and was proportional to the amount of cyanocobalamin added (Fig. 1). When tested on human sera encompassing the entire normal range of vitamin B_{12} concentrations this radiometric microbiologic assay gave values comparable to the standard microbiological (turbidimetric) method using *L. leichm.* With this new method, the use of cyanide in the extraction procedure was shown not to be necessary. Standard quality control studies on human sera, including recovery experiments, and study of intra- and inter-assay precision were performed with satisfactory results.

TABLE 1. *Production of $^{14}CO_2$ by* L. Leichmannii *in the presence of CN-Cbl**

Substrate	Radioactivity added (μCi)	$^{14}CO_2$ production (metabolic index units)** control	200 PG CN-Cbl added***
D-(U-^{14}C) glucose	5	440	459
D-(1-^{14}C) glucose	1	305	350
DL-(1-^{14}C) ornithine	1	0	7
L-(U-^{14}C) arginine	1	0	900
L-(guanido-^{14}C) arginine	1	0	2080
L-(U-^{14}C) valine	1	0	0
L-(U-^{14}C) aspartic acid	1	0	0
L-(U-^{14}C) isoleucine	1	0	0
^{14}C-formate	1	220	230

* CN-Cbl = cyanocobalamin

** 100 metabolic index units is equivalent to 0.031 μCi of $^{14}CO_2$.

*** 100 PG CN-Cbl added for ^{14}C-labeled glucose.

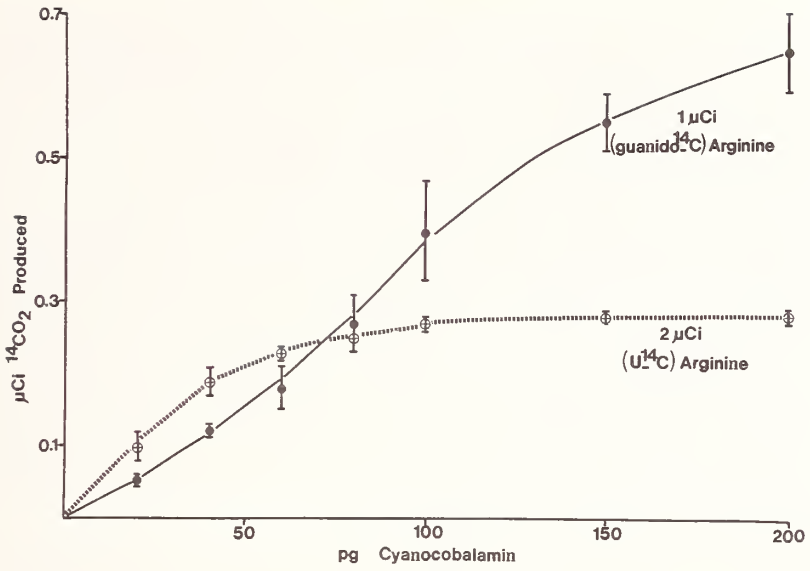

Figure 1. Effect of varying concentration of cyanocobalamin on metabolism of ^{14}C-arginine by *Lactobacillus leichamannii.* Incubation time, 16 hours. Each point represents mean of quadruplicate determination for guanido-^{14}C-arginine, and mean of triplicate determination for U-^{14}C-arginine.

Arginine is one of the amino acids essential for growth of *L. leichm.* The guanido carbon of arginine provides the basic structures of carbamyl phosphate [20]. The latter compound is involved in two major pathways: (1) pyrimidine biosynthesis, and (2) the production of ATP through the arginine dihydrolase pathway, in which carbamyl phosphate is coupled to ADP, producing CO_2, ATP, and NH_3 [21]. The $^{14}CO_2$ that we are measuring from guanido-^{14}C-arginine is probably evolved through this latter pathway. In the presence of vitamin B_{12}, the actively growing *L. leichm.* utilizes its supply of carbamyl phosphate for the more rapid biosynthesis of both pyrimidines and ATP. Thus we believe we are measuring a specific metabolic event, requiring biologically active forms of B_{12} rather than the relatively non-specific end point of total cell division.

The advantages of the radiometric microbiological assay over the turbidimetric assay are partly summarized in Table 2. The assay is simplified due to the fact that colored, turbid or precipitated debris do not interfere with the $^{14}CO_2$ output or detection and therefore the extract and precipitated material from the original sample do not need to be separated. Extraction of

TABLE 2. *Microbiological assays of vitamin* B_{12}

Turbidimetric method	Radiometric method
FOOD + EXTRACTING SOLUTION (CYANIDE)	FOOD + EXTRACTING SOLUTION + ASSAY MEDIUM
↓ AUTOCLAVE	↓ AUTOCLAVE
↓ REMOVE THE PRECIPITATE BY CENTRIFUGATION	↓ INOCULATE
↓ ADJUST THE pH AND VOLUME	↓ INCUBATE
↓ EXTRACTS + ASSAY MEDIUM	↓ MEASURE B_{12} PRESENT BY $^{14}CO_2$ PRODUCED
↓ AUTOCLAVE	
↓ INOCULATE	
↓ INCUBATE	
↓ MEASURE B_{12} PRESENT BY BACTERIAL GROWTH USING ABSORBANCE AT 700 mμ	

vitamin B_{12} bound to food could be accomplished in one autoclaving step in sealed 20 ml vials with the assay medium (3–5 min, 15 psi). It was these facts that led us to believe that this method might be uniquely applicable to food assays. After autoclaving, the vials are cooled, then immediately inoculated with the labeled arginine, the bacterial suspension, and then incubated for 16–20 hours at 37° C. The amount of $^{14}CO_2$ generated by *L. leichm.* is then automatically quantified by an ionization chamber (Bactec R301, Johnston Laboratories, Inc., Cockeysville, Maryland). This system automatically samples each vial in the following way: Vials to be tested are placed in one Bactec tray and the starting button is pressed, initiating a fully automated test cycle. Automatic sterilization of the vial top and sampling needles prevents bacterial contamination. The vials are sequentially tested by positive pressure flushing of sterile air into the vial (and thus the $^{14}CO_2$ into the instrument). The $^{14}CO_2$ is quantified by an ionization chamber. The level of radioactivity, as measured by the Bactec, in each of our assays is directly proportioned to the amount of the appropriate essential nutrient present. The results are automatically recorded on paper and also displayed in digital fashion on the front panel. The test cycle for 60 sample vials is completed in 60 minutes (1 minute/vial). The $^{14}CO_2$ is automatically and quantitatively trapped in an absorbant-filled chamber for easy and safe disposal.

Preliminary studies of powdered instant non-fat dry milk, evaporated milk, baby formula and various samples of strained baby food (beef, chicken, egg yolk) showed that this radiometric assay gave vitamin B_{12} values comparable to the standard microbiological assay (modified A.O.A.C. method) [2]. When we directly assayed the same food samples by both methods we obtained a correlation coefficient of 0.96 (Fig. 2). Results obtained on several baby food and preparations of milk by our radiometric microbiologic assay compared favorably in most instances with published values (Table 3). The sensitivity of this radiometric microbiologic method as determined by standard curves performed repeatedly over 1 1/2 years is in the range of 10 pg/mL. An unexpected finding was that all samples containing oatmeal which were tested had small, but highly consistent, detectable amounts of vitamin B_{12} activity present (Table 3).

Reproducibility of the radiometric microbiological assay for vitamin B_{12} in food was studied using a powdered non-fat dry milk and three different baby foods; each was analyzed in duplicate or triplicate 5 to 7 times within a period of 2 months. As shown in Table 4, the variance between

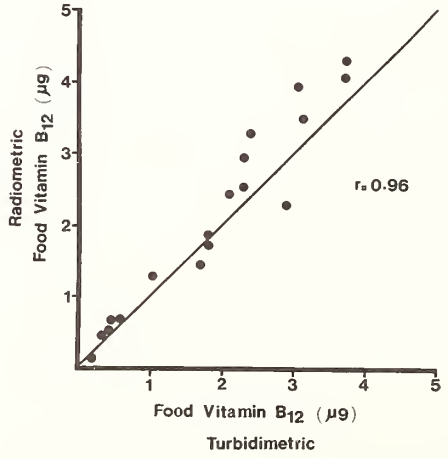

Figure 2. Comparison of radiometric and turbidimetric microbiological assays of vitamin B_{12} in food. Foods include various preparations of milk and baby foods (beef, chicken, egg yolk).

TABLE 3. *Radiometric microbiological assay*

Food	Samples of same lot no.	Total sub-samples	Vitamin B_{12} content (μg/100 g) Mean±S.D.	Published values	
Non-fat powdered milk	4	19	4.60 ±0.72	4.033±0.348	*
Evaporated milk	2	7	0.29 ±0.03	0.163±0.0146	*
Similac-Redi Nurse	1	4	1.93 ±0.23/L	1.5/L	†
BABY FOODS					
Beef, strained	2	12	1.53 ±0.36	0.58	Δ
Chicken, strained	3	20	0.94 ±0.24	1.16	Δ
Egg yolks, strained	5	17	2.37 ±0.63	1.62	Δ
Cereal and egg yolk	3	11	0.203 ±0.02	-	-
Oatmeal, pre-cooked, dry	1	6	0.07 ±0.02	0.0	Δ
Oatmeal with applesauce and banana	3	16	0.0069±0.003	0.0	Δ
Rice with applesauce and banana	3	9	0.0	0.0	Δ
Plums with tapioca	3	9	0.0	0.0	Δ

* Agriculture Handbook No. 8-1.
† Physician's Desk Reference, 31st Edition, 1977.
Δ U.S. Dept. of Agriculture Home Economics Research Report 36, 1969.

TABLE 4. *Radiometric microbiological assay*

Reproducibility study of vitamin B_{12} content in food

Assay No.	Sample I Milk, powdered (μg/3.2 oz pkg)	Sample II Beef, strained (μg/jar)	Sample III Oatmeal, strained (μg/jar)	Sample IV Chicken, strained (μg/jar)
1	4.15	1.525	0.01125	1.235
2	4.25	1.325	.006	1.30
3	3.53	1.34	.006	1.14
4	4.5	1.265	.006	1.125
5	4.57	1.215	.013	0.775
6	-	1.9375	.0135	0.70
7	-	-	-	0.825
Mean±S.D.	4.2±0.41	1.43±0.27	0.0093±0.004	1.01±0.24

repeated assays was small. Furthermore, the reproducibility of results obtained when different sub-samples of the same lot were assayed was satisfactory (Table 5).

Recovery studies using the radiometric assay and two different kinds of milk and seven different baby foods were done with the radiometric microbiologic assay. As shown in Table 6, the mean recovery when 30–50 pg of cyanocobalamin was added to foods of animal origin was 106.0±8.8 (1 S.D.), with a range of 95.0% to 115.0%. The mean recovery when the same amount of cyanocobalamin was added to food of vegetable or grain origin ranged from 63.6% to 68.6% with a mean of 66.3±2.1% (1 S.D.).

Three different "extraction" procedures were tried on these samples of baby food after they had been well honogenized using a conventional Teflon pestle homogenizer. These ranged from the relatively complicated A.O.A.C. method to no extraction; that is, simply diluting the homogenized food with distilled water, adding an aliquot of the diluted specimen directly to the

TABLE 5. *Radiometric microbiological assay*

Food	Vitamin B_{12} content (μg/100 g) (samples of same lot no.) #1	#2	#3	#4	#5	Total sub-samples
Non-fat powdered milk	4.23 ±0.39	4.70 ±0.46	4.6 ±0.54	4.8 ±1.2	-	19
Evaporated milk	0.298 ±0.05	0.296 ±0.02	-	-	-	7
Beef, strained	1.63 ±0.45	1.44 ±0.27	-	-	-	12
Chicken, strained	0.97 ±0.19	0.96 ±0.28	0.88 ±0.26	-	-	20
Egg yolks, strained	2.27 ±0.80	2.59 ±0.85	2.69 ±0.42	2.28±0.55	2.05±0.57	17
Cereal and egg yolk	0.203 ±0.016	0.21 ±0.03	0.193 ±0.016	-	-	11
Oatmeal with applesauce and banana	0.0069±0.003	0.0069±0.004	0.0069±0.003	-	-	16
Rice with applesauce and banana	0.0	0.0	0.0	-	-	9
Plums with tapioca	0.0	0.0	0.0	-	-	9

TABLE 6. *Radiometric microbiological assay*

Recovery of cyanocobalamin (30–50 pg) added to food Sample	% Recovery
Non-fat powdered milk, instant	110.4
Evaporated milk	95.0
Beef, strained	111.2
Chicken, strained	98.3
Egg yolk, strained	115.0
X±1 S.D.	106.0±8.8
Carrot, strained	67.0
Oatmeal with apples and bananas, strained	65.9
Oatmeal, precooked, dry	63.6
Rice, precooked, dry	68.6
X±1 S.D.	66.3±2.1

assay vial and proceeding with the assay. As shown in Table 7, our assay results for vitamin B_{12} content were essentially identical.

Further simplification of and important reduction in the technician time required for the radiometric microbiological assay was accomplished by (1) establishing that the meticulously cleaned glassware required for the standard turbidimetric assay was not necessary for this radiometric assay; (2) that previously lyophilized cultures of *L. leichm.* could be used in this assay. Comparison of vials which had previously been soaked and then rinsed multiple times against unwashed vials using the radiometric assay on a variety of different foods gave essentially identical results ($r=0.99$, Fig. 3).

Lactobacillus has been successfully lyophilized for use in other microbiological assays [22,23]. Simultaneous radiometric microbiologic assays using the standard maintenance culture routine versus immediate inoculation at the time of assay with previously lyophilized cultures and

TABLE 7. *Radiometric microbiological assay of vitamin B_{12} in foods. Comparison of three extraction procedures*

Food	Vitamin B_{12} (μg/100 g) A	B	C	n
Chicken, strained*	1.013±0.18	1.08±0.30	0.943±0.31	5
Beef, strained*	1.347±0.12	1.49±0.34	-	5
Egg yolk, strained*	2.71 ±0.42	2.29±0.55	2.07 ±0.58	3

A—AOAC extraction procedure. Aliquot amounts of diluted samples were then assayed.

B—Extraction in B_{12} assay medium. Aliquot amounts of diluted samples were then assayed.

C—Diluted in deionized water (no extraction). Aliquot amounts of diluted samples were then assayed.

Comparison of procedures A–B and A–C in all 3 foods gave no significant difference, $p>0.2$ to $p>0.5$.

* Samples of the same lot no. were used.

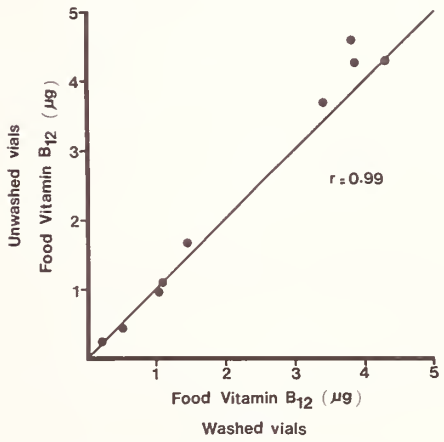

Figure 3. Radiometric microbiological assay. Comparison of washed versus unwashed vials. Foods tested included various preparations of milk and baby foods (beef and chicken).

five different food samples are shown in Figure 4 ($r=0.98$). Lyophilization of *L. leichm.* thus eliminates the routine maintenance procedures usually required for use of this organism for assay of vitamin B_{12} (Table 8), again materially shortening technician pre-assay preparatory time.

Similar radiometric microbiological assays for folacins and niacin have been developed and shown to be sensitive, reproducible and specific. This new semi-automated methodology seems immediately applicable to the assay of these essential nutrients in human foodstuffs. These new methods combine the biologic specificity of the microorganism with the precision and automation of measuring a physical event as the end point (i.e., quantification of evolved $^{14}CO_2$).

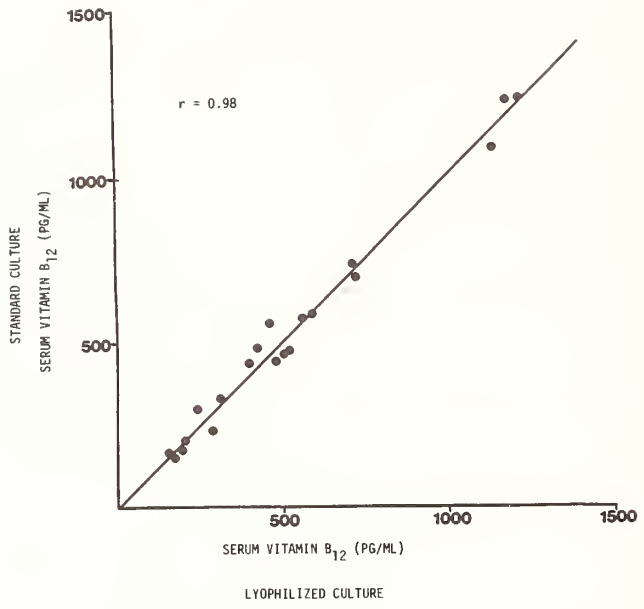

Figure 4. Radiometric microbiological assay. Comparison of use of previously lyophilized cultures inoculated into test vial immediately before beginning assay incubation period to the conventionally stored and sequentially inoculated bacterial suspension which must be prepared before each assay. Foods tested included various preparations of milk and baby food (beef, chicken, egg yolk).

TABLE 8. *Alternative methods of maintaining test culture organism*

Lyophilization technique	Standard technique
	Stock cultures of *Lactobacillus leichmannii* are kept as stab inoculum in micro assay culture agar. These are subcultured monthly in micro assay culture agar.
	↓
	Prior to day of assay, subculture into micro inoculum broth and grow for 16–18 hours at 37 °C.
	↓
	On morning of assay, subculture from 16 hour grow to fresh micro inoculum broth. Grow for 6–7 hours at 37 °C
	↓
Lyophilize bacteria and seal the ampoules. Store at 4 °C.	Centrifuge bacteria. Wash three times with assay medium.
↓	↓
On day of assay, break open ampoule, resuspend, dilute and immediately inoculate assay vials.	Resuspend, dilute and inoculate assay tubes or vials.

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GAS LIQUID CHROMATOGRAPHIC ANALYSIS OF PANTOTHENATES AND PANTHENOL

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Gas Liquid Chromatographic (GLC) methods are described for the analysis of calcium and sodium pantothenates and panthenol in pharmaceutical preparations. In one method the acetate derivatives are prepared using a pyridine: acetic anhydride mixture. The residue is dissolved in chloroform and injected onto a column packed with 2% neopentyl glycol sebacate. In a second method trimethyl silyl ester-ethers of the pantothenate compounds are prepared using a mixture of silylating agents. GLC column packings of 5% SE-30 and 3% OV-1 were used. The application of these methods to pharmaceutical preparations is discussed.

Key words: Gas/liquid chromatography; panthenol; pantothenates; pharmaceutical preparations.

I. Introduction

Calcium pantothenate is a member of the vitamin B complex included in many vitamin preparations. An official method using microbiological techniques has been published by the A.O.A.C. [1]. There are also a large number of chemical methods in the literature which have been used to measure the pantothenic acid content of pharmaceutical preparations [2–7]. Most of the above methods are relatively time-consuming; therefore GLC methods were developed in FDA laboratories with a view toward obtaining the specificity, accuracy and precision of the chemical methods with the shorter time-requirements of GLC methods.

Unmodified polyhydroxy and carboxylic organic compounds, such as panthenol and pantothenic acid and its salts, are not volatile enough for direct GLC and both have a tendency to decompose at high temperatures or are not soluble enough in the common organic solvents. However, these problems are overcome by converting the compounds to volatile derivatives such as acetates or trimethylsilyl ethers for GLC analysis.

This paper describes the GLC determination of the acetate derivatives of pantothenic acid ethyl ester and panthenol as well as the trimethyl silyl ester-ethers of these compounds. The application to pharmaceutical products is discussed.

A number of multivitamin preparations containing calcium pantothenate have been analyzed by preparing trimethylsilyl derivatives. Using a 3% OV-1 on 100/120 mesh Gas Chrom Q column packing at 200 °C, a single peak was observed at the same retention time as the standard solutions. The sample peaks were analyzed quantitatively by comparison with a standard curve. One sample, which had been stored for several years, had a label claim of 20 mg/tablet. The analysis showed a calcium pantothenate content of 19.0 mg/tablet. Other samples were also in agreement with the label claim. This phase of the project has not been completed. Additional samples should be analyzed and a recovery study of a standard added to samples will be performed.

II. Experimental Detail

Instrumentation—A model 5000 Barber-Colman gas chromatograph fitted with a high temperature hydrogen flame-ionization detector and 5-mV, 2-s, 11-inch strip recorder, was used for the analysis of the derivatives.

Materials—The following were used: d-pantothenyl alcohol, d-pantothenic acid calcium salt and dl-pantothenyl alcohol; (Sigma Chemical Co., St. Louis, MO); dl-pantothenic acid (K and K Laboratories, Plainview, NY); d-pantothenic acid sodium salt (Mann Research Laboratories, New York, NY); trifluoroacetic anhydride (TFAA), propionic anhydride, and valeric anhydride (Eastman Organic Chemicals, Distillation Products Industries, Rochester, NY); bis(trimethylsilyl)acetamide (BSA) (Supelco, Inc., Bellefonte, PA); bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Inc.); trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) (Applied Science Laboratories, Inc., State College, PA); dimethyl sulfoxide (Crown Zellerbach Corp., Chemical Products Division, Camas, WA); chloroform, spectrophotometric (J. T. Baker Chemical Co., Phillipsburg, NJ); and hexane (n-hexane, pure grade) (Phillips Petroleum Co., Special Products Division, Bartlesville, OK). All other solvents and reagents used were reagent grade and required no further purification.

Preparation of Derivatives-Esterification—The methyl and ethyl esters of pantothenic acid were prepared from calcium pantothenate; the procedure of Stoffel et al. [8] was used for the preparation of the methanolic and ethanolic HCl solutions. In a typical experiment, 54.4 mg of calcium pantothenate (or its equivalent of sodium pantothenate) and 5 mL of 2.5% (w/w) ethanolic HCl were stirred in a small round-bottom flask with a stirring bar for 1.5 h at room temperature. The ethanolic HCl solution was removed by flash evaporation.

Acetylation—The pantothenic acid ethyl ester and panthenol were converted to the acetate derivatives by treating each compound with a 1:1 mixture of acetic anhydride and pyridine. The starting material was placed in a small round-bottom flask containing a stirring bar and 3 mL each of acetic anhydride and pyridine were added. The mixture was stirred for 1 h at room temperature. The acetylating reagents were removed by flash evaporation at 40–50 °C. The residue was taken up in 10 mL chloroform and filtered. A suitable aliquot was injected onto the chromatograph.

Preparation of Trimethylsilyl Derivatives—The diether monoester trimethylsilyl derivatives of pantothenic acid were prepared from d- and dl-pantothenyl alcohol. Standard solutions of the trimethylsilyl derivatives were prepared as follows: a 50 mL volumetric flask with a 24/40 stopper and stirring bar was charged with 146.9 mg of d-sodium pantothenate and 125.0 mg of d-pantothenyl alcohol. To these compounds was added 5 mL of a 4:2:2 mixture of bis(trimethylsilyl) acetamide-trimethylsilylimidazole-trimethylchlorosilane. The reaction mixture was stirred for 10 min at room temperature and 0.2 mL dimethyl sulfoxide was added. The mixture was stirred for another 5 min and then diluted to volume with benzene.

GLC—After an investigation of a number of column packings and column conditions for the analysis of the acetate derivatives the following were selected: 244×0.4 cm U-shaped Pyrex glass column packed with a liquid phase of 2% neopentyl glycol sebacate (NPGSB) on 110/120 mesh Anakrom ABS. Operating conditions were: column 230 °C, detector 280 °C, injector 280 °C, and carrier gas (argon) 60 mL/min. In the case of the trimethylsilyl derivatives, the selection was a 244×0.4 cm i.d. U-shaped Pyrex glass column packed with a liquid phase of 3% OV-1 (w/w) on 110–120 mesh Anakron ABS. Operating conditions were: column 200 °C, flash heater 280 °C, detector 280 °C, and carrier gas 80 m/min. Compressed air and hydrogen flow rates used with the flame ionization detector were 460 and 36 mL/min respectively. Peak areas were determined by triangulation (peak width at half-height × peak height) = peak areas.

Preparation of Samples—Multivitamin tablets were ground in a mortar and pestle. An aliquot of the powder containing 25 mg of calcium pantothenate was transferred to a 50 mL beaker and extracted 3 times with ethyl ether, which was removed by decanting. This step was necessary to remove the fat-soluble vitamins present in the formulation. The powder was dried on a steam bath and in a vacuum oven. Then, 1 mL of a 3:3:2 mixture of trimethylsilylimidazole, bis-trimethylsilylacetamide and trimethylchlorosilane was added. The mixture was stirred 10 min at room temperature, 1 mL of dimethyl sulfoxide added and the mixture stirred for 5 min at room temperature. The mixture was transferred to a 10 mL volumetric flask and filled to the mark with benzene.

III. Results and Discussion

The structure of the acetate derivatives of the standard materials was verified by IR and NMR spectroscopy. The structure of the trimethylsilyl derivatives was confirmed by NMR and mass spectrometry after conditions of silylation were found that produced only one reaction product per compound in quantitative yield.

Response data for the derivatives of panthenol triacetate and the pantothenate (ethyl ester) diacetate using the 2% neopentyl glycol sebacate column were obtained by injecting 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, 10.0 and 15.0 μg amounts. The mean peak area of six analyses for each amount injected was determined and plotted. The response was linear as the quantity of compound was increased. Excellent elution peaks were obtained. Sensitivity was determined using the flame ionization detector, an attenuator setting of 1, and electrometer output of 1×10^{-10} A and a 0-1 mV recorder. The sensitivity was 5–8 nanograms.

Similar experiments were performed on the trimethylsilyl derivatives of pantothenic acid and panthenol. These data show that the response was linear as the quantity of compound was increased. The limit of detection was 2–4 nanograms. However, quantitative measurements at these low levels was difficult because of the low signal-to-noise ratio.

A number of multivitamin preparations containing calcium pantothenate have been analyzed by preparing trimethylsilyl derivatives. Using a 3% OV-1 on 100/120 mesh Gas Chrom Q column packing at 200 °C, a single peak was observed at the same retention time as the standard solutions. The sample peaks were analyzed quantitatively by comparison with a standard curve. One sample, which had been stored for several years, had a label claim of 20 mg/tablet. The analysis showed a calcium pantothenate content of 19.0 mg/tablet. Other samples were also in agreement with the label claim. This phase of the project has not been completed. Additional samples should be analyzed and a recovery study of standard added to samples will be performed.

IV. Summary

GLC can be used to detect and analyze pantothenic acid salts and panthenol as acetyl derivatives. Trimethylsilyl ether derivatives are also amenable to GLC analysis and are adaptable to pharmaceutical products. Studies are underway to apply the GLC analysis to pharmaceuticals containing pantothenates and panthenol. A need exists for high-purity standards of various forms of pantothenic acid and panthenol.

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QUANTITATIVE ANALYSIS OF SIMPLE CARBOHYDRATES IN FOODS

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Liquid Chromatography and gas-liquid chromatography methods were developed to separate the mono-, di-, tri-, and tetrasaccharides commonly found in foods. The liquid chromatography system uses an anion-exchange column to separate the common food sugars as borate complexes. Sugars in the column eluate are quantified colorimetrically by reaction with p-hydroxybenzoic acid hydrazide subsequent to hydrolysis with sulfuric acid. In the gas-liquid chromatography system, the peracetylated aldononitriles or trimethylsilylated oximes of sugars are chromatographed on a column packed with either 3% SP2250 or 3% OV-17.

Preparation and extraction of samples for two chromatography procedures and derivatization for gas-liquid chromatography are discussed. The combination of the two chromatography systems for the determination of sugars in foods appears to be very useful. One or both of these analytical systems will allow the sugar content of foods to be determined in detail.

Key words: Carbohydrates; fructose; galactose; gas-liquid chromatography; glucose; lactose; liquid chromatography; maltose; raffinose; ribose; stachyose; sucrose; sugar analysis.

I. Introduction

Simple carbohydrates not only have specific roles in nutrition, but are also associated with certain health problems such as obesity, cardiovascular diseases, diabetes, hypertriglyceridemia, dental caries, hypertension, and various carbohydrate intolerances [1,2]. However, comprehensive and reliable data on carbohydrates content in foods are lacking [3].

Although there have been many studies of the ion-exchange chromatographic analysis of sugars (for a recent review see Jandera and Churacek [4]), the published methodology leaves room for improvement. The analyses of the common food sugars are often time consuming (6–18 hours), subject to artifacts, nonspecific, and insensitive to some sugars. We have developed a new ion-exchange chromatographic procedure for the analysis of the common food sugars as their borate complexes. It has the potential of being automated and avoids the use of dangerous reagents such as acetonitrile and concentrated sulfuric acid. Common food sugars can be routinely analyzed in 2 hours with this procedure.

Since Sweeley et al. [5] introduced a simple and rapid procedure for preparing volatile trimethylsilyl (TMS) derivatives of sugars, gas-liquid chromatography has become an important technique for carbohydrate analysis [6]. However, a chromatogram of a sugar TMS ether-glycoside will show multiple peaks due to the presence of various isomeric and anomeric forms of each sugar in solution. These multiple peaks often overlap when complex mixtures of sugars are chromatographed. Several investigators [7,8] have converted the sugars into oximes before trimethylsilylation. We have combined these approaches with the use of aldononitrile acetate derivatives [9], and the resultant combination has proved to be a useful system for the analysis of food sugars.

The combination of liquid and gas-liquid chromatography is able to separate and quantify mono-, di-, tri-, and tetrasaccharides in foods. Details of each system will be discussed as well as the application to a common food.

II. Procedures

A. Extraction of Sugars from Foods

Procedures for extraction depend on the type of food and the analyses performed. Analysis of sugars in soft drinks requires only concentration or drying the sample before derivatization for the gas-liquid chromatography system or desalting for the liquid chromatography system. Examples of methods used for the extraction of the sugars from some solid foods are shown in Figure 1. Different extraction procedures probably will be needed for other foods.

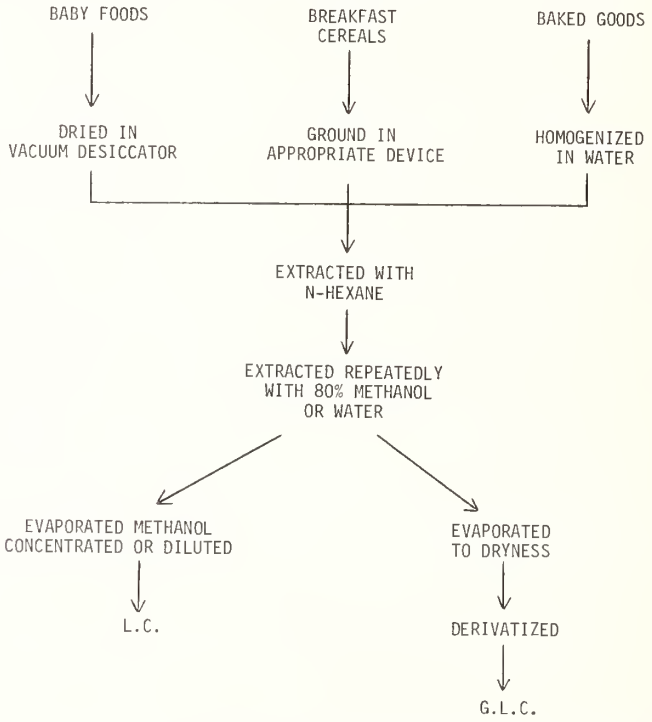

Figure 1. Extraction procedures for food sugars to be analyzed by the liquid (LC) and gas-liquid (GLC) chromatography.

B. Liquid Chromatography

For liquid chromatography [10], an aqueous solution of a food extract or standard mixture containing ribose as an internal standard was first desalted on two columns (0.9×2.0 cm) in series that were packed, respectively, with Dowex AG-50 and Dowex AG-3. The mixture was then diluted with Tris-HCl (0.1 mol/L), borate (0.05 mol/L), and sodium chloride (0.02 mol/L) buffer at pH 9.5; applied on an Aminex-A25 column (0.6×27 cm); and eluted with the same buffer. The eluate was mixed inline with sulfuric acid (1 mol/L), heated to 100° for 1 minute, and then reacted inline with p-hydroxybenzoic acid hydrazide (7.5 mM) in 1M NaOH at 100° for 5 minutes. The colored product was cooled in a 15° bath, and its absorbance measured at 400 nm.

C. Gas-Liquid Chromatography

For preparation of trimethylsilylated oximes, a methanol extract of food (10–15 mg) was dried and mixed with 1 mL of a pyridine solution containing 3 mg phenyl-β-D-glucopyranoside and 25 mg hydroxylamine hydrochloride in a Teflon-lined screw-capped tube. The mixture was heated at 75° for 30 minutes; after brief cooling, 1 mL hexamethyldisilazane and 0.1 mL

trifluoroacetic acid were added and mixed vigorously for 30 seconds. The mixture was allowed to stand at room temperature for at least 30 minutes and was then centrifuged to precipitate the unreacted hydroxylamine hydrochloride. An aliquot of the supernatant was injected into a gas-liquid chromatograph.

For preparation of aldononitrile acetates, the cooled mixture of oximes was treated with 1 mL acetic anhydride and heated at 90° for 60 minutes. The mixture was evaporated to dryness under a stream of nitrogen at 60°. The residue was extracted with 1 mL chloroform; washed once with 1 mL water and dried over sodium sulfate. An aliquot of the chloroform solution was injected into a gas-liquid chromatograph.

Replicate samples with varying amounts of known sugars were used routinely to establish recoveries.

Details of the gas chromatographic conditions are as follows:

Gas chromatograph:	Hewlett-Packard 5840A equipped with an automatic sampler
GLC column:	6′×1/8″ stainless steel column packed with 3% SP2250 or 3% OV-17 on 80/100 mesh Supelcoport
Column temperature:	150–325° programmed at 10°/min
Detector temperature:	300°
Injection port temperature:	200°
Carrier gas and flow rate:	Helium, 30 mL/min

III. Results and Discussion

Both the liquid and gas-liquid chromatographic methods described above are applicable for the simultaneous analysis of sugars commonly found in foods (including fructose, galactose, glucose, lactose, maltose, raffinose, ribose, stachyose, and sucrose). Under the conditions described for our liquid chromatography system, the sucrose peak appeared first, with a retention time of 13 minutes, and glucose emerged last after 135 minutes. The order of elution for all eight sugars is shown in Figure 2. Separation of the oxime TMS ethers of reducing sugars and TMS ethers of nonreducing sugars on gas-liquid chromatography is shown in Figure 3. Raffinose had the longest retention time of 15 minutes. In general, retention times of TMS ethers are proportional to their molecular weights. Figure 4 is a chromatogram of oxime TMS ethers of some less common sugars which occasionally may be found in certain foods; of these, 2-deoxyribose, galactose, and melibiose show two components. In Figure 5, we show the well-resolved peaks of some selected pentoses and hexoses as their aldononitrile acetates.

We have developed a liquid chromatography system that is practically automated, analyzes most common food sugars rapidly and does not require hazardous reagents. We have also adapted a gas-liquid chromatography method that can analyze mixtures of sugars with wide ranges of concentrations. With two such operable but different techniques available, we can verify analytical data within the laboratory and select the method according to the amounts and types of sugars in a given food sample. The gas-liquid chromatography system can be used for preliminary qualitative analyses and quantitative analyses of more complex mixtures, and the liquid chromatography system can be used in routine analysis of samples having relatively simple mixtures of sugars in high proportions. The results of the analyses of one soft drink are given in Table 1. A fresh sample was analyzed on the day of purchase and shelved and frozen samples from this same bottle were stored in sealed glass ampules for later testing. The unopened sample was from a second bottle obtained on the same day but stored unopened at room temperature for 30 days.

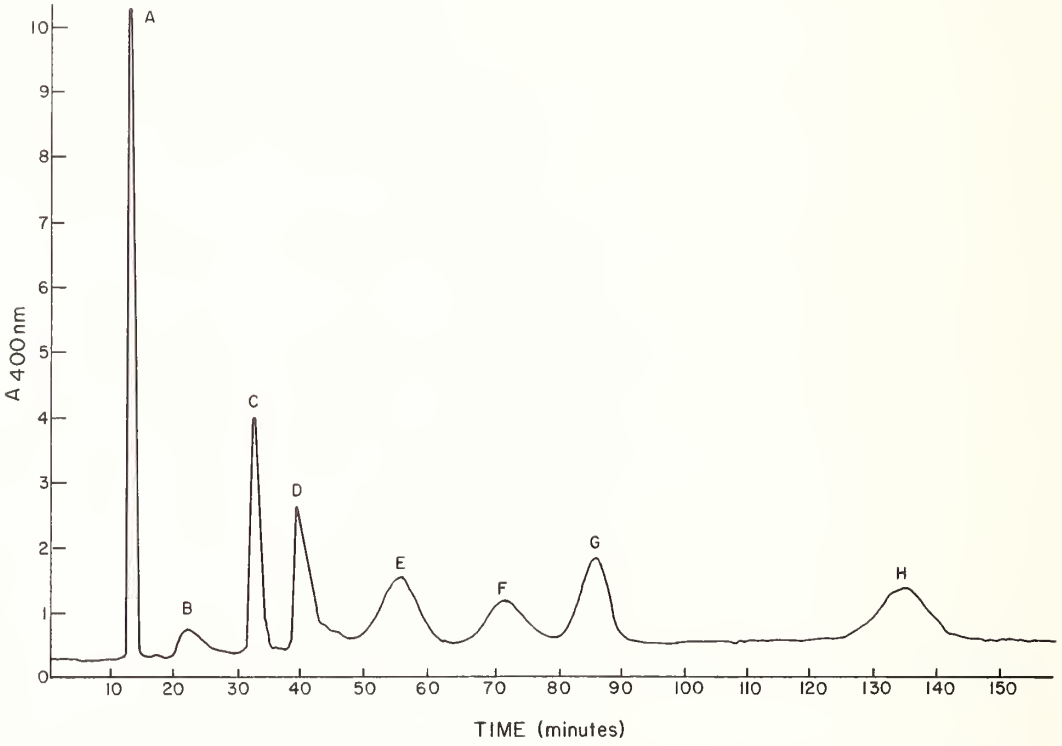

Figure 2. Liquid chromatogram of borate complexes of sugar standards. A, sucrose; B, lactose; C, ribose; D, mannose; E, fructose; F, galactose; G, xylose; H, glucose.

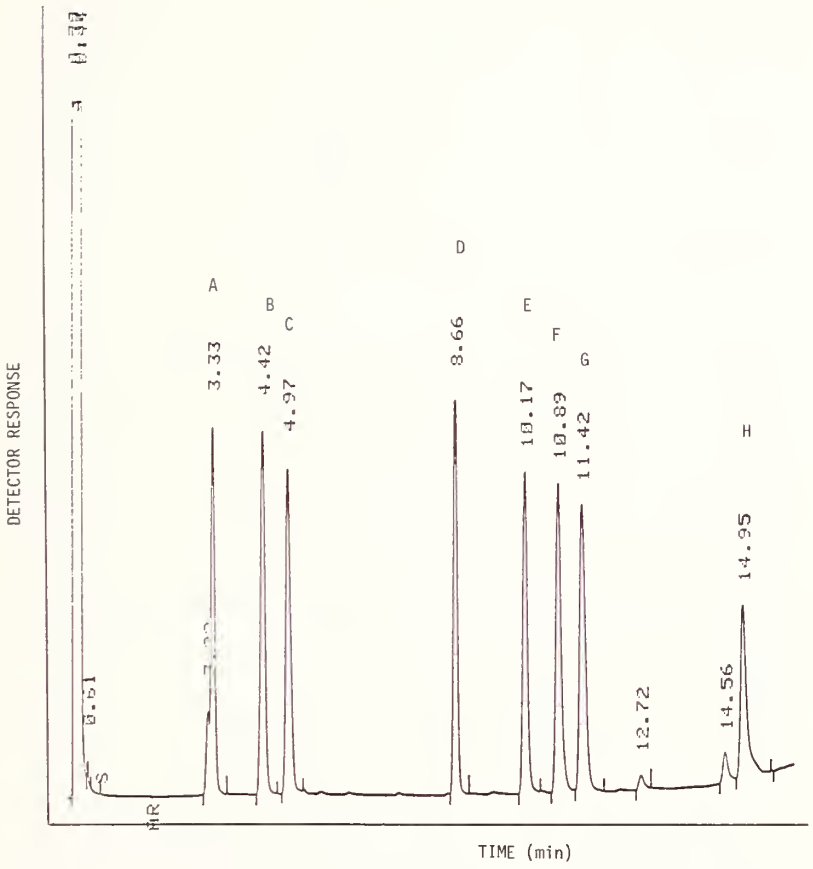

Figure 3. Gas chromatogram of oxime TMS ethers of reducing sugar standards and TMS ethers of nonreducing sugar standards. A, ribose; B, fructose; C, glucose; D, phenyl-β-D-glucopyranoside; E, sucrose; F, lactose; G, maltose; H, raffinose.

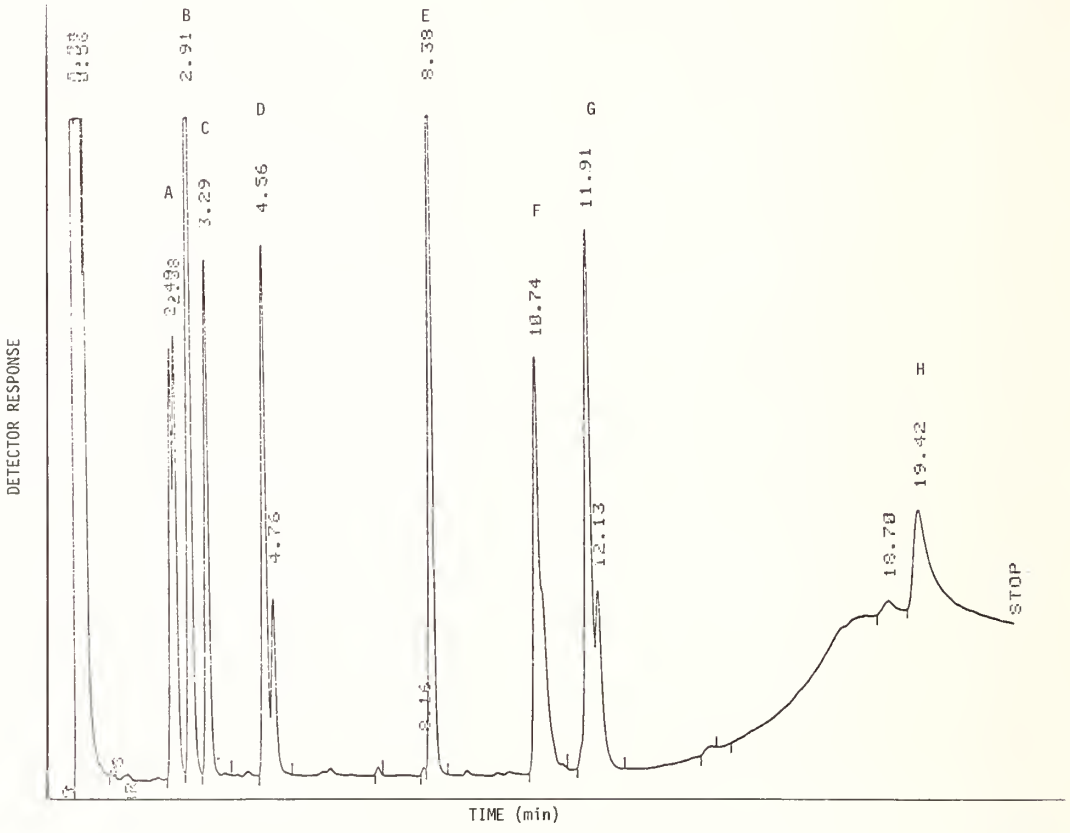

Figure 4. Gas chromatogram of oxime TMS ether of reducing sugar standards and TMS ethers of nonreducing sugar standards. A, 2-deoxyribose; B, arabinose; C, rhamnose; D, galactose; E, phenyl-β-D-glucopyranoside; F, cellobiose; G, melibiose; H, stachyose.

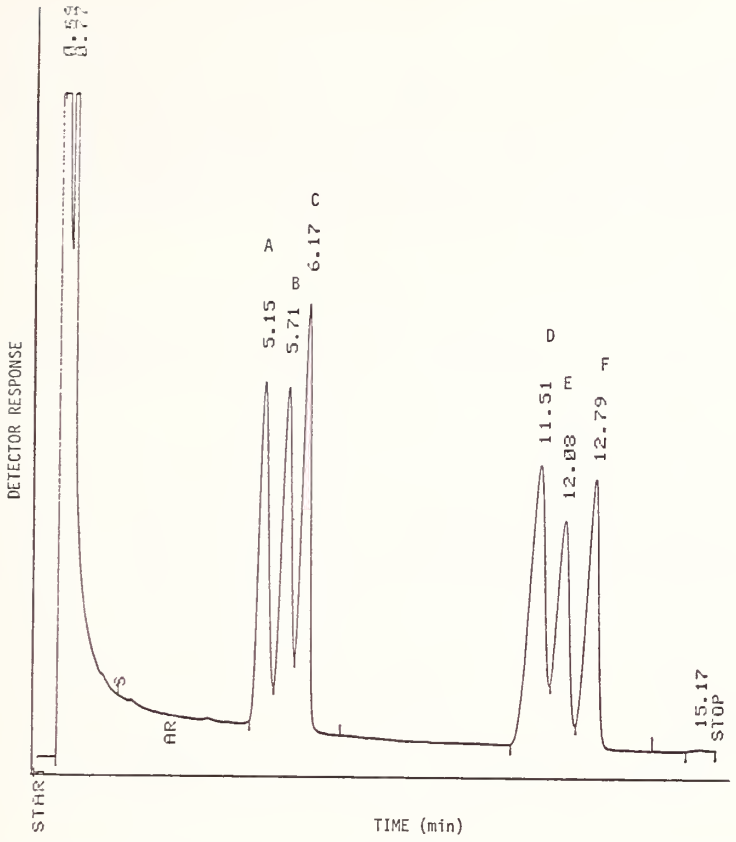

Figure 5. Gas chromatogram of aldononitrile acetates of sugar standards. A, ribose; B, arabinose; C, xylose; D, mannose; E, glucose; F, galactose. The column temperature was programmed from 180–250° at a rate of 2° per minute.

TABLE 1. *Sugar analysis of soft drink (mg/mL) by liquid (LC) and gas-liquid (GLC) chromatography*

Sugar	Fresh		30 days later					
			Unopened		Shelved		Frozen	
	LC	GLC	LC	GLC	LC	GLC	LC	GLC
Sucrose	88.0	85.6	33.8	30.6	31.3	31.9	87.0	93.6
Fructose	10.0	17.2	41.0	40.3	45.5	44.9	12.3	13.5
Glucose	10.0	17.2	39.0	38.9	44.0	43.0	14.5	13.8
Total sugar	108.0	120.0	113.8	109.8	120.8	119.8	113.8	120.9

The preliminary data demonstrate that the liquid and gas-liquid chromatography methods give comparable results. Furthermore, it is apparent that the storage conditions affect the levels of different sugars in the samples. The appropriate conditions for the storage of food samples prior to sugar analysis will probably have to be determined for each food or food group.

Studies of the appropriate sugar extraction procedures for different foods are now underway in our laboratory, and we hope to begin routine analysis of a large variety of foods for their individual sugar contents.

IV. Acknowledgment

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Mention of the trade mark on a proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

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DETERMINATION OF VITAMINS E AND K IN FOODS AND TISSUES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Foods and tissues were extracted with hot isopropanol and acetone. Low polarity lipids, including vitamins E and K, were obtained by addition of hexane, then water, to the filtered extract. Tocopherols (α, β, γ, and δ) and tocotrienols were separated by high performance liquid chromatography (HPLC) on LiChrosorb Si60 in hexane containing either 0.2% isopropanol or 5% diethyl ether. High sensitivity and specificity were obtained by using a fluorometric detector. Vegetable oils, margarines, meat, liver, grains, milk, plants and complete meals were successfully analysed by this method. In the analysis for vitamin K, lipids were first filtered through a short column of hydroxyalkoxypropyl Sephadex (HAPS). Triglycerides were reduced in some samples (milk, vegetable oils) by low pressure chromatography in hexane on a long column of HAPS (120 cm$\times$1 cm). Crude or purified lipids were then chromatographed on a preparative HPLC column (25 cm$\times$1 cm) 5 μ LiChrosorb Si60 in hexane containing 0.03% isopropanol. A fraction containing phylloquinone was collected and rechromatographed on a LiChrosorb Reverse Phase column (25 cm$\times$3.2 mm) using a 30 minute gradient of 15% water in methanol to methanol. Phylloquinone levels (μg/g) were measured in a variety of foods including peas (0.36), cabbage (0.46), spinach (2.44), green beans (0.47), lettuce (1.95) and cow's milk (0.018).

Key words: High performance liquid chromatography; phylloquinone; tocopherols; tocotrienols; vitamin E; vitamin K.

I. Introduction

Naturally occurring forms of vitamin E consist of a family of 6-chromanols, the tocopherols (α, β, γ, and δ), and the related, triply unsaturated, tocotrienols (Fig. 1). Hitherto, analysis for vitamin E usually involved saponification, paper, column or thin-layer chromatography (TLC) and quantitative measurements based on the reducing properties of the chromanols in the Emmerie-Engel reaction [1]. Gas-liquid chromatography has recently been employed in the analysis of foods after saponification, TLC and derivatization [2].

A fluorometric method was developed for α-tocopherol which was applicable to a wide variety of foods [3]. Unsaponifiable lipids were chromatographed under low pressure on hydroxyalkoxypropyl Sephadex (HAPS). We have modified this procedure by utilizing high performance liquid chromatography (HPLC) and now all forms of vitamin E can be measured with minimal preliminary purification; this improved method is described below.

The structures of substances with vitamin K activity are shown in Figure 2. Phylloquinone (abbreviated PK-4 to indicate 4 isoprene units in the side chain) occurs in plants. The menaquinones (MK4–MK13) are biologically active naphthoquinones with unsaturated side chains of varying lengths. They are produced by bacteria and they are deposited in animal tissues. They are obtained from food and also from microorganisms in the gut. Although the K-vitamins have been determined chemically during biochemical research with plants and microorganisms [4], few analyses have been performed for nutritional purposes. A method has been described for determining phylloquinone added to infant formula [5] and menaquinones in liver have been

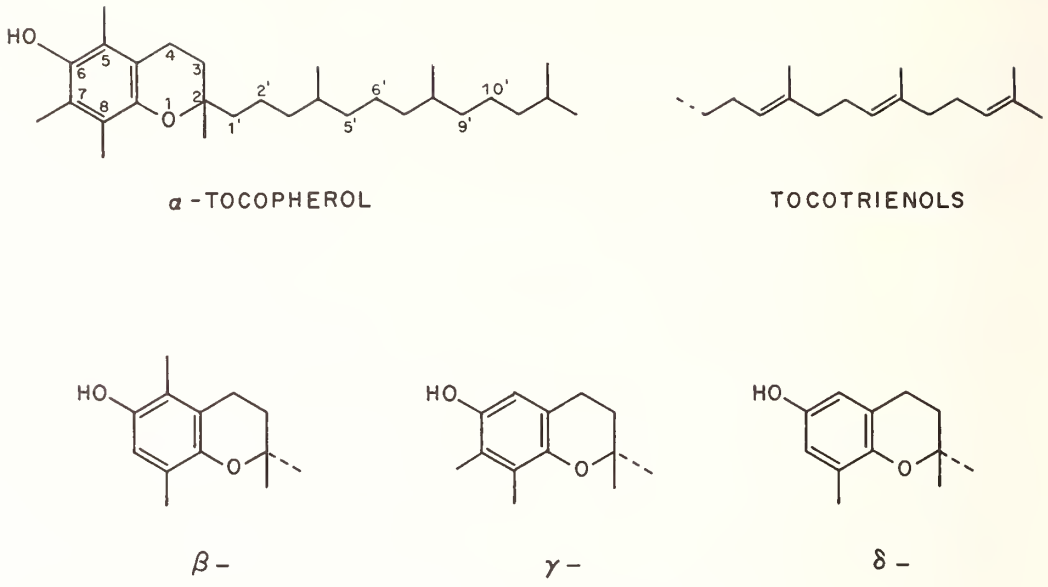

Figure 1. Structures of tocopherols and tocotrienols.

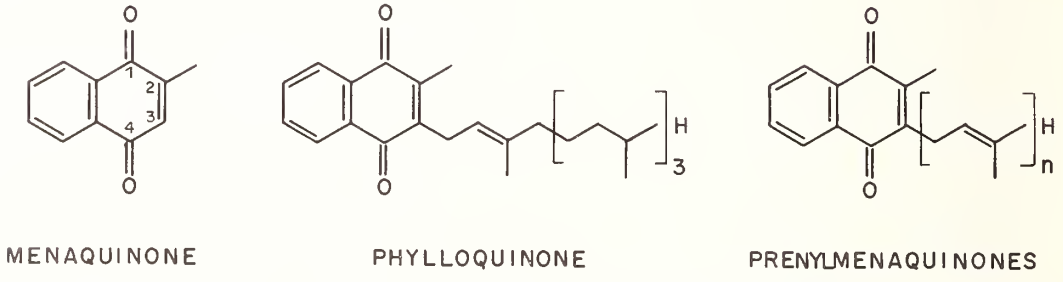

Figure 2. Structures of phylloquinone (PK4) and menaquinones (MKn).

examined in a series of research projects [6]. Otherwise, most of the remaining information concerning the vitamin K content of foods was obtained many years ago in bioassays [7].

We have recently applied HPLC to the separation of K-vitamins in foods; a method for determining phylloquinone using this technique is described below.

II. Materials and Methods

A. Solvents and Reagents

Hexane and methanol were of HPLC quality. Hexane for HPLC solvent systems was prepared by mixing equal volumes of water-saturated hexane with dry hexane fresh from the bottle. Ether was distilled from reduced iron. Other solvents were glass distilled immediately before use. HAPS was prepared from Sephadex G-25 superfine (3).

B. Extraction of Foods

A 10 g sample of food was digested for 10 minutes in 100 mL isopropanol in the cup of a Virtis 45 homogenizer and then the mixture was homogenized for 1 minute. Acetone (50 mL) was added and the extract was filtered through glass-fibre paper (Whatman GF/A) into a 500 mL separatory funnel. The residue was rinsed with acetone (50 mL) and then rehomogenized 1 minute

with 100 mL acetone. The extract was filtered as before into the separatory funnel and the residue was washed with 50 mL acetone. Hexane (100 mL) was added to the pooled extracts and the funnel was swirled to mix the solvents. Water (100 mL) was added, the funnel was swirled gently, and the phases were allowed to separate. The hexane layer was transferred to a second funnel and the lower aqueous phase was extracted twice with 100 mL portions of hexane. The pooled hexane extracts were washed twice with water (100 mL) and then were evaporated under reduced pressure.

C. HPLC Analysis for Vitamin E

Lipids were chromatographed in hexane (2 mL/min) containing 0.5% isopropanol or 5% diethyl ether on a column (25 cm×3.2 mm) of LiChrosorb Si60 (5 micron). A Perkin-Elmer MPF-3 spectrophotofluorometer equipped with a flow-cell was used as a detector; the excitation wavelength was set at 290 nm (9 nm slit) and the emission at 330 nm (18 nm slit). A 310 nm high-pass filter was placed in the emission beam.

D. Analysis for Vitamin K

1. Removal of Polar Lipids Using HAPS

A short column of HAPS (3 cm×1 cm) was prepared by adding a slurry of the packing in hexane to a glass column blocked at the base with glass wool. Lipids (up to 0.5 g) were applied to the column in hexane and were eluted with hexane containing 1% isopropanol. The elution was continued until the main chlorophyll band reached the base of the column. A fresh column was used for each sample.

2. Chromatography on HAPS

Lipids (up to 0.5 g) were chromatographed at low pressure in hexane on HAPS using an absorption detector set at 254 nm. The system was identical to that described for the analysis of vitamin D (9) except that the length of the column was 120 cm. A fraction containing phylloquinone and lower menaquinones was cut using the retention times of standards as a guide.

3. HPLC on Wide Columns of Silicic Acid

Lipids (up to 50 mg) were chromatographed on a 25 cm×1 cm column of LiChrosorb Si60 (5 micron) in hexane containing 0.03% isopropanol. The solvent was pumped at 10 mL/min by an Altex 100 pump fitted with a preparative head. An absorption detector was used at 262 nm. A fraction containing phylloquinone was collected for rechromatography using the retention times of standards as a guide.

4. Reverse-Phase Chromatography

Purified lipids were chromatographed on a LiChrosorb "Reverse-phase" column (25 cm×3.2 mm) using a 30 minute gradient of 85% to 100% methanol flowing at 1 mL/min. An absorption detector was used at 262 nm.

III. Results and Discussion

A. Extraction of Vitamins

The extraction procedure was applicable to a wide variety of foods and tissues. Hot isopropanol was effective in extracting non-polar lipids. The addition of acetone not only completed the extraction but also reduced the viscosity of the solvent mixture and speeded

filtration. Troublesome emulsions were never encountered and the extracted lipids were readily soluble in hexane. Tests with spinach confirmed that less than 1% of the tocopherols remained in the residue after application of the procedure.

B. Analysis for Vitamin E

The separation of tocopherols and tocotrienols was readily achieved by HPLC; thus γ and β tocopherols, which are difficult to distinguish by TLC, were completely resolved. γ-Tocopherol was adequately separated from β-tocotrienol (Fig. 3). The high sensitivity and specificity of the fluorometric detector eliminated the need for the purification steps, such as saponification, used in other methods; this saved time and avoided losses. Vegetable oils were dissolved in hexane (5 mg/mL) and 20 μL of the solutions were chromatographed in 0.2% isopropanol in hexane. This system separated the tocopherols and also resolved plastochromanol-8, which was found in small amounts in many oils, from γ-tocopherol (Fig. 4). Margarines were analysed similarly after dissolution in hexane.

In the analysis of lipids from foods and plants, 5% ether in hexane was preferred as solvent system. Ether separated all forms of vitamin E from each other more effectively than isopropanol and, more importantly, it improved the separation of broad unidentified interfering peaks from α-tocopherol in low-potency samples (Fig. 5). Results obtained with various kinds of foods are listed in Table 1.

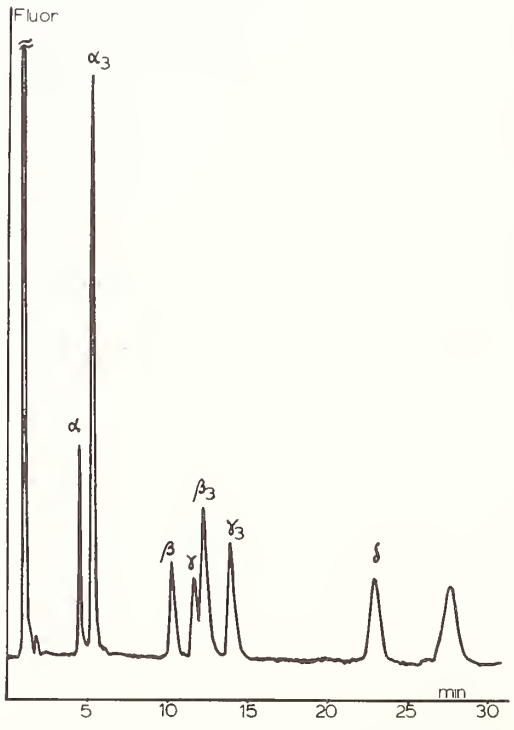

Figure 3. HPLC chromatography of barley oil (100 μg) mixed with 20 ng each of α, β, γ and δ-tocopherols in LiChrosorb Si60, 5 μ. The barley oil contained α, β, and γ-tocotrienols (marked α_3, β_3 and γ_3). Solvent system: 0.2% isopropanol in hexane; 2 mL/min; fluorometric detector.

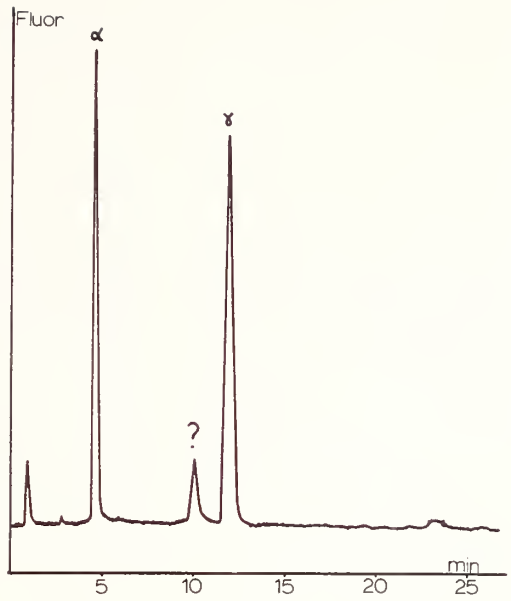

Figure 4. HPLC of rapeseed oil (200 μg) on LiChrosorb Si60, 5 μ. The small peak eluted before γ-tocopherol has been identified as plastochromanol-8. Solvent system: 0.2% isopropanol in hexane; 1.5 mL/min; fluorometric detector.

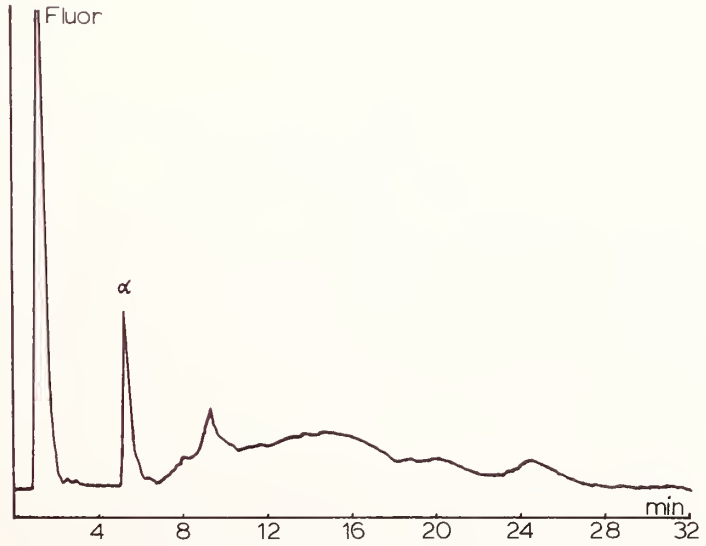

Figure 5. HPLC of lipids from winter milk. γ-tocopherol is separated. Solvent system: 5% ether in hexane; 2 mL/min; fluorometric detector.

TABLE 1. *Levels of tocopherols (μg/g) found in various foods*

	Tocopherol			
	α	β	γ	δ
Butter	18.7	-	0.52	-
Bread	0.56	2.32	2.59	0.09
Milk	0.37	-	?	-
TV dinner (beef)	2.94	2.94	5.37	2.42
TV dinner (chicken)	2.18	2.12	11.24	4.55
Spinach (fresh)	28.90	-	12.94	0.10
Spinach (stored 6 months frozen)	0.78	-	0.40	-
Beef steak	2.20	-	-	-
Rapeseed oil	197.0	-	585.0	8.8

C. ANALYSIS FOR VITAMIN K

Four steps were developed for the isolation of the K-vitamins but not all of them were needed in the analyses of many foods. The first short HAPS column was always utilised to eliminate polar lipids and chlorophylls. The long HAPS column was useful in the separation of phylloquinone from up to 0.5 g triglyceride. Phylloquinone and lower menaquinones were eluted after the bulk of the fat (Fig. 6); however, low-polarity menaquinones (i.e., larger than MK10) were discarded in this step. This column was used only in the analyses of samples rich in fat, such as milk.

The large HPLC silicic acid column separated phylloquinone from menaquinones (Fig. 7) and ubiquinones. Usually many unidentified peaks were encountered and the vitamins could rarely be distinguished. A fraction was collected at the appropriate time to include phylloquinone. It was rechromatographed in the reverse-phase system which was capable of separating individual menaquinones and phylloquinones (Figs. 8 and 9). The levels of phylloquinone found in various foods are shown in Table 2.

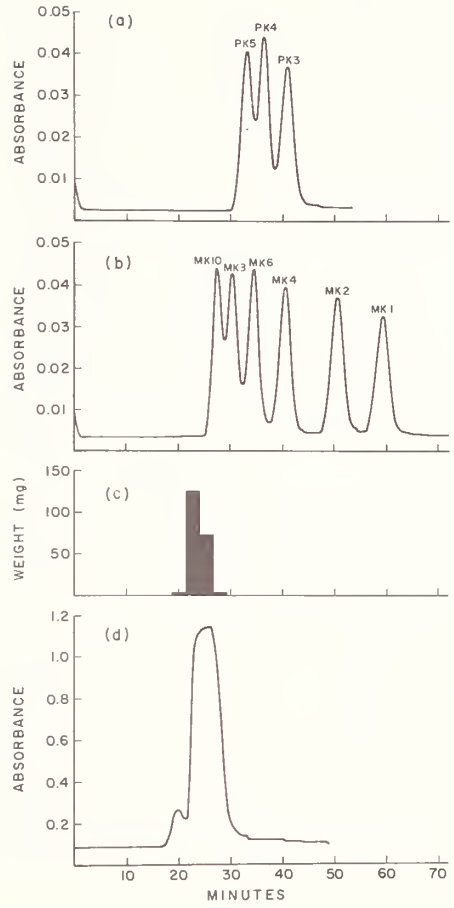

Figure 6. Chromatography of standards on 120 cm column HAPS in hexane (1 mL/min). a. phylloquinones 3, 4 and 5; b. menaquinones 1–10; c. soybean oil (250 mg); d. soybean oil (250 mg), absorption at 254 nm. During chromatography of a sample, the eluent was collected between 30 and 60 minutes.

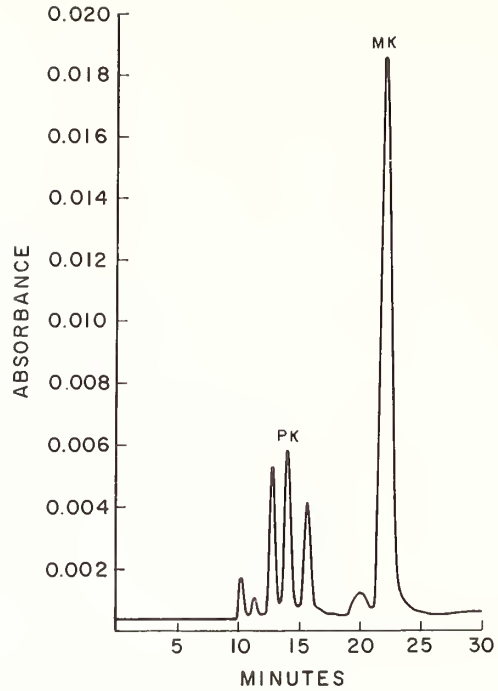

Figure 7. HPLC of phylloquinones and menaquinones on 25 cm × 1 cm LiChrosorb Si60, 5 μ. Solvent system: 0.03% isopropanol in hexane; 10 mL/min; absorption detector at 262 nm.

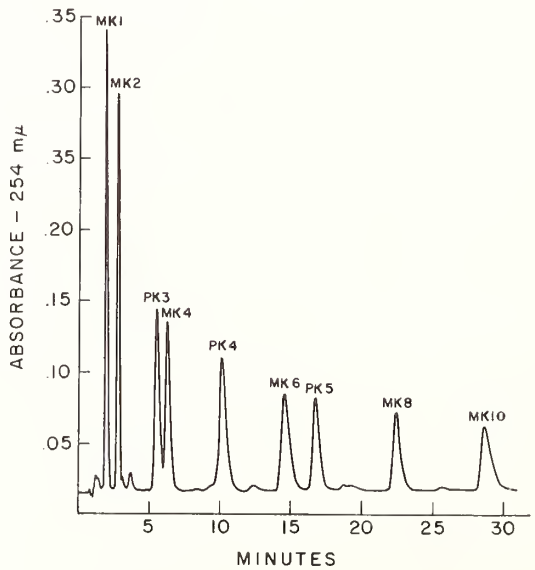

Figure 8. HPLC of phylloquinones and menaquinones on 25 cm × 3.2 mm LiChrosorb Reverse Phase. Solvent system: 30 minute gradient 85% aqueous methanol to methanol; 1 mL/min; absorption detector at 262 nm.

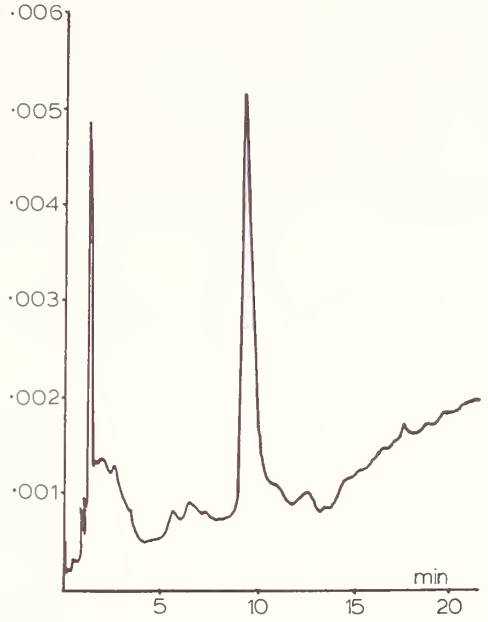

Figure 9. Detection of phylloquinone in lettuce. Conditions as in Figure 8.

TABLE 2. *Levels of phylloquinone (μg/g) found in various foods*

	Phylloquinone
Spinach	2.44
Lettuce	1.95
Green beans	0.47
Peas	0.36
Cabbage	0.46
Milk	0.018
Potatoes, cucumber, beets, cauliflower, orange, apple	<0.05

IV. Conclusion

The application of HPLC to the analyses of vitamins has enabled previously difficult and tedious determinations, such as for vitamin E, to be performed routinely. Analyses rarely attempted in the past, such as for vitamin K, can now be performed on common foods.

V. Acknowledgments

We wish to thank Drs. V. Gloor and G. Brubacher of F. Hoftman-LaRoche & Co., Ltd., Basle for gifts of samples of menaquinones and phylloquinones which greatly facilitated our research.

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CURRENT STATUS OF LIPOXIDASE METHOD FOR DETERMINING 9,12-di-*cis*-POLYUNSATURATED FATTY ACIDS

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The enzyme lipoxidase isolated from the soybean exhibits a specificity for the *cis, cis*-methylene interrupted configuration in a straight carbon chain. MacGee in 1959 reported the development of a procedure for measuring *cis, cis*-methylene interrupted polyunsaturated fatty acids based on lipoxidase. The Canadian Food and Drug Directorate modified the MacGee method and adopted the modified procedure for legal use. The United States Food and Drug Administration through the Association of Official Analytical Chemists (AOAC) and others through the International Union of Pure and Applied Chemistry have been studying collaboratively either the MacGee method or individualized versions of the MacGee Method. After a number of collaborative attempts, the AOAC in 1977 completed a satisfactory study. The method has been adopted as an "official interim first action method" by the AOAC. Five oleo-margarine stocks in blind duplicates were analyzed by 17 laboratories located in the United States, Canada, England, Australia, The Netherlands, and Sweden. The method that is being adopted is limited to the official assays of fats and oils and does not include general food extracts. A collaborative study of the additional modifications of the basic method so that food extracts may be analyzed is planned.

Key words: Fats; lipoxidase; oils; PUFA; 9,12-di-*cis*-polyunsaturated fatty acids.

I. Introduction

The need for a rapid, easy-to-use method for the measurement of the 9,12-di-*cis*-polyunsaturated fatty acids (*cis*-**PUFA**) as a practical requirement, has dramatically increased within government regulatory and food industry quality assurance laboratories with the advent of recent labeling requirements [1]. The "fatty acid" regulation specifically requires the Canadian FA-59 lipoxidase method for determining the *cis*-**PUFA** content of food products. The FA-59 method is an adaptation of the MacGee lipoxidase method [2] for measuring the *cis*-**PUFA** content of fats and oils.

The enzyme lipoxidase occurs in plant materials. The enzyme had been identified and studied in soybean [3–7], pea [8], alfalfa [9], navy bean [3], urd bean, mung bean, wheat and peanut [3] in some detail prior to 1958. Sumner and Dounce [3] in 1939 observed that carotene oxidase from soybean, navy bean and other beans not only added gaseous oxygen to the double bonds of carotinoids to form peroxides but also does the same thing to unsaturated fatty acids. Carotene oxidase was discovered in 1928 by Bohn and Haas (per Sumner and Dounce [3]). Andre and Hou [10] first used the term lipoxidase in 1932. Reiser and Fraps [11] determined the lipoxidase content of 13 different kinds of beans and found their lipoxidase activity to be 3–24% of that in soybeans. Miller and Kummerow [12] reported the lipoxidase activity of soybeans to be 200 times as active as wheat germ. Sumner [13] found soybeans to be only 40 times as active as wheat germ whereas Blain and Todd [14] reported soybeans to be 20–30 times as active as wheat germ. These variations may be explained by the observations of Irvine and Anderson [15] indicating large variations in the lipoxidase content between wheat varieties.

The earlier lipoxidase experiments utilized the ultraviolet absorption of the conjugated diene hydroperoxides resulting from lipoxidase catalyzed oxidation of *cis*-**PUFA** to measure the concentration of the enzyme in the presence of excess substrate [16]. The studies following 1955

have been mainly concerned with measuring the substrate concentration by oxidation in the presence of excess enzyme with the work of MacGee [2] being the first major report applying the concept for measuring *cis*-**PUFA** in fats and oils. He observed that the extinction coefficients of the diene hydroperoxides at 234 nm are the same for all the essential fatty acid series. Also, the mechanism of the chemical reactions were fully described schematically. A specific extinction coefficient of 78.2 was determined for 1 g of *cis*-**PUFA** in 1-cm light path at 234 nm. The calibration curve, based on a series of concentrations of saponified cottonseed oil containing about 50% *cis*-**PUFA**, obeyed Beer's Law. MacGee later reported that the absorption obeys Beer's Law between 5 and 25 μg substrate [17].

Zmachinski et al. [18] in 1966 demonstrated that the lipoxidase method is equally as reliable as the combined use of the spectrophotometric and thiocyanometric methods for estimating the *cis*-**PUFA** content of hydrogenated fats containing residual dienes. They concluded that the lipoxidase procedure is the method of choice due to its simplicity and speed. Beare-Rogers and Ackman [19] in 1969 reported that the marine polyenoic acids were suitable substrates for lipoxidase. Their lipoxidase results agreed well in most cases with gas chromatographic results and indicated that only one peroxide group was formed in polygenoic fatty acids with one obstensibly suitable 1,4-pentadiene system. The latter result is in agreement with Hamberg and Samuelsson [20] who earlier observed that the 13-hydroperoxide was formed and that the oxygen function was introduced at the W6 position in the fatty acid substrates and these were required to contain the *cis, cis*-1,4-pentadiene system with the methylene group in W8 position. Dolev et al. [21] previously had demonstrated the existence of only the 13-hydroperoxide thus implying a high degree of enzyme specificity.

Waltking and Zmachinski [22] compared UV spectrophotometry, GLC and the lipoxidase method to determine fatty acid composition of liquid nonhydrogenated oils subjected to heat abuse. All of the methods gave substantially the same analytical results with the exception of GLC peak normalization in lieu of direct or internal standard calibration procedures. They concluded that when the question of nutritional value is involved, the measurement of the essential fatty acids (*cis*-**PUFA**) by the lipoxidase method is more valid. In another study [23], Waltking found that the higher polyunsaturated fatty acid values obtained by GLC, column chromatography and the IR spectrophotometric methods contained the isomeric forms of linoleic possessing no essential fatty acid properties as well as the *cis*-**PUFA.** The agreement between the GLC and lipoxidase results was $\pm 1\%$ for oils containing only the *cis*-**PUFA.** The precision of the lipoxidase method (0.18–2.14 S.D.) was comparable to 0.11–2.82 S.D. reported for GLC [24].

The U.S. regulation [1] specified that the Canadian FA-59 method [25] would be used. The U.S. regulation was not restricted to measurements of *cis*-**PUFA** content in fats and oils but to foods in general. When we attempted to make application of the FA-59 method to general food lipid extracts, we were unable to measure the *cis*-**PUFA** content due to a cloudiness caused by suspended material in the cuvette. So as a consequence, the FA-59 method had to be modified to remove the interference that was especially peculiar to food extracts. The suspended material was identified as mainly saturated fatty acids consisting of palmitic, stearic and a trace of oleic. The modified methodology was released as an interim method for regulatory purposes in 1974 [26] and the research and development study published in 1977 [27]. A centrifuging step was introduced into the procedure that removes the white suspended material from the reading solution prior to the final UV reading.

A collaborative study of the MacGee method was carried out in 1972 [28]. Large deviations from the mean values and poor replication occurred which were attributed to inexperience with the lipoxidase method. Another collaborative study involving 17 laboratories and 5 oleomargarine stocks in a blind duplicate experimental design was conducted in 1977 [29].

The method outlined under experimental is the lipoxidase procedure as developed for fats, oils and food extracts [26,27].

II. Methodology

A. Definition

This method is applicable to the enzymatic determination of the *cis, cis*-methylene interrupted polyunsaturated fatty acids (triglycerides) in fat extracts of fats, oils and foods.

B. Principle

The fatty acids in the sample are saponified to potassium salts. The salts of the *cis, cis*-methylene interrupted polyunsaturated fatty acids are then conjugated and oxidized to hydroperoxides by atmospheric oxygen in the presence of the enzyme lipoxidase. The weight in grams of total *cis, cis*-methylene interrupted polyunsaturated acids per 100 g of sample is calculated from the absorbance of the conjugated diene hydroperoxide measured at 234 nm.

C. Reagents

1. Potassium Borate Buffer, 1.0 mol/L, pH 9.0

Dissolve 61.9 g H_3BO_3 and 25.0 g KOH in ca 800 mL distilled H_2O by stirring and heating. Cool to room temperature and adjust pH to 9.0 by adding 1.0 mol/L HCl or 1.0 mol/L KOH as required. Dilute to 1000 mL with distilled H_2O and mix.

2. Potassium Borate Buffer, 0.2 mol/L, pH 9.0

Dilute 200 mL of 1.0 mol/L potassium borate buffer to 1000 mL with distilled H_2O and mix.

3. Lipoxidase Stock Solution

Dissolve 20 mg of lipoxidase, 50,000 units/mg, soybean, (ICN Nutritional Biochemicals, Cleveland, Ohio 44128 or its equivalent) in 10 mL of ice-cold 0.2 mol/L potassium borate buffer.

(a) Dilute Lipoxidase Solution

Mix 2 mL of stock solution with 8 mL of ice-cold 0.20 mol/L buffer. If a large number of analyses are to be performed, 5 mL of stock solution mixed with 20 mL of ice-cold 0.2 mol/L buffer giving 25 mL of dilute lipoxidase solution is more desirable.

(b) Boiled Dilute Lipoxidase Solution

Transfer 4 mL of dilute lipoxidase solution to a 10 mL vol flask and hold in boiling H_2O for 5 minutes.

4. Alcoholic KOH, 0.5 mol/L

Dissolve 1.40 g KOH in 95% ethanol and dilute to 50 mL with 95% ethanol. Prepare fresh each day.

D. Procedure

1. Preparation of Sample

(a) Vegetable oils

Weigh accurately 100 mg of sample and transfer with *n*-hexane into a 100 mL volumetric flask, which has just been flushed with nitrogen. Dilute to volume with *n*-hexane. Transfer 1.00 mL of *n*-hexane solution to a 100 mL volumetric flask and completely evaporate the solvent under a stream of nitrogen.

(b) Margarines

A 20 mL aliquot of the petroleum ether total lipid extract previously prepared from a chloroform: methanol (2:1, v:v) lipid extract is quantitatively transferred to a 100 mL volumetric flask. Experience with a specific product may indicate that a different size aliquot is more desirable. The petroleum ether is evaporated (must be completely removed) and the residue taken up in *n*-hexane and further diluted with pure grade *n*-hexane to reach a desired final concentration.

Transfer 1.00 mL of the *n*-hexane solution to a 100 mL volumetric flask and completely evaporate the solvent under a stream of nitrogen.

(c) Shortenings, Meats, Solid Fats and other Food Stuffs (moderate to high **PUFA** content)

Follow the instructions provided for margarines under (b) above. If cloudiness appears at any dilution step, abort this procedure and follow the procedure for products having a low **PUFA** content as outlined under (f) below. Also, if the **PUFA** content is too low to obtain satisfactory spectrophotometric readings, revert to (f) below.

(d) Saponification of Samples

Add 2 mL of 0.5 mol/L alcoholic KOH solution to the solvent-free sample in the 100 mL volumetric flask, mix 5 hours or overnight. After saponification is complete, add 20 mL of 1.0 mol/L potassium borate buffer solution and 50 mL of distilled water, mix then add 2 mL of 0.5 mol/L HCl and dilute to volume with distilled water.

(e) Determination of the **PUFA** Content

Pipette 3.00 mL of the saponified fatty acid solution into each of four test tubes (13×100 mm). To the first two tubes (blanks) add 0.10 mL of the boiled (inactive) dilute enzyme solution and mix well. To the third and fourth test tubes (duplicate samples) add 0.10 mL of the (active) dilute enzyme solution, mix and let the test tubes stand exposed to air at room temperature for 30 minutes. Transfer the contents of the tubes to matched 1 cm quartz cuvettes and place in the spectrophotometer. Adjust the instrument with the blank samples and measure the absorbance of the reacted samples at 234 nm.

(f) Determination of the **PUFA** Content of Special Samples (shortenings, meats, solid fats, margarines and other food stuffs containing low percentages of **PUFA**)

The procedure is followed as outlined under (e) "Determination of the **PUFA** Content" except the 3.00 mL of the saponified fatty acid solutions are placed in polypropylene (Nalgene) centrifuge tubes rather than standard test tubes and the respective enzyme solutions are added to the appropriate tubes. The tubes are placed in a centrifuge and spun at 12,000 G for 30 minutes to remove a white suspended material, which is present in many of these solutions. Immediately decant the supernate into 1 cm quartz cuvettes, let samples and blanks stand, at room temperature, in the presence of atmospheric oxygen for 20 minutes. The absorbance value is then determined on the UV spectrophotometer.

2. Calculations

(a) Standard curve

A 100 mg quantity of trilinolein (99% pure, Nu Chek Prep, P. O. Box 172, Elysian, Minnesota, or its equivalent) is weighed and transferred to a 100 mL volumetric flask with *n*-hexane. A 10 mL aliquot of the solution is quantitatively transferred to a 100 mL volumetric flask and diluted to volume with *n*-hexane. Aliquots of 1 mL, 3 mL, 6 mL and 9 mL are quantitatively transferred to separate 100 mL volumetric flasks. The aliquot in each volumetric

flask is reduced to dryness with a stream of nitrogen. Pipette 2 mL of 0.5 mol/L KOH into each triglyceride containing volumetric flask and store in the dark for 5 hours or overnight. To each saponified sample add 20 mL of 1.0 mol/L potassium borate solution, 50 mL of distilled water and 2 mL of 0.5 mol/L HCl: then dilute to final volume with distilled water. Pipette 3.00 mL of the prepared fatty acid solution from each of the standard samples into four separate test tubes. To the first and second tubes add 0.1 mL of the boiled enzyme and mix well. To the third and fourth tubes add 0.1 mL of the unboiled (active) enzyme, mix well and allow to stand at room temperature for 30 minutes. Repeat this procedure for each of the four standard sample levels, read and record the absorbances at each level. A standard curve (Lambert-Beer plot) is drawn using absorbance vs. μg/mL for each level of standard.

(b) Sample

Trilinolein, g/100 g product$=W\times DF\times 10^{-4}$ where $W=\mu$g *cis*-**PUFA**/mL sample, obtained from the calibration plot, and DF=dilution factor.

III. Results and Discussions

The parts of the above methodology relating to fats and oils were collaboratively studied under the joint auspices of the A.O.A.C. and Am. Oil Chem. Soc. [29]. The application to general food extracts will probably be collaboratively studied during the latter part of 1978 and early 1979. Two comparisons were made in the study, i.e., 1) the method as outlined above for fats and oils and 2) the same procedure except that the enzyme is reduced 50% to the level used by MacGee [2,17], saponification is carried out overnight and acetone used in lieu of *n*-hexane for standards and samples. The latter procedure uses the modifications as proposed by Waltking [23]. Seventeen industrial, governmental and university laboratories from Australia, Canada, England, The Netherlands, Sweden and the United States participated in the study. The results, based on actual values obtained for each sample, reproducibility C.V.% and repeatability indicated that there were no significant differences between the two procedures. A low *cis*-**PUFA** oil (Palm) exhibited much higher repeatability C.V.%, i.e., 9.7 and 12.2, than did a high *cis*-**PUFA** oil (Corn), i.e., 2.3 and 4.6. The methods were marginal as tested for the low *cis*-**PUFA** oil. The method has been recommended to the A.O.A.C. as an interim first action method. The A.O.A.C. has not completed its evaluation, thus the status of the method is not known at the time of writing this report.

There are two areas associated with this method in which the NBS Standards Program can be of considerable service. The first item is to provide authentic, well documented trilinolein specifications that may be cited for reference purposes. The second item is to provide uniform, well defined lipoxidase specifications as to plant source purification procedure, activity, etc., that can be cited for the purposes of this method. The soybean lipoxidase is generally the most available lipoxidase from commercial supply houses. However, there is considerable lack of information furnished by the vendors regarding their individual offerings (Table 1). These descriptions are representative of the cross section of information available when consulting the offerings of various vendors. All of these are U.S. suppliers but generally, a similar problem exists with European supplies available commercially [30]. Levin [30] has observed, as the present investigators have, that a minimum of 50,000 units/mg is required for the method to perform properly. The reference cited by vendor B, i.e., Koch et al. indicated that there are at least two lipoxidase enzymes in soybeans; one functions with the free acid and the other one functions with the triglyceride. Their procedure produces a number of fractions from the soybean and the specifications developed should include sufficient description so that there is no doubt as to which enzyme fraction is to be used as the source. The only other vendor citing a preparation procedure, uses an entirely different methodology.

TABLE 1. *Lipoxidase descriptions in supplies catalogs*

Vendor	Information Furnished
A	Lipoxidase (soybean) freeze dried, linoleate: O_2 oxidoreductase (1.13.11.12), activity: min. 50,000 units/mg dry wt., one unit catalyzes an increase of 0.001 A_{234}/min, pH 9.0, at 25 °C with linoleic as the substrate (Theorell, H., Bergstrom, S., and Akeson, A., *Pharm. Acta. Helv.* **21,** 318 1946).
B	Lipoxidase, soybean (1.99.2.1): 1 unit causes an increase of absorbance at 234 nm of 0.001/min at 25 °C, due to linoleate oxidase; The "fatty acid" lipoxidase described by Koch et al., *Arch. Biochem. Biophys.* **78,** 165 (1958); A salt-free, lyophilized powder; Store at 5 °C; Stable for 1–2 years; 8,000 units/mg.
C	Lipoxidase: from soybean; purified, salt-free lyophilized; stable; 50,000 units/mg; 1 unit increases absorbancy 0.001/min at 234 nm, pH 9.0, 25 °C due to linoleate oxidation.
D	Lipoxidase (soybean) salt free lyoph. pwdr. prox. 50,000 units/mg material.
E	Lipoxidase, soybean: Lyophilized, salt-free; 1 unit increases absorbancy, 0.001 min, 234 nm, pH 9.0, 25 °C caused linoleate oxidation; 50,000 units/mg.
F	Lipoxidase.

IV. Summary

A collaboratively studied lipoxidase method for determining the *cis*-**PUFA** content of fats and oils is available. The method has not been collaboratively studied for use with food lipid extracts. A need exists for appropriate NBS specifications for the enzyme lipoxidase and for the reference standard trilinolein.

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TRACE ANALYSIS IN AGRICULTURAL PRODUCTS. METHODS FOR HYDRAZINES, CARBAMATES, N-NITROSODIETHANOLAMINE AND OTHER COMPOUNDS

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Agricultural crops are often exposed to chemical agents such as growth regulators, herbicides and pesticides. As a result of processing and other factors, these chemical agents undergo degradative reactions which lead to compounds considered toxic, carcinogenic or otherwise hazardous. Such compounds, however, are present in trace quantities and their analyses and/or monitoring require specialized techniques. Hydrazines, for example, have been found in tobacco and mushrooms. In the case of the former, the presence of hydrazine may be related to the use of the chemical suckering agent, maleic hydrazide. Determination of both the hydrazine and its suspected precursor is facilitated by reaction with a halogenated acylating or alkylating agent, and subsequent use of electron-capture gas chromatography. In addition, hydrazine has been determined by mass-fragmentographic techniques, and most recently thermal energy analysis (TEA) has been proposed for its determination.

A number of N-nitrosamines have been shown to result from the reaction of various pesticides and nitrite. N-Nitrosodiethanolamine, for example, arises from the reaction of nitrite and diethanolamine (found in certain pesticide formulations) or triethanolamine; this nitrosamine is difficult to determine because of its polarity. In this case, therefore, the analytical scheme involves methanol extraction, column chromatography and trimethylsilylation, followed by combined gas chromatography-mass spectroscopy. A ^{14}C-internal standard is also included to monitor the analytical scheme. We can now analyze N-nitrosodiethanolamine by HPLC/TEA as well.

Other chemical compounds likely to be found in agricultural products in trace quantities as the result of pesticide application (or processing) are carbamates and halogenated hydrocarbons. The former require solvent partition and column chromatography prior to gas chromatography/mass spectroscopy. Chemical ionization mass spectroscopy is especially helpful here. Halogenated hydrocarbons may be determined by electron-capture gas chromatography; however, in the case of vinyl chloride, prior reaction with Br_2 facilitates the analysis.

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Key words: Carbamates; chemical ionization; electron-capture; hydrazines; maleic hydrazide; mass spectroscopy; nitrosamines; nitrosodiethanolamine; silylation; thermal energy analyzer (TEA); tobacco.

I. Introduction

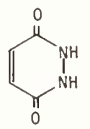
For a number of years, we and others have been engaged in elucidating the chemical composition of tobacco and tobacco smoke in an effort to rationalize their carcinogenic activity as demonstrated in animal bioassay systems [1–6]. Although most of the 2500 compounds now known to occur in tobacco and tobacco smoke are native to tobacco, or are formed from native tobacco constituents during processing, storage or combustion, a significant number have also been shown to arise as a result of agricultural chemicals applied to tobacco plants in the field, or flavorants and other additives applied to the unfinished product during manufacture [7–11].

In the course of our studies, therefore, we have had to develop analytical methods for various specific compounds, or classes of compounds associated with tobacco, and as many of these materials are present in trace amounts, together with many other substances, the methods developed required both enrichment steps and rather sensitive detection devices [12]. As a case in

point, the present paper will discuss a number of compounds isolated and quantitated in tobacco that possibly arise from the application of the chemical suckering agent, maleic hydrazide, to field-grown tobacco. The significance of maleic hydrazide, as an agricultural chemical, however, is not limited to tobacco, because maleic hydrazide is used on other crops as well, including potatoes, onions and citrus.

II. Hydrazines

In some of our initial studies with maleic hydrazide (MH), we were concerned that MH might be a precursor for hydrazine (Fig. 1)., the latter arising from MH as a result of hydrolysis of MH, or through thermal degradation during tobacco combustion [13]. Hydrazine is, of course, a rather reactive molecule, and the method we developed for hydrazines involved reacting these compounds with pentafluorobenzaldehyde, obtaining a derivation suitable for electron-capture-gas chromatography (Fig. 2). In the case of tobacco smoke, the smoke was immediately passed through an aqueous solution containing the aldehyde. In the case of tobacco, an aqueous extract of the tobacco was reacted with the aldehyde. Subsequent steps in the method involved thin-layer or column chromatographic clean-up of the desired extract, followed by gas chromatography (Fig. 3). In this manner, we found hydrazine and 1,1-dimethylhydrazine in various tobacco products (Table 1). It should be noted that the identification of these compounds is confirmed by mass-spectroscopic techniques.



MALEIC HYDRAZIDE

MH-30

CHEMICAL SUCKERING AGENT FOR TOBACCO AND OTHER CROPS

Figure 1. Maleic hydrazide.

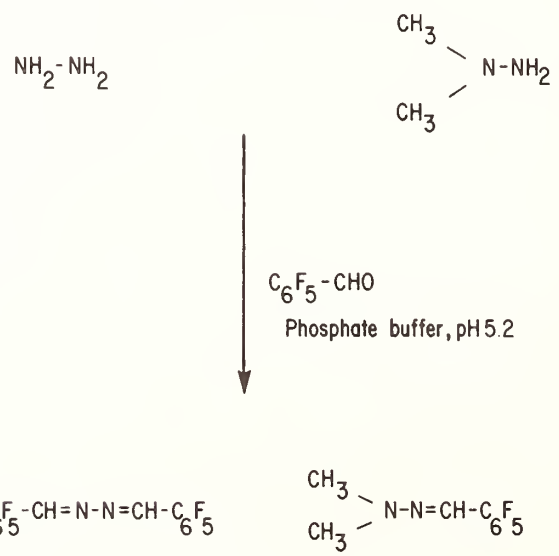


Figure 2. Reaction of hydrazines with pentafluorobenzaldehyde.

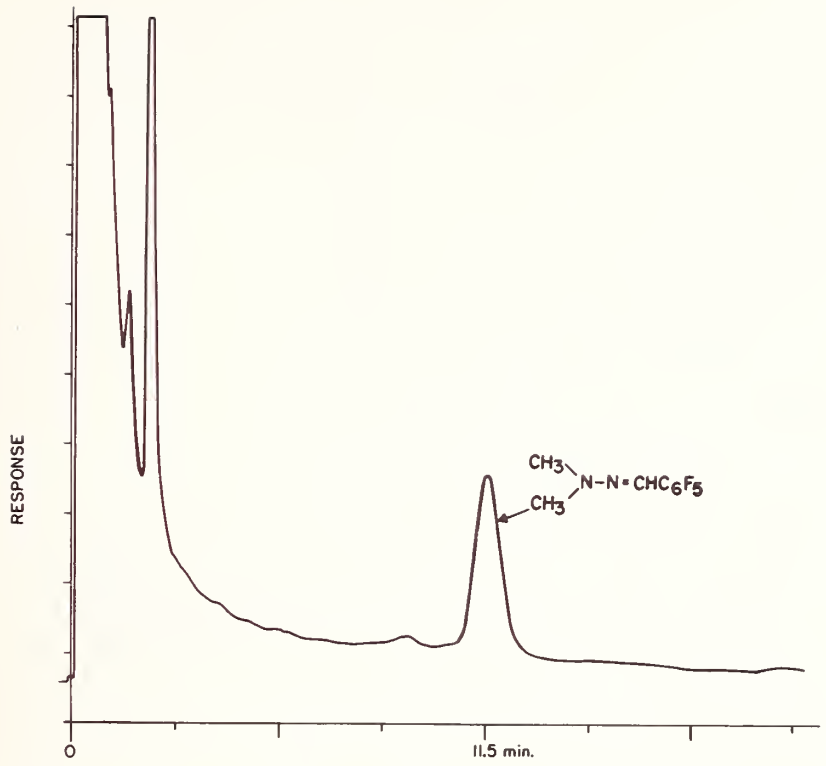


Figure 3. Gas chromatogram of tobacco isolate showing 1,1-dimethylhydrazine as the derivative pentafluorobenzaldehyde.

TABLE 1. *Levels of hydrazines in tobacco [13,25]*

(ppb)

	NH_2-NH_2	CH_3 CH_3 >N-NH_2
Cigarette tobacco[a,b]	96±3	60±6
Chewing tobacco[a]	41	98
Snuff	37	97
Bright tobacco	41	147
Burley tobacco	62	0

[a] Commercial products
[b] MH residues: 5–10 ppm

Interestingly, in subsequent studies we learned that the hydrazine and 1,1-dimethylhydrazine present in tobacco products do not appear to be related to the use of MH on tobacco [13], even though MH formulations have been shown to contain trace amounts of hydrazine as a contaminant from the manufacturing process (Table 2). Rather, hydrazines may be present in tobacco as such, or may have precursors other than MH. Pyrolysis studies indicated the possible nature of some of these precursors (Table 3) [13].

It turns out that MH is a rather stable compound, not easily hydrolyzed. On the other hand, as will be discussed below, on thermal degradation, it decomposes principally to isocyanic acid and acetylene, and possibly to diimide and bis-ketene [14,15]. Only at relatively low pyrolysis temperatures does MH yield any hydrazine [13].

TABLE 2. *Hydrazine content (ppm) of commercial MH formulations*

Sample No.[a]	Hydrazine
1	7.9
2	0.5
3	2.4
4	84.3
5	3.8
6	39.1
7	0.3
8	7.6

[a] U.S.D.A. designations
Samples 1–3, from Tifton, Ga.
Samples 4–6, from Lexington, Ky.
Samples 7,8, from Tennessee

TABLE 3. *Hydrazine as a product of pyrolysis [13]*

Substance pyrolyzed	Weight (g)	Temp. (°C)	Hydrazine produced Theory (g)	Isolated (μg)
Maleic hydrazide	2.0	870	0.6	0.8
Maleic hydrazide	2.1	400	0.6	37.1
Glycine	2.0	870	0.4	2.2
Diglycylglycine	0.8	870	0.2	1.5
Urea	1.7	870	0.9	1.1

In passing, it should be noted that hydrazines are becoming increasingly important environmental agents because they find extensive application in agriculture, industry and medicine. More than 35 hydrazines, including the two present in tobacco, are animal carcinogens [16,17]. Moreover, we have examined another agricultural chemical, succinic acid-2,2-dimethylhydrazide, also known as Alar-85, for carcinogenicity [18]. Administered to mice, in 2% aqueous solution (the same concentration as used by farmers when spraying apple orchards), this material induced lung and blood vessel tumors in mice. In aqueous solution, this hydrazide could hydrolyze to 1,1-dimethylhydrazine, also an animal carcinogen [17]. We did, in fact, show that Alar-85 does hydrolyze to 1,1-dimethylhydrazine in substantial amounts over a 24 hour period (Fig. 4). For this measurement, we used the analytical procedure described earlier for hydrazines. In addition, in our laboratory we have tested several hydrazines as bacterial mutagens, and have obtained positive results. In addition to their observed biological activity, hydrazines are of interest with regard to their mechanism of action *vis a vis* carcinogenesis [17].

Before leaving the subject of hydrazines, we should note that hydrazines have also been reported to occur naturally in certain species of edible mushrooms. These are generally present as hydrazones, in which form they may not be toxic (Table 4). The most common of these is gyromitrin, the N-methyl-N-formylhydrazone of acetaldehyde. Although in this bound form methylhydrazine may not be toxic, or carcinogenic, in the free form it is rather toxic and carcinogenic in animal and other bioassays [19–21]. Whether or not the N-methyl-N-formylhydrazone of acetaldehyde (ethylidene gyromitrin) is metabolically converted to methylhydrazine is an area of concern.

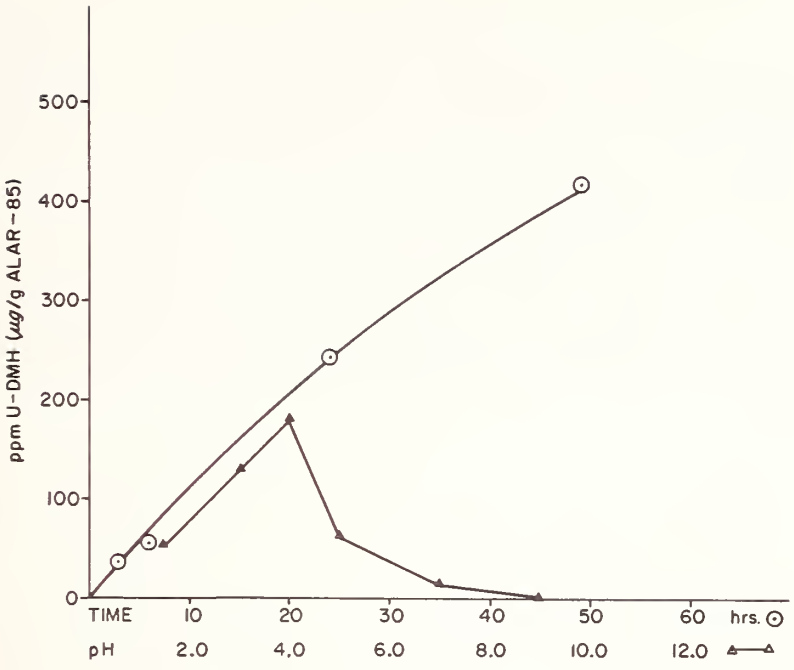


Figure 4. Hydrolysis of Alar-85 (succinic acid-2,2-dimethylhydrazide) to 1,1-dimethylhydrazine (U-DMH).

TABLE 4. *Hydrazines in mushrooms*[a,b] *as N-methyl-N-formyl hydrazones*

$$CH_3—\underset{\displaystyle CHO}{\overset{\displaystyle |}{N}}—N=CH—R$$

R–	Abundance, mg/kg
CH_3–	50
CH_3–CH_2–	1
CH_3–$(CH_2)_2$–	1
CH_3–$\underset{\displaystyle CH_3}{\overset{\displaystyle |}{CH}}$–$CH_2$–	2
CH_3–$(CH_2)_3$–	1
CH_3–$(CH_2)_4$–	1
CH_3–$(CH_2)_6$–	<1
CH_3–$(CH_2)_4$–CH=CH–	
trans	<1
cis	<1

[a]Species: *Gyromitra esculenta* (False morel).
[b]Data from Pyysalo and Niskanen, *J. Agric. Food Chem.* **25**, 644 (1977).

III. Carbamates

Although MH does yield some hydrazine and other compounds under pyrolysis conditions, the major thermally-degradative pathway for MH appears to be via two pathways, one of which leads to isocyanic acid and acetylene (Fig. 5) [14]. Like hydrazine, isocyanic acid (HNCO) is a very reactive compound, and in the presence of amines and alcohols gives rise to substituted ureas and carbamates, respectively. We considered the fact that tobacco and tobacco smoke contain volatile alcohols, such as methanol and ethanol [22], and that any isocyanic acid generated from MH should react with these compounds to give rise to either methyl or ethylcarbamate (urethan). In model pyrolysis studies, the co-pyrolysis of either alcohol with MH led to the formation of the corresponding carbamate (0.1–0.3% yield). Therefore, we developed the scheme outlined in Figure 6 for the analysis of these carbamates in tobacco products. The carbamates were eventually

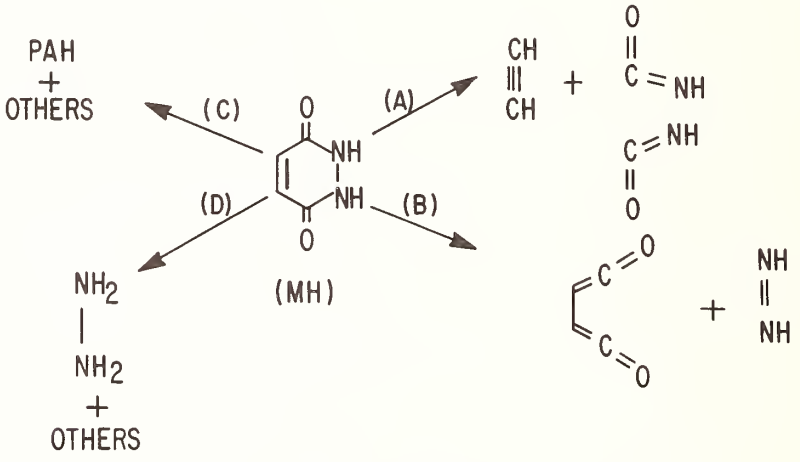


Figure 5. Products from the thermal degradation of maleic hydrazide (MH).

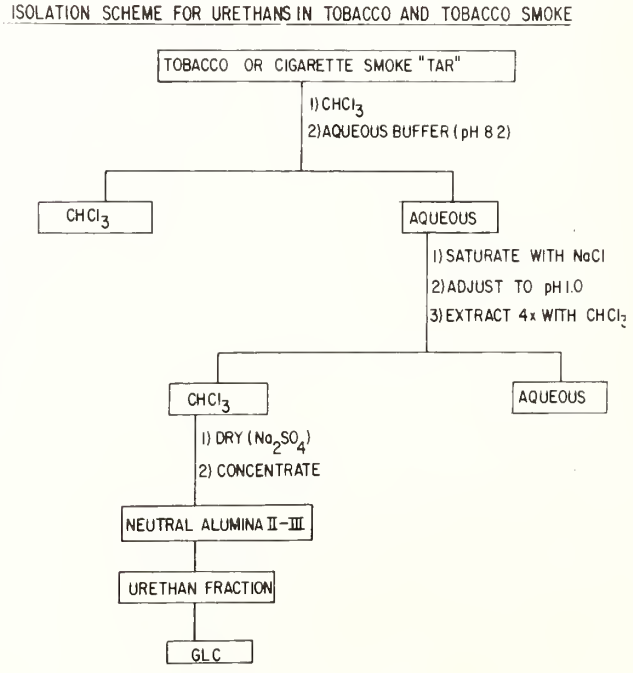


Figure 6. Scheme for analysis of urethan.

detected by gas chromatography (Fig. 7). Confirmation of identities was achieved by gas chromatography-mass spectrometry (Fig. 8). Chemical ionization-mass spectroscopy, in this case, proved to be most useful, although electron-impact mass spectroscopy provided workable spectra as well.

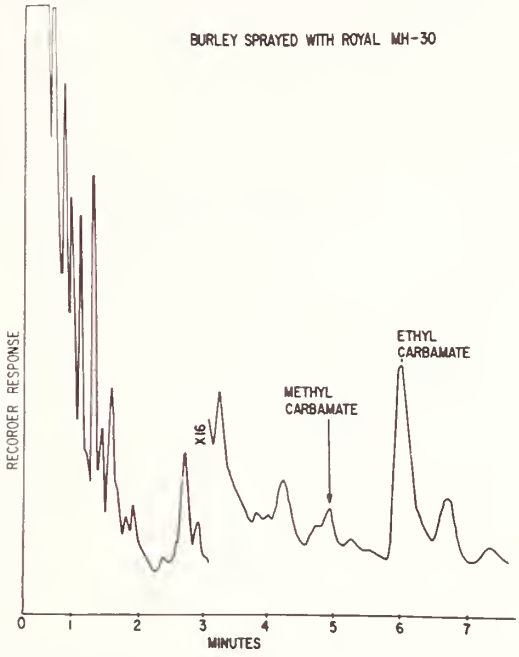


Figure 7. Gas chromatogram of tobacco isolate containing ethyl and methyl carbamates.

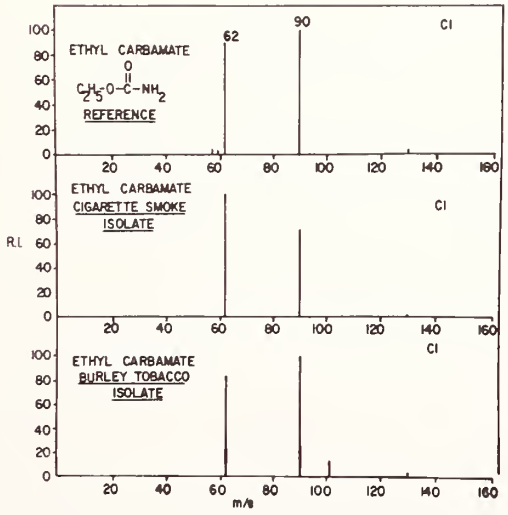


Figure 8. Chemical-ionization mass spectra of isolated and reference ethyl carbamate.

Data for levels of carbamates in tobacco and tobacco smoke are shown in Tables 5 and 6. Note that only urethan (ethyl carbamate) was detected in the smoke. Again, the significance of these findings is that urethan (ethyl carbamate) is an animal carcinogen; it is an initiator of skin tumors, and a lung carcinogen in mice [23]. Moreover, methyl carbamate was shown to be active in a mammalian cell short-term bioassay [24]. It should also be pointed out that, based on the data we obtained, the presence in tobacco of methyl and ethyl carbamates, like hydrazine, does not appear to be related to the use of MH on tobacco.

TABLE 5. *Carbamates in tobacco [15]*

ng/g

Tobacco[a]	Methyl carbamate	Ethyl carbamate
Treated with MH	29±9	290±36
Untreated	45±5	375±25

[a] Burley tobacco

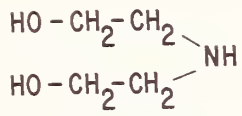
TABLE 6. *Urethan in cigarette smoke ng/cigarette [15]*

Tobacco treatment	Urethan
Off shoot T[a]	30,25
MH-treated I	35,25
MH-treated II	25
Hand-suckered I	20
Hand-suckered II	40,35
Commercial cigarette[b]	35

[a] Experimental chemical suckering agent.
[b] 85 mm, non-filter (U.S.). All other cigarettes were also 85 mm, non-filter.

IV. N-Nitrosodiethanolamine

A third aspect of our studies with the agricultural chemical maleic hydrazide involves an apparently unrelated compound, N-nitrosodiethanolamine (NDELA). The presence of this compound in tobacco, however, is found to be associated with the use of certain formulations of MH that contain diethanolamine (Fig. 9). This amine when added to MH formulations by the manufacturer serves as a solubilizing agent, forming the diethanolamine salt of MH. Otherwise, MH is very insoluble in water, and not amenable for use as an aqueous spray by farmers. What is important here is that diethanolamine, in the presence of nitrite, is easily nitrosated to the corresponding N-nitrosamine (Fig. 10). Tobacco and other plant products contain nitrate/nitrite, and, therefore, one should expect to find this nitrosamine in tobacco products to which formulations of MH (containing diethanolamine) had previously been applied. We then developed an analytical method for the determination of NDELA (Fig. 11), which we applied to tobacco. As in many of our analytical methods, we used a ^{14}C-labelled internal standard, U-^{14}C-NDELA, which we synthesized [25]. NDELA is a somewhat polar substance, and its isolation from tobacco required extraction with methanol, column chromatography, silylation, and gas chromatography



DIETHANOLAMINE
(Solubilizing Agent for MH)

Figure 9. Diethanolamine.

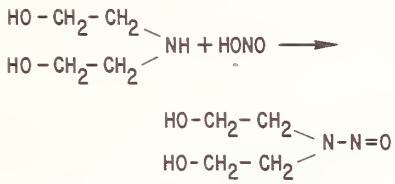


Figure 10. Formation of N-nitrosodiethanolamine (NDELA) from diethanolamine (DEA).

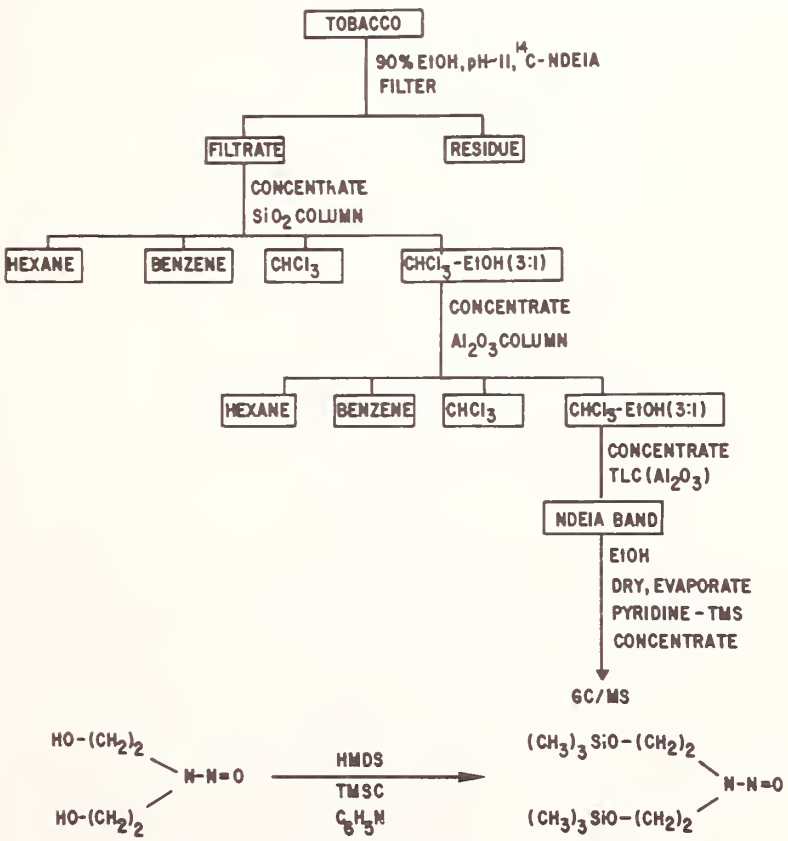


Figure 11. Scheme for the analysis of N-nitrosodiethanolamine.

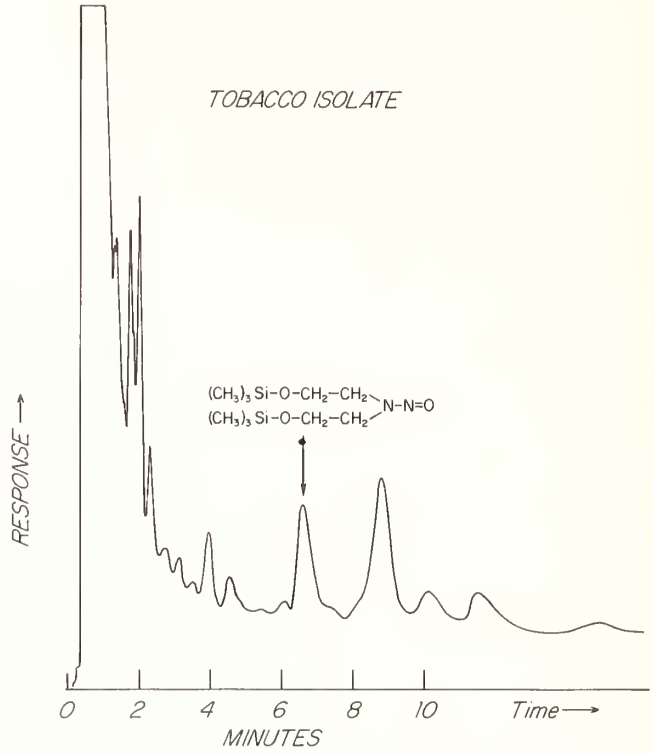


Figure 12. Gas chromatogram of tobacco isolate containing silylated NDE1A.

TABLE 7. *N-nitrosodiethanolamine in various tobaccos* [25]

Tobacco	Amount (ng/g)
Burley[a]	173(176,163,181,170)
Flue-cured[a]	145
Flue-cured[b]	<0.1[d]
Commercial blend[c]	104

[a] Treated with MH formulation containing diethanolamine.
[b] Not treated with any MH formulation.
[c] Taken from a U.S. cigarette.
[d] Limit of detection.

(Fig. 12). We confirmed its identity by use of mass spectroscopy. Levels of NDE1A that we found in tobaccos (Table 7) show that the presence of this nitrosamine is indeed related to the use of certain formulations of MH on tobacco. Although many model studies have shown that appropriate pesticides can be nitrosated in the laboratory [26], our data demonstrate, for the first time, a nitrosation of a pesticidal formulation occurring in the crop. We are now aware that diethanolamine and related amines are used in other agricultural chemicals, in cosmetics and shampoos, and in metal cutting and grinding fluids and, therefore, their conversion to the corresponding nitrosamines is certainly something we are concerned about [27]. In carcinogenesis studies, we observed the formation of nasal cavity and tracheal tumors in hamsters to which NDE1A was administered [28]. Previously, NDE1A was observed to be a hepatocarcinogen in rats [29].

The method we initially used for the analysis of NDE1A was basically a gas chromatographic method. We are currently determining this nitrosamine in tobacco, cosmetics and cutting fluids by use of the thermal energy analyzer (TEA), a detection system specific for nitrosamines [30]. Using this approach, we have confirmed our previous observations with regard to the presence of NDE1A in specific tobacco samples [31,32]. We should also point out that this nitrosamine is also amenable to detection by high pressure liquid chromatography (HPLC) in conjunction with UV detection (254 nm), and in fact, it is now possible, by means of an appropriate interface, to use HPLC in combination with the thermal energy analyzer. Using such a system, and a minimum of "clean-up," we detected small amounts of NDE1A in certain MH formulations as well (800 ppb NDE1A in MH-30).

In our studies with MH then, we examined the possibility that MH formulations could serve as precursor for hydrazine, urethan and N-nitrosodiethanolamine. Of the three, only N-nitrosodiethanolamine is apparently associated with the use of certain MH formulations.

V. Other Compounds

In addition to the foregoing compounds, methods for which we have discussed in some detail, our studies in tobacco carcinogenesis have necessitated the search for many other compounds. For example, we recently completed an extensive analysis of the naphthalenes present in tobacco smoke. These compounds are the products of the limited combustion of organic matter, and would of course, be expected to occur in cooked foods as well. Using ^{14}C internal standards again, solvent partition, column chromatography, we isolated a "naphthalene" fraction which contained the compounds listed in Table 8. These were resolved and identified by gas chromatography-mass spectrometry, and were quantitated directly from the gas chromatogram [33].

In a similar manner, we isolated from both tobacco and tobacco smoke a series of quinones that are common to agricultural products [34]. In this case, in addition to the usual gas

TABLE 8. *The naphthalene fraction of cigarette smoke [33]*

Peak No.	Mol. Wt.	Designation	Amount (per cigarette)
1	130	1-Methylindene	
2	128	Naphthalene	2.8 μg
3	146	Ethylbenzofuran(s)	
4	144	Ethylindene(s)	
5	142	2-Methylnaphthalene	1.2 μg
6	142	1-Methylnaphthalene	1.0 μg
7	154	Biphenyl	83.1 ng
8	156	2-Ethylnaphthalene 1-Ethylnaphthalene	33.4 ng
9	156	2,6-Dimethylnaphthalene 2,7-Dimethylnaphthalene	63.7 ng
10	156	1,3-Dimethylnaphthalene 1,6-Dimethylnaphthalene 1,7-Dimethylnaphthalene	155 ng
11	152	Acenaphthylene	
	156	1,4-Dimethylnaphthalene 2,3-Dimethylnaphthalene 1,5-Dimethylnaphthalene	
12	156	1,2-Dimethylnaphthalene	
14	168	4-Methylbiphenyl	
	156	1,8-Dimethylnaphthalene	
15	170	Trimethylnaphthalenes	

TABLE 9. *Quinones in tobacco/tobacco smoke [34]*

	Tobacco (ppm)	Smoke (ng/cgt)
2,3,6-Trimethyl-1,4-naphthoquinone	<1	220±17
2,-Methyl-9,10-anthraquinone	36.0	190±36
9,10-Anthraquinone	3.0	88±13
2,3-Dimethyl-1,4-naphthoquinone	5.2	34±2
Tetramethyl-1,4-benzoquinone	n.d.[a]	35

[a] Not detected.

chromatographic techniques, we also utilized mass fragmentography for purposes of quantitation. The quinones we determined are shown in Table 9, and include benzoquinones, naphthoquinones and anthraquinones.

VI. Conclusion

In this paper, we have presented several examples of the analytical techniques we have developed with regard to our studies in tobacco carcinogenesis. They represent methods for only a few of the many classes of compounds present in tobacco products, but each compound or class of compounds studied usually requires development of a new method, or modification of an existing one. Our analytical studies of course, are performed in conjunction with our carcinogenicity studies, and together they help provide an understanding of tobacco carcinogenesis.

VII. Acknowledgments

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CRITERIA FOR PURITY OF FOOD ADDITIVES USED IN BIOLOGICAL TESTS

Experience with Saccharin and Amaranth (FD&C Red No. 2)

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The presence of traces of certain impurities in food additives used for various biological tests could significantly influence the results of biological testing and give an erroneous impression of the toxicity of the food additive. After a brief review of the literature, the authors summarize their results about the chemical purity of saccharin and Amaranth samples used in biological tests. Criteria for establishing and identifying the purity of chemicals used in biological tests are suggested.

Key words: Amaranth; Ames test; criteria for purity; food additives; mutagenicity; naphthylamines; saccharin; saccharin impurities; *Salmonella typhimurium*.

I. Introduction

A variety of impurities are known to occur in many organic chemicals. In most instances, these impurities occur as traces or are innocuous compounds, biologically inactive and of little technological significance for the quality of the given chemical. Chemicals used as drugs, analytical reagents, certain organic solvents, standards or chemicals used for special purposes are normally required to meet specific criteria for their purity. Chemicals used in the food industry are no exception. While the importance of eliminating impurities from drugs has been well acknowledged, the possibility of unwarranted side-effects from traces of impurities in food additives has been only recently recognized.

In a number of tests where animals were fed for extended periods with food additives or agricultural chemicals which could find their way into the human diet, certain toxic, teratogenic or even tumorogenic effects were observed. After painstaking investigations, the reason for these unwanted effects was sometimes found to be due to the presence of small amounts of certain impurities. Usually, the presence or even existence of such impurities was not known or expected, and because of their very small amounts, they were not chemically detected before the beginning of the biological tests. This brings us to the point of the importance for specifications, regulations and criteria of methodology for complete analytical identification of the purity for substances used in biological tests. Lack of or disregard of complete information for the purity, or for the profiles of the chemical impurities in the material to be biologically tested could not only limit the usefulness of toxicological data for regulatory purposes, but in addition can provide an erroneous impression of its toxicity.

In recent years, a number of short term bioassays have been developed, with the aim to test carcinogenic potential of chemicals by determining their mutagenic properties. Short duration, low cost, reproducibility and possible correlation with carcinogenicity, make these methods increasingly attractive. The sensitivity of some mutagenicity tests is about 10^3 to 10^5 times greater than

corresponding carcinogenicity tests in rodents [1]. Thus, the presence of very small amounts (in part-per-billion level) of biologically active impurities in otherwise innocuous food additives, might significantly influence the results of the mutagenicity testing of that food additive.

To illustrate the significance of this aspect in analytical toxicology, we will use a few examples from recently published literature.

Impurities in synthetic food additives or agricultural chemicals are usually a by-product of the synthetic process and may arise from side reactions of the main process, or from incomplete reactions, or from the reaction of contaminants already present in the starting materials.

A classic example of the significance of this problem can be illustrated by the highly toxic 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which appeared in trace amounts in some samples of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). TCDD, a very potent hepatotoxin and teratogen even at microgram levels, is produced as a by-product during the preparation of 2,4,5-T. The initial concern for the possible hazard to humans exposed to 2,4,5-T was precipitated by teratologic studies when 2,4,5-T was administered to pregnant mice and rats. The incidence of fetal abnormalities was slightly higher in the treated animals than in the control animals. Later, tests indicated that these abnormalities were caused by about 25 ppm of TCDD which was present as a contaminant in the 2,4,5-T sample used in this particular test [2].

In the February 1978 issue of the Cancer Research, a paper was published from Dr. Bruce Ames' laboratory [3] describing the importance of impurities in a mutagenicity assay using Salmonella. In one example, it was found that an estimated 0.25% of impurity was responsible for all the mutagenic activity observed with 7-hydroxy-2-acetylaminofluorene (7-OH-2-AAF). The impurity 2-acetylaminofluorene (2-AAF), a potent carcinogen, was eliminated by HPLC [4]. It is interesting to note, that the same sample of 7-OH-2-AAF was tested in thorough animal tests for carcinogenicity and the results were negative. Thus, this is an example where the bacterial tests were more efficient than the animal tests in detecting small amounts of potent carcinogens [3].

Another example (reported as personal communication by R. Gustafson) describes the case of a new research compound with potential utility, which in short term bioassays was found to be weakly mutagenic. About 30 samples from a variety of batches were tested but not all of them possessed mutagenic properties. Because a few of the batches were nonmutagenic, a mutagenic impurity was suspected. Eventually, it was found that mutagenicity really was due to an impurity which was successfully eliminated through recrystallization [3].

II. Purity of Amaranth and Saccharin Used in Biological Tests

In recent years, saccharin and Amaranth have been the subjects of intensive biological and chemical tests at the Health Protection Branch in Ottawa. All samples of both chemicals, used in various biological assays, were analysed according to the existing standards and they were accepted or certified as "food additive grade." However, more precise chemical analysis of the same samples revealed qualitative and quantitative differences in the impurities in different samples.

A. Variations of Impurities

The question of impurities in food additives is often complicated by the fact that the same additive is sometimes prepared by different manufacturing processes, using a variety of starting materials. For preparation of saccharin, there are about four methods, two of them being widely used. The main production of saccharin in the U.S.A. starts with anthranilic acid (Maumee procedure), while in Europe and Japan the starting material is o-toluenesulfonamide (o-TS) [4]. Even with the same manufacturing process, the nature and the concentration of impurities often vary from lot to lot [5]. One impurity of special interest in saccharin was o-TS. The variation of this impurity in different lots of saccharin produced a few years ago was between 2.5–5,050 ppm [6]. Saccharin recently produced in the U.S.A. by the Maumee procedure has no detectable o-TS.

Variations in the organic solvent soluble impurities from different saccharins have already been reported [7].

The impurities found in Amaranth present an even bigger problem. When organic solvent impurities from nine different samples of commercial Amaranth were subjected to thin layer chromatography, up to a dozen impurities were visible under UV light. Different batches of Amaranth show qualitative and quantitative differences in impurities.

The impurity problem is more complicated when the manufacturer, for one reason or another, is forced to change the supplier of his starting materials, or to introduce some "small" correction in the process. This could eliminate or introduce a new "set" of impurities in the final product, even though the basic procedures and purification of the final product are unchanged.

B. Choice of Analytical Procedure for Determination of Purity

The important problem for the analytical toxicologist is: what type(s) of analysis should be employed to ascertain the purity of food additives used in biological tests? Specifications for chemical methods only deal with known impurities for which sensitive analytical procedures have already been developed. At this point we can ask ourselves a number of questions, e.g.

- are chemical tests sufficient or are some additional biological assays also required?
- are sensitive biological tests superior to chemical analysis?
- is it more important to monitor impurities of known identity, even in relatively higher amounts, but physiologically inactive? or
- is it more important to investigate and assess the potential for biological activity (i.e., mutagenicity) of small amounts of unexpected impurity?

Before trying to give any reasonable answer to these important questions, we will summarize some results obtained recently in our laboratories.

The total amount of impurities, without identifying them, but expressed as "percentage of organic solvent solubles" or "ether extracts" may be an important characteristic of the quality of a particular sample of a food additive, but from the toxicological point of view it is nondescriptive.

The impurities and quantities permitted in Amaranth certified as "food additive grade," are presented in Table 1 [8].

Table 1. *Specifications for food additive grade Amaranth (Canada, U.S.A., FAO/WHO)*

Pure dye (Amaranth)	Not less than 85.0%
Volatile matter (at 135 °C)	Not more than 10%
Water insoluble matter	Not more than 0.5%
Ether extracts	Not more than 0.2%
Chlorides and sulfates of sodium	Not more than 5.0%
Mixed oxides	Not more than 1.0%
Subsidiary dyes	Not more than 4.0%

We were particularly interested in looking at those "not more than 0.2% of ether extractable" impurities. We developed a sensitive method for isolating and identifying the presence of some aromatic amines from commercial Amaranth. We found in some older samples of commercial Amaranth, variable amounts of α- and β-naphthylamines. The amount of β-naphthylamine did not exceed 1.25 ppb, while α-naphthylamine was found in higher concentrations in some samples. Since sensitive methodology for identifying these two amines did not exist previously, traces of these amines could have unknowingly been present in some of the Amaranth samples used in biological tests. The presence of traces of β- or α-naphthylamines, or other free aromatic amines, or subsidiary dyes which could produce these aromatic amines after

intestinal biotransformation, could be the reason for the variable results in biological tests with Amaranth.

The positive teratogenic and carcinogenic tests on rats with Amaranth reported in 1968 were done with "40% Amaranth paste" without any chemical characterization of the impurities except to say "it contained in the dried sample 65.7–75.2% of chemically pure dye" [9].

We tested the mutagenic effect of purified Amaranth using a few strains of the Ames Salmonella with a few different tissue activation systems. The end result was that the Amaranth did not show mutagenic activity [10].

C. Interactions in Some Biological Tests

Two interesting observations occurred during the course of these studies [10]. It was observed first: that not only β-naphthylamine but also α-naphthylamine are potent mutagens if proper conditions are used (uninduced hamster liver activation for α-naphthylamine and Aroclor-induced rat liver activation for β-naphthylamine); secondly, the mutagenicity of naphthylamines was suppressed by Amaranth. Figure 1 shows the effect of Amaranth upon mutagenicity of α-naphthylamine in the presence of uninduced hamster liver activation. The suppression of mutagenicity of β-naphthylamine in the presence of Aroclor 1254 induced rat liver activation is shown in Figure 2. The absolute amount of β-naphthylamine is shown on the abscissa. Thus 10 μg

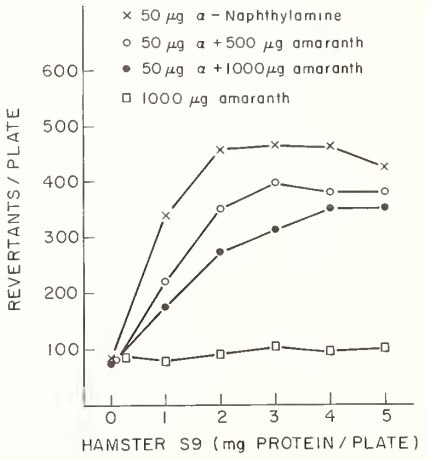

Figure 1. Interaction between α-naphthylamine and Amaranth in the Ames/Salmonella test [11].

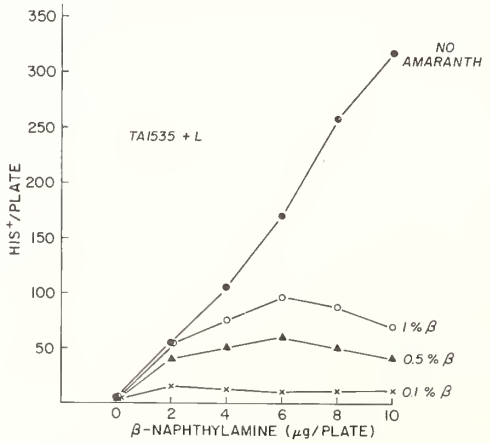

Figure 2. Interaction between β-naphthylamine and Amaranth in the Ames/Salmonella test [11].

of β-naphthylamine alone gives rise to a large number of revertants. However, in the presence of 9.99 mg of Amaranth, the mutagenicity of 10 μg β-naphthylamine was totally suppressed. In other words, one could finely tune the Salmonella assay for detection of pure naphthylamines and then test commercial Amaranth at high doses, but one could not conclude from negative results that the Amaranth sample was not contaminated with naphthylamines [10].

D. Mutagenic Tests with Saccharin and its Impurities

After earlier equivocal results in carcinogenicity tests of saccharin in rats, a number of investigators tested the mutagenic potential of various commercial saccharin samples. Kramers in 1975 [11] suggested that the variable results in mutagenicity assays might be related to the occurrence of varying amounts of mutagenic impurities in commercial saccharin.

In our Ames tests commercial saccharin was not mutagenic, however, small amounts of unidentified organic solvent soluble impurities in some samples of saccharin were mutagenic, as presented in Table 2.

We demonstrated the presence of mutagenic activity in some, but not all, samples of sodium saccharin produced by three different procedures. This mutagenicity was due to a very small amount of unidentified impurities. A few impurities which were identified, i.e., o-TS, were not mutagenic [12].

Also, mutagenicity was not affected by the presence or absence of saccharin in the bioassayed sample.

Table 2. *Mutagenic activity of saccharin impurities [13]*

Process	Sample	Org. solvent solub. impurity mg/kg	Mutagenicity	Times tested
Maumee	*S-1022*	*13*	+	16
	GS-1233	5	–	2
	6368	13	+	1
	S-1469	20	+	3
	GSC-0129	15	+	3
	F, G, H, I, J	1-5	–	2 each
Remsen-Fahlberg	QA-80	86	–	1
	QB-125	305	–	1
	191010	45	+	3
	"Hicks"	212	–	2
"New"	K	38	+	1

Four of the samples of saccharin we studied for mutagenic activity had been subjected to cancer bioassays in rats. From Table 3 it is obvious that any attempt to correlate carcinogenicity with mutagenicity of specific samples of saccharin is not possible at this time.

Our very recent attempts at isolating the mutagenic impurity from saccharin have not been very successful. A number of lots of very recently produced saccharin possess very little total impurities (in one lot there was about 1 ppm of total organic solvent soluble impurities) and they are not mutagenic.

TABLE 3. *Correlation between carcinogenicity of samples of saccharin in the rat and the Ames Salmonella mutagenicity of their impurities*

Sample		Mutagenicity	Carcinogenicity	
S-1022	M	+		
			+	2 Generation
GS-1233	M	-		
QA-80	R.F.	-		
			+	2 Generation
QB-125	R.F.	-		
191010	R.F.	+	-	1 Generation
"Hicks"	R.F.	-	± ?	1 Generation

TABLE 4. *Exclusions of artifacts during the extraction of mutagenic impurities from saccharin*

1. Histidine—independence of "Revertans": *Confirmed*
2. Organic solvent control: *Not mutagenic*
3. Second extract: *Not mutagenic*
4. Extract using different solvents: *Mutagenic*
5. The extracted residue redissolved in DMSO: *Mutagenic*
   Ethanol: *Mutagenic*

E. ARTIFACTS

It is essential to take all possible precautions to avoid introducing artifactual mutagenic activity during extraction processes. Precautions taken with saccharin impurities are summarized in Table 4. The possibility for artifactual effects of the solvents used for the preparation of extracts should be given special attention. We had analysed a number of solvents for their total impurities and mutagenic activity. We found that some possess measurable amounts of impurities which are easily detectable on GLC as presented in Figure 3. Impurities in some organic solvents could exceed the amount of the impurities isolated from the food additive [13].

III. Recommendations

Although these guidelines may represent an idealistic point of view for an analytical toxicologist, in practice some of them may not always be feasible or even necessary for some compounds. For example, it is unacceptable to suggest that all food additives to be tested in a short-term (2-day) mutagenicity assay be characterized for purity. However it is equally irresponsible to claim that a food additive is mutagenic or biologically active in any assay without establishing identity and purity of the test chemical.

The characterization of purity and information about impurities in the tested sample should be reported more completely, than was the case with some tests in the past. The identification should not end with quotation of the name of the product, its production lot number, the name of the manufacturer and a general statement about "total impurity content." Whenever possible every sample of food additive used in any toxicological, biological, short- or long-term bioassay, should be identified as completely as possible. A reasonable approach should prevail in determining the rigidity for a complete toxicological evaluation of these impurities.

Ideally, before long-term biological tests with a food additive are initiated, a panel of experts should agree upon the analytical definition of purity for that particular food additive. If possible, a single batch of material should be made available for use in all biological and chemical

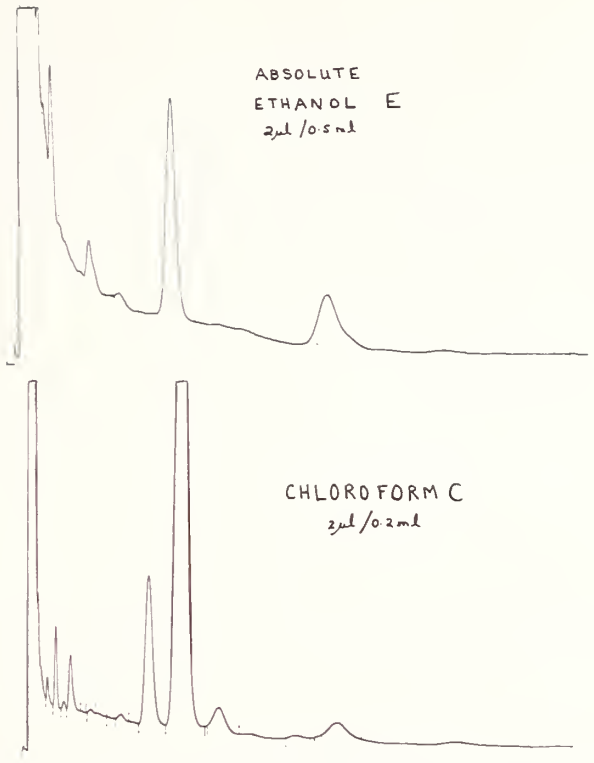

Figure 3. Gas liquid chromatogram of impurities from organic solvents [13].

tests. This sample should be therefore designated as a "reference sample" for any impurity-related questions of that food additive prepared by a given procedure.

The best way to identify the purity of food additives seems to be to combine a series of chemical analyses, such as HPLC, GLC, TLC, PC, and MS (Fig. 4).

All of these procedures may not always be necessary. Sometimes the use of a specific analysis should be sufficient for determining the presence or absence of a certain biologically-active impurity.

If impurities are found, they should be isolated, separated, concentrated, identified chemically if possible, and tested biologically. Which biological tests should be performed is an open question that should be influenced by the amount of the impurity, as well as by the specificity, level and pattern of use of that food additive.

The isolated impurities should be tested using a variety of bioassays. The Ames Salmonella test seems to be a very promising short term bioassay for carcinogenic potential by detecting mutagenic activity. Impurities should be tested for their biological activity either as such, and in the presence of small amounts of the tested food additive.

Every effort should be made to establish the chemical structure of the isolated impurities. The synthetic pathway and possible impurities in the starting material should be considered in making a list of possible by-products. This should help to identify the unknown.

A special type of bioassay testing should be considered if it is suspected that a biologically-inactive impurity could become toxic after intestinal biotransformation.

If the chemical identity of the impurities is not availab'e, they should be characterized by obtaining "a profile of the impurities" of each tested sample by subjecting the impurities to a series of chemical analyses (TLC, PC, HPLC, etc.), combined with biological (mutagenic) short-term assays.

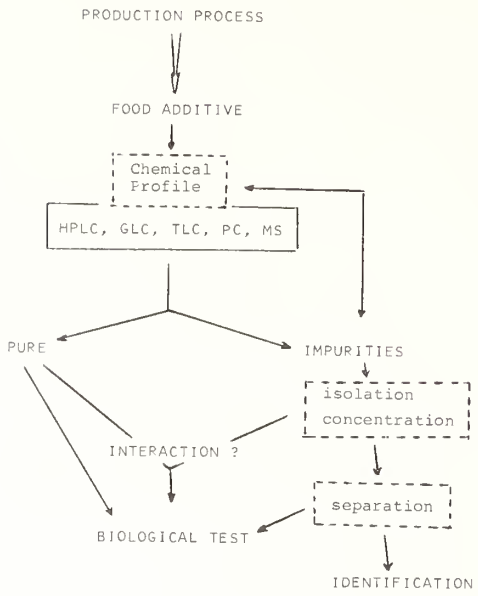

Figure 4. Schematic presentation for the criteria for purity of food additives used in biological tests.

Tests for chemical purity of food additives used in biological tests should be carried out using a few different lots from several different manufacturers, keeping in mind that each tested lot should be a genuine representative of the sample(s) normally consumed by the general population.

During these operations care must be taken to avoid introducing any artifactual impurities.

Only samples of compounds which are free of biologically active impurities should be used for long-term animal studies. For those compounds where traces of some impurities cannot be eliminated, the biological tests should be considered with samples containing these impurities, providing a thorough description and/or characterization of the impurities is made. Good Laboratory Practices legislation [14] should ensure that many of these recommendations are practiced.

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ADVANCES IN THE ANALYSIS OF NATURAL FOOD TOXICANTS

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An overview of the latest developments in analytical methods devised specifically for the detection of selected naturally occurring food toxicants—mycotoxins, natural plant toxins and seafood toxins—is presented. Special emphasis is placed upon the need for careful attention to sampling and sample preparation, the types of analytical techniques available for qualitative detection and quantitation, the need for confirmative techniques, and the requirement for high quality standard materials.

Key words: Food toxicants; mycotoxins; review; seafood toxins.

I. Introduction

The scientific literature is replete with reports concerning the actual or probable association between the occurrence of toxicants in foods (specifically mycotoxins, phytoalexins and other naturally occurring plant toxins, and seafood toxins) and acute or subacute poisonings of man and animals on ingestion of such foods. In order to assess the potential for harm to the human population due to the presence of such toxicants in foods, one must consider at least two factors: the degree of exposure to the toxicant (occurrence, incidence, level of contamination) and the toxicological properties of the material, both acute and subacute (Table 1) [1]. This paper is restricted to a review of some of the newest analytical methods and techniques which have been developed and applied to the detection of various toxicants in foods, and emphasizes the importance of strict attention to sampling and sample preparation, and the need for good reference standards. The manner in which regulatory actions are influenced by analytical detection methods will be alluded to.

TABLE 1. *Assessment of the potential for harm associated with man's exposure to food toxicants*

Toxicity information	+ Exposure of Man = Assessment of Risk
• Chemical structure	• Food commodities
• In vitro testing	• Levels of contamination
• Acute, subchronic and chronic testing	• Frequency and amount of human consumption
• Other biological information	• Length of exposure

A review of the literature reveals that sensitive analytical methods have been developed for detection of a wide variety of toxicants in foods, including mycotoxins in corn, rice, grains, peanuts, tree nuts, edible tissues from food-producing animals (meat, milk and eggs), spices, and fruits [2–4], for naturally occurring plant toxicants in tomatoes, potatoes, beans, carrots, peas, and other vegetables [5,6], and for seafood toxins such as paralytic shellfish poison in clams, ciguatoxin and tetrodotoxin in fish and the like [7–9].

II. Sampling and Sample Preparation

The first steps in the analysis of a foodstuff for the presence of toxic contaminants, and the steps all too often not considered carefully, involve obtaining the sample and preparing the sample for analysis. Associated with each of these steps is a relative error, which when added to the error associated with the actual determinative measurement results in the total analytical error expected for the analysis (Fig. 1) [10]. For example, if the contamination in the food lot is heterogeneous such as a small percentage of subsets (i.e., individual kernels or nuts), then the sampling error would be large if the analytical sample taken is small. On the other hand, if the analytical sample size taken from the lot is increased, then the sampling error will be decreased until, in theory, it

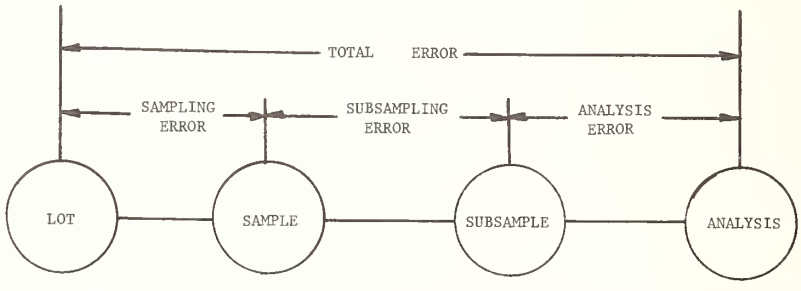

Figure 1. Relative error associated with sampling, sample preparation (subsampling) and analysis. (Whitaker, T. B., *Pure & Appl. Chem.* **49**, 1709–17 (1977).)

disappears when the total lot is analyzed. Of course, to analyze the entire lot is seldom practical nor desirable. If the contamination is homogeneous throughout the lot (i.e., well-mixed liquid products) then the sampling error will be small.

Sample preparation often involves reducing the sample size through quartering, riffling or by grinding, comminution with solvent, etc. Each of these techniques has associated with it a subsampling error. Techniques have been developed for reducing this type of error also; one of the best examples of this was the development of the Dickens-Satterwhite subsampling mill [11] for grinding and subsampling peanuts.

The errors associated with each stage of the analysis are taken into account when food lots are analyzed for regulatory purposes. In the case of peanuts the plan developed [10] and in current use for detection of the mycotoxin, aflatoxin, involves the following: A lot of peanuts (40,000–100,000 lbs.) is sampled using an automatic sampler, trier or some such device; the sample size taken is 3×21.8 kg (3×48 lbs.). The extent and use of each sample is directly dependent on the level of contamination found. One 21.8 kg sample is comminuted in a Dickens mill which automatically separates out an 1100 g subsample. The entire sub-sample is used in the analytical method. Two analyses are made on the initial sample extract; if the average of the two analyses is <16 ppb, the lot is accepted, if >75 ppb, the lot is rejected. If the average indicates levels of aflatoxin between 16 and 75 ppb, then the second 21.8 kg sample is processed as above and analyzed. Again an average, now of four analyses, is calculated; the acceptance–rejection limits are now set at <22 ppb and >38 ppb respectively. If necessary, the third sample is processed for

those samples showing 22–38 ppb aflatoxin. The acceptance–rejection limits (for an average of 6 analyses) is now set at 25 ppb.

Similar sample—sample preparation—analysis procedures must be developed for each commodity under study. A great deal of effort has been devoted to the development of such procedures; a review of sampling plans and collaboratively studied methods for detection of mycotoxins in foods has been published [12].

III. Mycotoxins

Historically, qualitative and quantitative assay procedures for mycotoxins have been based upon the use of thin layer chromatography (TLC) and gas-liquid chromatography (GLC) with authentic samples of the individual, pure mycotoxins being used as reference materials. Several excellent reviews are available describing these methods [13–15], including an excellent review of analytical methods for detection of aflatoxins in foods (paper by Nesheim, NBS symposium). The presence of the toxin was usually verified by derivative formation or through bioassay. Recent advances in instrumental techniques have resulted in a dramatic improvement in the methodology, particularly with reference to accuracy, lower detection limits, speed of analysis, ease of confirmation, etc. It is these major advances in methodology which are discussed below.

One of the most striking changes observed in the methodology for detection of mycotoxins has been the movement toward use of high performance liquid chromatography (HPLC) techniques. This has been due primarily to the dramatic improvements in recent years in HPLC instrumentation and column technology. The major advantages of HPLC are high accuracy, excellent sensitivity, ease of adaptation to a wide variety of compound types, ease of adaptation to confirmatory techniques and high potential for automation. Many thermally unstable compounds may be analyzed without difficulty using HPLC without derivatization (as is the case with GLC).

A number of HPLC procedures have been developed specifically for detection and quantitation of aflatoxins in a wide variety of foods; some of these methods are listed in Table 2.

TABLE 2. *High performance liquid chromatographic methods for the analysis of aflatoxins as contaminants in food commodities and biological fluids*

Mycotoxin	Commodity/substance	Limit of Detection	Detector	Reference
Aflatoxin Q_1, M_1, B_{2a}, and aflatoxicol H_1	Urine	5–200 ng	UV	[16]
Aflatoxin B_1, B_2	Cottonseed products	5–100 ng/g	UV	[17]
Aflatoxin B_1, B_2, G_1, G_2, M_1, M_2	Corn (B_1), standard solutions (B_2, G_1, G_2, M_1, M_2)	20–50 ng/g	UV	[19]
Aflatoxin B_1, B_2, B_1, G_2	Standard solutions	1–2 ng	UV	[18]
Aflatoxin B_1, B_2, G_1, G_2	Peanut butter	<1 ng/g	Fluorescence	[21, 22]
Aflatoxin B_{2a} (B_1), B_2, G_{2a} (G_1), G_2	Peanuts, almonds, ground coffee, corn, pistachio nuts, seeds grains, choc. peanut candy	1–15 ng/g	Fluorescence	[55]
Aflatoxin B_{2a} (B_1), B_2, G_{2a} (G_1), G_2	Wine, fruit juice	0.02 ng/mL	Fluorescence	[60, 61]
Aflatoxin B_1, B_2, G_1, G_2	Olive oil	1 ng/g	Fluorescence	[63]

Originally, these procedures involved use of an ultraviolet (UV) detector set at 350–365 nm. Unfortunately these methods suffered from interference from UV-absorbing substances in the food extracts leading to relatively high detection limits (>10 ng/g) [16–19]. With the advent of sensitive HPLC fluorescence detectors, the detection limits improved dramatically. A method was developed [20] in which aflatoxins B_1 and G_1 are converted into the even more intensely fluorescent hemiacetal derivatives (B_{2a} and G_{2a}). This method has a limit of detection for aflatoxin in corn as low as 0.75 ng/g, and an absolute detection limit on pure standard material as low as 10 pg.

More recently it has been found that one may pack the liquid flow-through cell of the fluorescence detector with a silica gel adsorbent. The aflatoxin-containing eluate from the analytical column on flowing through this column causes the silica gel to fluoresce and this fluorescence (due to aflatoxin adsorbed onto the silica gel) is measured [21]. A commercial, fluorescence detector based upon this principle is now available. The principle advantage of this technique of course, is the fact that the aflatoxins are measured directly and no derivative need be prepared. The lower detection limit using this column is estimated to be <1 ng/g [22].

A recent development in HPLC detectors involves the use of a laser light source [23]. In this technique a 4 μl suspended droplet of solvent eluting from the analytical column serves as a "windowless" cell. The 325 nm line of a He-Cd laser (8 mw) is used; the laser beam is focused on a small spot inside the droplet. This technique has been used in the fluorescence detection and quantitation of aflatoxins (as the hemiacetal derivatives); as little as 750 fg aflatoxin in 4 μl solvent are readily detected. The lower limit of detection in corn was reported to be *ca.* 2 ng/g.

A considerable effort has been made in the application of HPLC techniques to the detection of mycotoxins other than aflatoxins in foods. Some of these methods are found in Table 3. The methods for patulin, penicillic acid and zearalenone have been applied successfully in small surveys, and in the case of penicillic acid and zearalenone, these surveys resulted in the first reported natural occurrence of these materials in foods. In each case the eluate fraction containing the mycotoxin was collected and examined by mass spectrometry to successfully confirm the presence of the mycotoxin. In the case of zearalenone [24] confirmation was also accomplished by sequential HPLC analysis of the sample using four different excitation wavelengths. In this confirmation procedure the fluorescence response is compared to that obtained from standard zearalenone under identical conditions.

Emphasis on HPLC procedures does not mean that other analytical techniques are being neglected. In the last few years, TLC methods have been further developed to meet specialized analytical requirements. For example, an extremely sensitive, two dimensional analysis of eggs for

TABLE 3. *High performance liquid chromatographic method for the analysis of mycotoxins other than aflatoxins as contaminants in food commodities and biological systems*

Mycotoxin	Commodity/substance	Limit of detection	Reference
Patulin	Apple juice	11 ng/ml	[65]
Patulin	Apple butter	15 ng/g	[64]
Penicillic acid, ochratoxin A & B	Corn	25 ng/g	[62]
Sterigmatocystin	Corn, oats	25 ng/g	[58]
Sterigmatocystin (metabolites)	Culture extract	–	[56]
Sterigmatocystin and O-methylsterigmatocystin	Standard solution	10 ng	[57]
Xanthomegnin	Corn	750 ng/g	[59]
Zearalenone	Corn meal	10 ng/g	[24]

aflatoxin B_1 has been published [25,26] and used in surveys. The lower limit of detection was reported to be <1 ng/g. The method is especially useful when extremely difficult sample extracts are to be analyzed, i.e., extracts which contain fluorescing materials which interfere in one-dimensional TLC methods. The method has been collaboratively studied with excellent results [27].

Another area of mycotoxin research in which TLC techniques are being applied is that of multi-mycotoxin detection procedures. For example, Seitz and Mohr [28] have developed a TLC procedure capable of detecting aflatoxin and zearalenone in corn at the 5 and 200 ng/g level respectively. Carrying the idea to its extreme, Durachova et al. [29] published a TLC procedure for the systematic analysis of 37 mycotoxins. The procedure presents a relatively inexpensive technique using a combination of different solvent systems and a series of spray reagents for characterization of the mycotoxins.

A great deal of effort has also been placed in the development of rapid, rugged, economical screening procedures for field use for detection of aflatoxins using small silica gel packed glass columns (minicolumns). The lower detection limits observed are currently in the range of 5 ng/g [30].

Mycotoxin detection methods based upon GLC also continue to be developed, especially for those mycotoxins not readily detected and quantitated by other methods, e.g., the trichothecene mycotoxins which contain generally no UV absorbing/fluorescent chromophore. Several GLC methods for various trichothecenes have been published recently [31,32] which have lower detection limits in the 50–125 ng range. Some of these methods have been coupled with mass spectrometric methods for added specificity. Recently an excellent GLC method has been developed in our laboratory which has a lower limit of detection for T-2 Toxin in corn of 100 ng/g and for diacetoxyscirpenol of 200 ng/g [33].

A more recent development in the area of mycotoxin research has been the attempt to develop radioimmunoassay (RIA) procedures for various mycotoxins (aflatoxins, ochratoxins). In the case of aflatoxins, Lawellin et al. [34,35] have developed an enzyme-linked immunosorbent assay for aflatoxin in biological fluids which permits detection of <10 pg of aflatoxin B_1/mL. An immunoassay was developed by Langone and Van Vunakis [36] which was reported to detect levels as low as 750 pg/mL aflatoxin B_1 in urine and 1 ng/g in corn or peanut butter. In our laboratories Yang and Ueno have developed an RIA technique capable of detecting as little as 10 pg/mL aflatoxin in urine [37,38]. These low detection limits are of considerable interest when sample size is severely limited (as for example in a liver biopsy sample).

Finally, an RIA procedure has been developed for detection of ochratoxin A at the 20 ng/g level in biological fluids [39,40].

The use of mass spectrometry for absolute confirmation of the presence of a particular mycotoxin has been used widely and a computer-based data bank of mass spectral data has been established for this purpose [41]. This technique has been used to confirm the natural occurrence of aflatoxins, patulin, penicillic acid, zearalenone and T-2 toxin in foods. Haddon et al. [42] have presented two procedures for the MS confirmation of aflatoxins in agricultural products. In one procedure, electron ionization mass spectra are obtained following isolation of the mycotoxin by preparative TLC; these spectra are then compared with standard spectra. The disadvantages of this technique are that extensive sample clean-up is required and the limit of detection is *ca.* 10–50 ng of TLC—pure aflatoxin. The second procedure involves high resolution selected ion monitoring (HRSIM) with a reported detection limit of 0.1 ng aflatoxin B_1 or M_1. The advantage of this procedure over the former seems to be that less sample clean-up is required and the sensitivity is better. Both techniques were applied to milk and urine samples.

The potential application of field desorption MS to the screening of crude food extracts for mycotoxins has been studied [43]. It was found that such extracts can be successfully screened using FD-MS; however, the technique is far from routine and many questions relative to clean-up, detection limits, etc., are yet to be answered.

Salhab et al. [44] have recently reported on a GLC-MS procedure for direct analysis of sterigmatocystin in wheat, rice, barley, and corn. Gel permeation chromatography was used to clean-up the extracts before introduction into the GLC-MS system. Levels of sterigmatocystin as low as 1 ng/g were estimated when using the mass fragmentography mode; using 3 ion monitoring detection limits of *ca.* 5 ng/g were reported.

IV. Natural Food Toxicants

Many substances naturally occurring in foods are toxic; however, these materials are normally found at extremely low levels in most food plants. Few good analytical methods have been developed for detection and quantitation of these toxicants. During recent years it has become apparent that certain compounds (phytoalexins) are produced and concentrated in plants as a result of infection by plant pathogens. Other components are produced by the plant in response to stress (mechanical injury, chill injury, exposure to heavy metal salts, etc.). Many of these "stress metabolites" are acutely toxic; again few analytical methods have been developed for detecting and quantitating these materials in food plants. Two excellent reviews relating to the significance of phytoalexins and stress metabolites in food plants are available [45,46]. Some of the research efforts published recently and currently in progress aimed at developing sensitive analytical methods for detection of these food toxicants are outlined below.

Ipomeamarone is one of several toxic furanoterpenoid compounds produced in sweet potatoes in response to stress; it is a potent hepatotoxin. A TLC method was published recently [46] for the qualitative determination of this compound in damaged sweet potatoes; Ehrlich's reagent is used for visualization. Quantitation may be accomplished by GLC, the lower limit of detection was reported to be 2 ng/g.

The sweet potatoes also produce a series of potent lung toxins in response to stress conditions; a sensitive GLC analytical method for the detection of one of these—4-ipomeanol—is under development. The method has an anticipated detection limit of *ca.* 20 ng/g [41].

Several metabolites having the pterocarpan nucleus are produced by stressed green beans; one of these, phaseollin, is known to be a potent hemolytic agent toward mammalian red blood cells [48]. Development of a quantitative GLC method for this material is currently underway.

Certain glycoalkaloids are present as natural components of white potatoes. Under certain stress conditions the amounts of these compounds increase to toxic levels. Many procedures have been developed for estimating the total alkaloid content and particularly for solanine and chaconine, the major glycoalkaloids of potatoes [49]. A procedure has been published recently which describes the qualitative and quantitative analysis of the permethylated derivatives of glycoalkaloids by GLC [50]. This procedure has not yet been adapted to the analysis of foods for glycoalkaloids.

Prior to 1972, interest in compounds produced in plants as a result of stress was centered around the biochemical basis of disease resistance in plants. Consequently, the necessary analytical tools for effective evaluation of the potential health hazard due to the presence of such stress metabolites in foods has not been fully researched. Research in this area is badly needed.

V. Seafood Toxins

It has long been known that certain seafoods (shellfish and finfish) are, at times, extremely toxic, each year many cases of seafood poisoning are reported. The toxins responsible for such poisonings are extremely potent. It is clear that a public health hazard does exist with respect to such toxins in seafoods, and that as greater use is made of seafoods, the potential health hazard will increase unless some way of conveniently detecting and subsequently controlling the presence of these toxins is found.

It has become apparent that there are three major types of seafood poisons: (1) ciquatera poison found in many food fish (jacks, groupers, mackerels, snapper, etc.); (2) paralytic shellfish

poison (PSP) found in mussels, clams and oysters and produced by algae on which the shellfish feed; and (3) puffer poison found in certain species of puffer fish (tetrodotoxin). Most methods for the detection of these three classes of toxins are bioassay procedures, i.e., the mouse bioassay for saxitoxin, the mouse intraperitoneal and mongoose feeding test (ciguatoxin), and the mouse intraperitoneal test (tetrodotoxin). Simple, rapid specific analytical methods for detection of these toxins are clearly needed for effective quality control of such seafood toxins. In recent years a start has been made in carrying out the research required in order to develop such methods.

Ciquatoxin(s) represents the most difficult challenge since the chemical structure of the toxin(s) is unknown. However, an RIA method has been reported recently for detection of ciguatoxin [51]. The method needs further development.

There has been some success in the development of chemical methods for detection of PSP (saxitoxins and *Ganyaulax tamerensis* toxins). Bates and Rapoport [52] have described a fluorometric method for detection and quantitation of PSP based upon extraction of the shellfish with acid, purification of the extract on an ion exchange resin, alkaline hydrogen peroxide oxidation of PSP to a purine which is then detected and quantitated by ultraviolet absorbance or fluorescence. The detection limit has been reported to be *ca.* 0.3 μg/100 g shellfish meat.

A somewhat more sensitive method for detection of PSP has been reported by Buckley et al. [53]; it is an *in situ* TLC-fluorometric procedure. The procedure involves the application of 1% hydrogen peroxide to the developed TLC plate followed by heating and scanning of the plate with a fluorometer. Unfortunately, the procedure does not work with crude extracts; considerable extract purification is required.

Gershey et al. [54] have described a convenient colorimetric procedure for detection of saxitoxin. The procedure is based on the reaction of 2,3-butanedione with the guanidine liberated on acid oxidation of saxitoxin. The test requires thorough sample extract clean-up since interferences from other guanidine-containing compounds can occur. A detection limit of *ca.* 0.5 ng/g was reported. To the authors' knowledge, there is currently no suitable chemical assay for tetrodotoxin.

VI. Reference Standards

All of the analytical techniques described in this paper require access to good quality reference standards. In the case of the mycotoxins these are obtained without difficulty; a list of known commercial sources of mycotoxins is found in Table 4. These materials, when received, should be carefully checked for integrity and purity.

Reference standards of phytoalexins and marine toxins are not readily available. With the exception of PSP, which may be obtained from the FDA, Cincinnati, OH, and tetrodotoxin, which may be obtained from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO., these must be obtained from researchers actively working with these materials.

VII. Summary

The relative importance of sampling, sample preparation, analytical methods, and reference standards as they apply to the analysis of foods should be carefully considered. Qualitative and quantitative analytical methods for mycotoxins have become in certain instances highly specific with the capability for extremely low levels of detection. Fluorescent and absorption properties have been used to improve the level of detection by HPLC analysis of several mycotoxins. Several methods are now available in which the detection limits are in the ng/g range. The use of laser-induced fluorescence detection, coupled with HPLC, permits the quantitation of aflatoxins B_1, B_2, G_1, and G_2 at levels as low as 750 fg. Enzyme-linked immunosorbent and radioimmunoassay techniques allow the detection of aflatoxin in the pg range. Analytical methods for phytoalexins and marine toxins are not as sophisticated as mycotoxin methods; however, the detection of selected compounds in ng quantities is achievable. The trend for the analysis of natural food

TABLE 4. *Sources of mycotoxins*

1. Aldrich Chemical Co., 940 St. Paul Avenue, W. Milwaukee, WI 53233
   - Aflatoxins, B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin, Patulin, Penicillic Acid, Diacetoxyscirpenol
2. Applied Science Laboratories, Inc., P.O. Box 440, State College, PA 16801
   - Aflatoxins B_1, B_2, G_1, G_2, M_1 (Reference standards)
3. Dr. M. Bachman, Commercial Solvents Corp., Terre Haute, IN 47808
   - Zearalenone
4. Dr. H. Burmeister, USDA/ARS, 1815 N. University St., Peoria, IL 61404
   - T-2 Toxin
5. Calbiochem, 10933 N. Torrey Pines, LaJolla, CA 92037
   - Aflatoxins B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin, Patulin, Rubratoxin B, Diacetoxyscirpenol
6. Eureka Labs., Inc., 401 N. 16th Street, Sacramento, CA 95184
   - Aflatoxin M_1
7. ICN-K&K Labs, 121 Express Street, Plainview, NY 11803
   - Aflatoxin B_1, B_2, G_1, G_2, Patulin, Diacetoxyscirpenol
8. Dr. L. Leistner, Federal Meat Research Institute, 865 Kulmbach, Germany, for European distribution only
   - Aflatoxin M (Reference standards only)
9. Makor Chemicals Ltd., Box 6570, Jerusalem, Israel
   - Aflatoxin B_1, B_2, G_1, G_2, ^{14}C-B_1, M_1, Ochratoxin A, Sterigmatocystin, Patulin, Penicillic Acid, Rubratoxin B, Diacetoxyscirpenol, Roridin A, T-2 Toxin, Verrucarin A, Zearalenone
10. Moravek Biochemicals, 15302 E. Proctor Avenue, City of Industry, CA 91745
    - ^{14}C and ^{3}H-labelled Aflatoxin, Patulin
11. Myco Lab Co., P.O. Box 321, Chesterfield, MO 63017, c/o Mr. Robert Sankey
    - Aflatoxins B_1, B_2, G_1, G_2, Zearalenone, T-2 Toxin
    - Diacetoxyscirpenol, Vomitoxin
12. Dr. A. E. Pohland, Food and Drug Adm., 200 C Street SW, Washington, D.C. 20204 (Reference standards only)
    - Aflatoxins B_1, B_2, G_1, G_2, Ochratoxins A, B, B Ethyl ester, Sterigmatocystin, Patulin
    - Penicillic Acid, Zearalenone, T-2 Toxin, Citrinin
13. RFR Corp., 1 Main Street, Hope, RI 02831
    - Aflatoxins B_1, B_2, G_1, G_2, Patulin, Penicillic Acid, Sterigmatocystin
14. Rijksinstitut voor de Volksgezondheid, P.O. Box 1, Bilthoven, The Netherlands
    - Aflatoxins B_1, (Reference standards only)
15. C. Roth, Postfach 1387, 7500 Karlsruhe 1, W. Germany
    - Aflatoxins B_1, B_2, G_1, G_2
16. Senn Chemicals, CH-8157, Dieseldorf, Switzerland
    - Aflatoxins, B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin
17. Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178
    - Aflatoxins B_1, B_2, G_1, G_2, Diacetoxyscirpenol, Ochratoxin A, Phalloidin, Patulin, Penicillic Acid,Rubratoxin B, Sterigmatocystin
18. Mr. R. M. Stubblefield, USDA/ARS, 1815 N. University St., Peoria, IL 61604
    - Aflatoxin M_1 and M_2
19. Supelco, Inc., Supelco Park, Bellefonte, PA 16823
    - Aflatoxins, B_1, B_2, G_1, G_2
20. Wale Corp., P.O. Box 27174, 1975 Ocean Ave., San Francisco, CA 94127 c/o Dr. Wallen Lee
    - Radiolabeled aflatoxins B_1, B_2, G_1, G_2, Aflatoxins Q_1, B_{2a}, Sterigmatocystin, Ochratoxin A, Patulin, Zearalenone, Penicillic Acid, Rubratoxin B, Diacetoxyscirpenol

toxicants is toward highly specific instrumental methods and away from the use of general non-specific bioassay procedures. Confirmatory methods based on MS techniques have been developed; the cost of instrumentation is the major drawback.

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ANALYSIS OF POTATO FOR GLYCOALKALOID CONTENT BY RADIOIMMUNOASSAY (RIA)

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Radioimmunoassay (RIA) provides a means of combining the specificity of immunology with the sensitivity of radiochemistry to efficiently and economically analyze for toxicants in food. The underlying principle of RIA is that of competitive binding. The basic methodology of RIA requires suitable antiserum as the source of specific antibodies, radiolabeled tracer, and a means of measuring precise quantities of reactants, separating the tracer that is bound to the antibodies from that which is not and quantification of the radioactive fraction(s). An antiserum highly specific for solanidine, the aglycone of the potato glycoalkaloid solanine, a potent cholinesterase inhibitor, has been produced in rabbits after immunization with a solanidine hemisuccinate-bovine serum albumin conjugate. The RIA procedure which utilizes such antiserum and dihydrosolanidine-^{3}H has a high degree of reliability and is capable of detecting as little as 150 pg of solanidine in a potato sample. This RIA procedure greatly reduces the amount of sample preparation, use of costly organic solvents, and increases the number of analyses per unit of time in comparison to conventional chemical methods of analysis for the total glycoalkaloid content in potato.

Key words: Potato alkaloids; radioimmunoassay; solanidine; solanine.

Glycoalkaloids are a class of nitrogen-containing steroidal glycosides found in potato tissues. In 1826, α-solanine was the first glycoalkaloid identified as a natural constituent of potatoes [1]. In 1954, α-chaconine was isolated and characterized as another potato glycoalkaloid [2]. These two are the predominant glycoalkaloids, although others have been found in lesser and varying amounts subject to genetic variety and different conditions of stress. The main difference between α-solanine and α-chaconine and other minor glycoalkaloids is the structure of the glycosidic side-chain (Fig. 1). Almost all of these are hydrolyzable to their common aglycone, solanidine.

Numerous accounts of solanine poisoning, resulting in intestinal discomfort and occasional deaths among humans and livestock have been reviewed by Bomer and Mattis [3] and Gull [4]. Poisoning has generally been associated with consumption of immature, greened, small, or well-

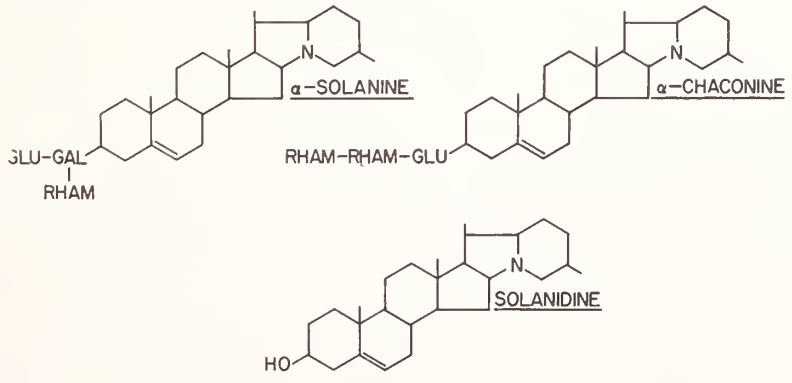

Figure 1. Structure of the two major glycoalkaloids that occur in potato (Solanum tuberosum). α-Solanine and α-chaconine have a common aglycone, solanidine, but differ from each other in the composition of their glycosidic side-chain.

sprouted tubers containing excessive amounts of solanine. The toxicity of solanine has been attributed to its inhibition of cholinesterase activity.

Values up to 38–45 mg solanine per 100 g have been obtained for potatoes implicated in fatal cases of human poisoning [5,6]. These compare with normal values of 3 to 6 mg per 100 g for non-poisonous potatoes. Current standards define values below 20 mg per 100 g as safe for consumption.

There are several published methods used for the analysis of solanine and other glycoalkaloids. One method involves the ammonia precipitation of glycoalkaloids and their spectrophotometric quantitation after complexation with antimony trichloride [7]. This method entails manipulation of large amounts of corrosive and toxic reagents. It also requires the presence of a double bond in the aglycone for any significant color formation.

Fitzpatrick and Osman [8] published a titrometric procedure for the analysis of total glycoalkaloid (TGA) content in potatoes. This procedure involves the measurement of the amino-nitrogen of the glycoalkaloid by nonaqueous titration with a solution of bromophenol blue and phenol after hydrolysis and cleanup of the sample extract. More recently Herb et al. [9] reported a gas-liquid chromatographic method for the quantitative separation of permethylated derivatives of various potato glycoalkaloids.

While each of these methods has varying degrees of advantage in regards to accuracy and reproducibility, all have a serious practical disadvantage of extended and involved extraction, hydrolysis, and careful cleanup procedures, which have been the bane of those concerned in the routine analysis of potatoes for consumption. For this reason radioimmunoassay (RIA) was considered as a possible method for analysis that would measure small quantities of glycoalkaloids with a minimum requirement for sample preparation.

I. Principles of Radioimmunoassay

A methodology called radioimmunoassay (RIA), which has existed since 1960, combines the specificity of immunology with the sensitivity of radiochemistry. The exciting aspects of RIA are that it provides highly sensitive, specific, precise, generally rapid, and relatively inexpensive techniques for the analysis of almost any substance that is of such low concentration in a sample to make detection by other means difficult, if not impossible. For these reasons, RIA has become a basic tool in medical research and clinical chemistry and is routinely used to analyze for a large number of substances such as hormones, vitamins, enzymes, drugs, metabolites, viral and cancer antigens, nucleic acid substances, and peptides, all of which would be difficult to analyze for by other methods.

The underlying principle of RIA is the same as that for other forms of competitive assay, and essentially that is competition between labeled and unlabeled substances for a limited number of binding sites on a specific reactor. Because of this competition for a specific number of binding sites on the reactor, there will be a progressive inhibition or decrease of binding of the labeled substance as the concentration of the unlabeled substance is increased. The concentration of the substance in the unknown sample is readily determined by comparing the degree of inhibition or decrease in the amount of labeled tracer that is bound by the specific reactor from the sample with that resulting when known amounts of the standard are added to control sample materials.

The basic methodology of RIA requires suitable reactants, a means of measuring precise quantities of the reactants, a technique for separating the labeled substance that is bound to the specific reactor from that which is unbound or "free," and a means to quantify the labeled fraction(s). In RIA the specific reactor is an antibody and is always present as a limiting factor. The labeled substance is labeled radioisotopically and is always present in a constant and known amount. The substance to be measured and its radiolabeled tracer compete for active sites on the antibody, thus the amount of the tracer-antibody complex formed is an inverse function of the concentration of the unlabeled substance present.

RIA is simple in principle but not always so in practice, primarily because of the nature of the reactants required for such assays. Antibodies, which are immunoglobulin proteins, are formed in animals as a part of their natural immune response to an antigenic stimulus such as a foreign protein. Substances such as pesticides or naturally occurring food toxicants, which in themselves are incapable of inducing antibody formation in animals but which when attached to ordinary immunogens such as proteins or polysaccharides induce antibody formation against themselves, are called haptens. The hapten therefore must be rendered immunogenic and this is accomplished by conjugating it to an immunogenic carrier molecule.

To achieve the most meaningful results, a well-conceived and pure immunogenic conjugate containing sufficient and appropriate hapten determinants provides the major key to a hapten-specific immune response. The means of covalently binding haptens to carrier proteins are varied. Regardless of the protein used, the same functional groups are available for the conjugation of the hapten. These include such functional groups as the carboxy, amino, imidazole, phenolic, sulfhydryl, and guanidino of the various amino acids. The functional groups of the hapten governs the selection of the method to be used for conjugating it to the functional group of the carrier protein. Theoretically it is possible, therefore, to render almost any small molecule immunogenic by proper manipulation of its functional groups, which might include amino, carboxyl, hydroxyl, ketone, or aldehyde.

II. Materials

A. Reagents

α-solanine, solanidine, stigmasterol, β-sitosterol, campesterol and cholesterol were provided by the Agricultural Research Division, U.S. Dept. of Agriculture, Eastern Regional Research Center, Philadelphia, PA.

Bovine Serum Albumin, crystallized (purified bovine albumin fraction V), Miles Laboratories, Inc., Elkhart, IN.

Phosphate Buffered Saline (PBS), pH 7.4: 24 mL 0.15 mol/L KH_2PO_4 and 76 mL 0.15 mol/L Na_2HPO_4 are added to 100 mL 0.9% NaCl solution.

Dextran-coated charcoal 10 g charcoal grade, and 200 mg dextran, (both radioimmunoassay grade, Schwarz-Mann, Orangeburg, NY) are stirred to homogeneity in 100 mL PBS, pH 7.4.

B. Apparatus

General Laboratory Centrifuge, Sorvall GLC-1, Ivan-Sorvall, Inc.
Liquid Scintillation Spectrometer, Mark 1, Nuclear-Chicago.
Pipetman adjustable micropipetters, 0–200 μL and 0–1000 μL.

III. Methods

A. Preparation of the Conjugate

The conjugate used to elicit antibody production against solanidine was prepared by refluxing it with succinic anhydride in pyridine for 72 hours. The resulting 3-0-hemisuccinyl solanidine was purified by preparative thin layer chromatography (TLC) on silica gel G using a chloroform:methanol (60:40) solvent system. A sample of the purified product was characterized after silylation by mass spectroscopy. The 3-0-succinyl solanidine was subsequently conjugated to bovine serum albumin (BSA) using the mixed anhydride method. Forty-five mg of the derivative dissolved in 10 mL of dioxane was mixed for 1 h with 10 mg of triethylamine. The mixture was cooled to about 10 °C before adding 13 mg of sec-butylchloroformate, stirred for 30 min; this mixture was then added all at once into a solution of 200 mg of bovine serum albumin (BSA) which was dissolved in 40 mL of a solution of water:dioxane (50:50), pH 9. Instant turbidity and

precipitation occurred and the mixture was stirred under ice bath conditions for 2 hours. The mixture was dialyzed against distilled water at 5 °C for 7 days, lyophilized and then characterized for purity and haptencarrier molecule ratio using the hydrolytic and titrometric method of Fitzpatrick and Osman [8]. The analysis revealed that the conjugate contained 4 solanidine residues per molecule of BSA.

B. Immunization and Preparation of Antiserum

Randomly bred white New Zealand female rabbits were immunized with 2 mg of the solanidine-BSA conjugate suspended by emulsification in 1 mL of a mixture composed of 3 parts of Freund's complete adjuvant and 1 part of saline solution. Initial injections were made in small increments intradermally on multiple shaved sites on the animals' backs. The animals, thereafter, were boosted via intramuscular injections with 1 mg of the conjugate at 4 to 6 week intervals. Rabbits were bled 10 days after each booster injection. Sera obtained from these bleedings were tested for antibody titer by examining their binding of the tritiated solanidine tracer.

Significant binding of tracer was exhibited after the second booster injection and increased until after the fifth booster injection where titer plateaued at its maximum for all subsequent booster injections. The antisera showing the highest titer of antibodies, as determined by the level of binding of radiolabeled tracer, were selected for use in the radioassay procedures. These antisera contained a heterogeneous population of antibodies with average association constants (Ka) ranging from 1.5 to 2.0×10^{12} L/mol and binding capacities in the range of 5.0×10^{-8} mol/L, as determined by the method of Nisonoff and Pressman [10]. That dilution of antiserum which bound $\sim$50% of the tracer was chosen as the working dilution in the analytical procedure—a final serum dilution of 1:25.

C. Standard Solanidine Solution

The insolubility of solanidine in water or in various combinations of water and ethanol or DMSO at a suitable concentration for the radioassays was surmounted by dissolving the chemical in 0.5% sulfuric acid, since the hydrosulfate salt of solanidine is soluble at physiological pH ranges which are also optimum for RIA. Fifty μL aliquots of the acid solution of solanidine was employed in phosphate buffered saline at pH 7.4 without any detectable precipitation.

D. Extraction of Alkaloids from Samples

Preparation and extraction of the potato samples was accomplished as described by Fitzpatrick and Osman [8]. While the lengthy cleanup step could be omitted for RIA, the potato extracts were subjected to the 2 h reflux step. For our purposes this consisted of macerating a 20 g aliquot of potato sample in a Waring blender with 100 mL of 2:1 methanol:chloroform for 5 minutes. The macerate was suction-filtered through a coarse fritted glass funnel. The tuber residue was returned to the blender and extracted again for 3 min with 70 mL with the same extraction solution. The mixture was filtered and the blender jar and residue were rinsed with the extraction solution until a final volume of 200 mL of the pooled filtrates and washings was reached. The extract was transferred into a separatory funnel to which 100 mL of 0.8% sodium sulfate solution was added and then shaken vigorously to separate the bisolvent system. The lower phase (chloroform) was discarded. The methanol phase was filtered and brought to volume in a 250 mL volumetric flask. Ten mL of the methanolic potato extract was refluxed with 25 mL of 2N sulfuric acid on a steam bath for 2 hr. After cooling to room temperature the reflux mixture was brought to volume with distilled water in a 250 mL volumetric flask. Aliquots of 50 μL of this solution were subsequently used in the RIA analyses.

E. RADIOLABELED TRACER

Purified solanidine was reductively tritiated across the 5,6 double bond to yield 5,6-[^{3}H-dihydro]-solanidine by the Amersham Corp. The specific activity of the product after purification by TLC was 12.0 Ci/mol.

F. RADIOIMMUNOASSAY PROCEDURES

All assays were conducted in duplicate by sequentially pipeting 40 μL of antiserum, 860 μL of phosphate-buffer-saline, pH 7.4, and 50 μL of the potato extract preparation or the standard solutions into 12×75 mm glass disposable tubes. After vortex mixing the mixtures were incubated at room temperature (24 °C) for 0.6 hour. Fifty μL of the tracer standard (~50,000 dpm/50 μL) was added, the mixture was again vortexed, and then incubated for 2 h at room temperature. Separation of the "free" radioactivity from the bound was accomplished by the adsorption of the "free" radiolabeled solanidine from the test mixture with dextran-coated charcoal. Five hundred μL of the charcoal suspension was added to each incubation tube, the contents in the tubes was vortex mixed, allowed to stand for 15 min at room temperature before being centrifuged at 1500 Xg for 10 minutes. The supernatant containing the antibody-alkaloid complex was transferred by pipet into liquid scintillation vials for radioassay in 10 mL of Handifluor cocktail medium. Controls were included to account for the efficiency of separation of "free" from bound radio labelled tracer by the dextran-coated charcoal (ddc-control) and to account for the radioactivity that bound to the antiserum (a/s control). The ddc-control contained the standard amount of radio labelled tracer, but no inhibitor or antiserum in the same volume of buffer used for the experimental samples. The a/s control consisted of the standard amount of radio labelled tracer and antiserum, but no inhibitor.

The average counts per minute (cpm) for the sample was corrected for efficiency and converted to disintegrations per minute (dpm). The percent of bound radioactivity was calculated according to the following equation:

$$\% \text{ Inhibition} = 1 - \frac{DPM_{sample} - DPM_{ddc\ control}}{DPM_{a/s\ control} - DPM_{ddc\ control}} \times 100$$

The quantity of solanidine in a sample was extrapolated from a standard curve that was constructed by plotting the concentration of solanidine on the abscissa of logit-log graph paper versus the percent inhibition on the ordinate. A typical standard curve is shown in Figure 2 and illustrates that as little as 150 pg of solanidine is detected by the RIA procedure. The quantity of solanidine found in the assay was then converted to express the TGA as mg of solanine/100 g of potato in the manner of Fitzpatrick and Osman [8].

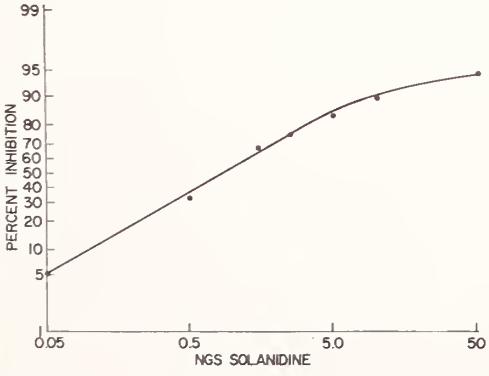

Figure 2. Standard curve for solanidine using 0.746 ng/mL of solanidine-^{3}H (~50,000 dpm) as the tracer and a final serum dilution of 1:25. Incubated for 2 h at 24 °C; separation by adsorption with dextran-coated charcoal.

IV. Results and Discussion

The antisera developed against solanidine showed a high specificity for the aglycone, solanidine, and a lower, although still substantial, affinity for the parent glycoalkaloid, solanine (Fig. 3). Only 0.35 ng of solanidine was required to cause a 50% inhibition of binding of the tracer to the antiserum, whereas 1.22 ng of solanine was required to cause the same effect. These values are sufficiently close to cause substantial error if analyses were made for one or the other compound exclusively. For instance, the first attempts to determine solanine by RIA yielded values 2 to 3 times higher than values obtained for the same samples by either the colorimetric or titrometric methods. Since solanidine is considered to be present in tubers at 10–15% of the total glycoalkaloid content, this discrepancy can be resolved then by the fact that a relatively small amount of the aglycone can exhibit more than twice the degree of inhibition than an equivalent amount of the glycoalkaloid.

On this basis it was decided to completely hydrolyze the glycoalkaloids in the tuber samples to solanidine and then to quantify the alkaloids as the aglycone. The values could then be expressed as mg solanine after making necessary molar conversions as described by Fitzpatrick and Osman [8].

Although α-chaconine has not yet been tested, it is expected that it will inhibit in about the same magnitude as solanine, since α-chaconine is also hydrolyzed to solanidine. Preliminary tests with stigmasterol, β-sitosterol and campesterol indicate that these steroidal components of potato show no significant inhibition levels as high as the 200 ng. Cholesterol shows no significant inhibition at the 500 ng level and about 20% inhibition at the 1000 ng level. Further tests may be appropriate to determine at what levels these compounds begin to display significant interference of tracer binding. However, most of these steroids are practically insoluble in aqueous media and undoubtedly are separated into the chloroform phase during the initial bisolvent extraction, and hence discarded.

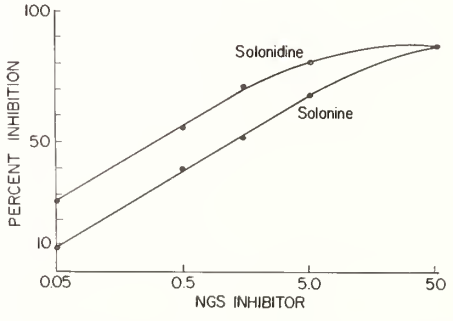

Figure 3. Inhibition by nonradioactive solanine and solanidine of binding of solanidine-^{3}H to an antiserum produced against a solanidine-BSA conjugate. Final serum dilution was 1:25.

The accuracy and reproducibility of the RIA method was determined by comparing the results by it to those obtained for the same samples by the titrometric method. A summary of these results is presented in Table 1. The RIA values, on the average, fall within 15% of the titrometric values. These results are especially encouraging in view of the fact that the RIA analyses were made on virtually crude potato extracts, whereas the analyses conducted by the titrometric method were made on samples that were exhaustively cleaned up.

A possible source of discrepancy and error may lie in the incomplete hydrolysis to the aglycone. Further study is needed to determine the optimal reflux time for the hydrolysis step. Aside from this sampling and dilution may also be a source of some error.

Although the efficiency and reproducibility of the extraction procedure is well proven to be of a high order by Fitzpatrick and Osman [8], there was still the possibility that plant material

TABLE 1. *TGA content found in potato samples by titrimetry and RIA*

Sample	mg/100 g fresh weight Titrimetry	RIA
Maine 1 (whole potato)	36.5	30.0
		32.6
		37.6
		33.0
Buckskin (whole potato)	7.2	7.4
		7.5
USDA #3 (liquid extract)	18.9	11.9
USDA #4 (liquid extract)	321.0	358.4
USDA #5 (liquid extract)	118.9	97.0
USDA #6 (dehydrated solid)	53.2	41.7
USDA #7 (dehydrated solid)	55.6	55.8
USDA #8 (dehydrated solid)	59.2	43.3

TABLE 2. *TGA recovered from potato samples fortified with solanine*

mg TGA originally in sample	mg solanine added	Theoretical total mg TGA	mg TGA found	% recovery
1.62	0.66	2.28	2.40	105.2
0.57	1.02	1.59	1.64	103.1
1.62	0.44	2.06	2.39	116.0
0.57	1.53	2.10	2.02	96.2

other than the related glycoalkaloids or the steroids tested could cause some interference in the RIA analyses. The 50 μL aliquot of potato extract used in the RIA analysis represents approximately 200 μg of plant, thus it was speculated that plant material other than glycoalkaloids or other steroids could cause some interference in the RIA method. This was tested by adding known amounts of solanine to potato samples whose TGA content was known and then analyzing the fortified samples by the RIA procedure. The results as shown in Table 2 indicate that there is only a minimum interference caused by plant material in the RIA analyses and that the recovery and detectability was excellent in fortified samples.

V. Conclusions

The results obtained thus far offer encouraging prospects that RIA can be utilized to provide a simple, fast, reliable, sensitive, specific, and comparatively inexpensive method for the analysis of glycoalkaloids in potato. One of its most attractive features is that it reduces to nearly one-half the time required per analysis as compared to other methods.

The sensitivity to subnanogram levels of glycoalkaloids by this method is a welcome feature, although not imperative. This sensitivity, however, may be utilized advantageously for possible application of RIA for the analysis of physiological specimens such as rumen, blood, and tissues in cases of suspected glycoalkaloid poisonings.

VI. Acknowledgment

Dr. S. F. Osman of the U.S.D.A. Regional Research Center, Philadelphia, Pennsylvania, is gratefully acknowledged for his helpful and gracious technical assistance and gifts of vital chemicals.

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ANALYSIS OF KEPONE® IN BIOLOGICAL SAMPLES

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A simple, rapid and quantitative method for the analysis of Kepone® in biological samples is presented. The sample is extracted with ethyl ether. After removal of the solvent, the residue is placed on a Florisil-based partition column and lipophilic materials are eluted with hexane. Kepone® is eluted with methanol-toluene and analyzed by gas chromatography using electron capture. Recoveries are 80–105%.

Key words: Analysis; biological; gas chromatography; Kepone®; partition chromatography; parts per billion.

I. Introduction

Pesticide analysis is not a new phenomenon in analytical chemistry. Yet pesticide residue analysis has changed as the limits of detection are pushed into the ppb and ppt ranges. Techniques must be developed that effectively remove interferences from the analysis. In the pesticide residue analysis of complex biological or organic matrices, the most difficult problem to solve is the removal of interferences. The method should be simple, rapid, and provide a matrix for quick, quantitative analysis of the pesticides.

Kepone®, $C_{10}Cl_{10}O$, is an insecticide developed in 1952. In 1975 it became newsworthy and its analysis became of great importance to many laboratories including ours. The removal of interferences in the electron capture G.C. method for Kepone® in biological materials soon became one of the toughest problems to overcome. In this paper we describe a method based upon a partitioning column described by Dale and Miles [1]. The method is simple, fast and quantitative for the analysis of Kepone® in biological materials. The limit of detection is less than 1 ppb. The cleanup procedure is very effective with recoveries in the 80–105% range at the 100 ppb level. The ease of the method is gaged by the lack of any special precautions or techniques involved. The method can be applied to any biological material including soils and sludges. We feel the method is far superior to other methods that utilize fuming sulfuric acid [2], multiple extractions [3], or activated Florisil adsorption chromatography [4].

II. Experimental

A. Reagents and Apparatus

1) Methanol, toluene and *n*-hexane were all distilled in glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). Ethyl ether, anhydrous, "Baker Analyzed" reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

2) Florisil PR, 60/100 mesh (Supelco Inc., Bellefonte, PA 16823). Anhydrous magnesium sulfate powder and anhydrous sodium sulfate (Matheson Coleman & Bell, Norwood, OH 45212).

3) Kepone® (Allied Chemical Corporation, Morristown, NJ 07960).

4) All bluefish and shrimp samples were purchased locally.

5) Gas chromatography. Instrument: Perkin Elmer Model 910 equipped with a ^{63}Ni electron capture detector. Column: 6′×2 mm i.d. glass packed with 3% SE-30 on gas-Chrom Q 80/100 mesh (Applied Science Laboratories Inc., State College, PA 16801). Operating conditions:

Oven 230 °C, injection port-250 °C, detector-350 °C, carrier gas 5% methane in Argon @ 30 mL/min.

6) Cleanup column: 14×300 mm buret equipped with a Teflon stopcock.

B. Preparation of Column

A slurry of 7 g Florisil PR in methanol saturated with hexane was added to the column containing a plug of glass wool. The column was washed with 20 mL methanol saturated with hexane. 5 g sodium sulfate was placed on top of the Florisil PR and the column was again washed with 20 mL methanol saturated with hexane. When the methanol reached the top of the sodium sulfate layer, 50 mL of *n*-hexane was added to the column and eluted until the hexane reached the top of the sodium sulfate.

C. Procedure

A 10 g sample, accurately weighed, was placed in a 125 mL Erlenmeyer flask containing 50 mL ethyl ether. The sample was homogenized using a Polytron homogenizer (Brinkman Instruments, Westbury, NY 11590). 5 g magnesium sulfate was added to the flask. The slurry was then added to a sintered glass funnel containing 5 g magnesium sulfate and filtered. The residue was washed with four 50 mL aliquots of ethyl ether. 10 mL *n*-hexane was added to the filtrate and the solvent removed in a rotary evaporator until approximately 5 mL remained. A drop of methanol was added to the flask and the contents quantitatively transferred to the column using three successive rinses with 2 mL aliquots of *n*-hexane. Any lipophilic materials were then eluted from the column with 50 mL of *n*-hexane and discarded. Kepone® was then eluted from the column with 40 mL toluene containing 15% (v/v) methanol. 5 mL of the eluate was injected into the gas chromatograph.

A 40 mL 100 ppb standard was prepared by adding 5 mL of a 200 ppm stock solution of Kepone® in methanol to a 40 mL eluate obtained from a blank column prepared as above.

If any interfering peaks were present in the chromatogram, the eluate was washed with three successive 5 mL portions of 1N NaOH. The combined NaOH portions were then acidified with 5N H_2SO_4 and extracted with three successive 5 mL portions of toluene. The combined toluene portions were successively placed in a 10 mL volumetric flask and the solvent removed under a stream of nitrogen until approximately 8 mL remained. One mL methanol was added to the flask and the solution brought to volume with toluene. 5 μL was injected into the gas chromatograph. A 400 ppb standard was prepared by adding 5 μL of the stock solution to 10 μL toluene containing 10% methanol.

III. Results and Discussion

Recoveries of Kepone® deposited directly on the column or from spiked, concentrated fish oils was found to be 95–100%. When samples were spiked before extraction, recoveries were found to be 80–105%. However, we found two critical factors affecting the results.

The effect of methanol on the reproducibility of electron capture response to Kepone® has been shown [4]. We have also found that changes in solvent composition can change the response by as much as 100% regardless of the methanol content. For this reason the solvent composition for a set of sample and standard solutions should be identical. When the Kepone® was eluted from the partitioning column with 15% methanol in toluene, we found the final eluate composition to be approximately 60/30/10-toluene/hexane/methanol. To ensure consistency, all standards were prepared daily from a blank column eluate.

We also found that the ether used for extracting must be ethanol free. When Burdick and Jackson ether (containing 2% v/v ethanol) was used to extract, a large amount of residual ethanol was found to remain in the flask after removal of the ether. If placed on the column as is, the

ethanol eluted some of the Kepone® off the column during the hexane wash. Recoveries were found to be 20–80%. Removal of the ethanol completely from the flask occasionally resulted in the loss of Kepone®. When ether containing BHT as the preservative was used, these problems were not encountered.

IV. Conclusions

We feel that this method is far superior to others currently in use. It utilizes a minimum number of simple, quick steps, requires no pretreatment of reagents or materials and is accurate and reproducible. We have found the performance of partition chromatography to be better and more reliable than that of adsorption chromatography or liquid/liquid extractions. The method is also sensitive. Concentrations of 10 ppb based on 10 g samples may be easily and accurately determined without further concentration of the column eluate. With concentration, levels of <1 ppb may be determined.

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PROGRESS IN THE AUTOMATION OF EXTRACTION AND CLEANUP PROCEDURES FOR DETECTING TRACE AMOUNTS OF PESTICIDES IN ENVIRONMENTAL SAMPLES

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The discovery of a class of reusable adsorptive silica gels [1] and the recent development of an effective commercial module for continuous evaporation to dryness [2,3] have opened new possibilities for the eventual complete automation of pesticide residue analysis.

Appropriate modules were assembled to index, blend, extract, pump, filter, partition, concentrate, and chromatograph (cleanup) fortified samples of vegetable, animal, or mineral material according to conventional schemes for residue analysis. The liquid effluent from this system was then collected by a fraction collector for residue determination by gas chromatography or alternatively, in favorable cases, the pesticide residues were measured directly by means of a moving-wire/element selective (chlorine, phosphorus, or nitrogen) detector [4,5]. The standard peristaltic pump used in AutoAnalyzer systems was found to provide sufficient pressure to give adequate solvent flow rates through silica gel cleanup columns. The performance and usable lifetimes of either charcoal or silica gel columns under continuous-flow solvent gradient systems (step gradient or linear gradient) were evaluated. The efficacy of the entire automatic extraction and cleanup system was tested using fortified samples selected to present specific problems such as alfalfa and tomato (pigments), beef fat (lipids), soil, and water. Although the results show that automated extraction and cleanup systems for pesticide residue and analysis are now both feasible and practical, much development work yet remains to optimize such systems for routine regulatory analysis.

Key words: Continuous flow system; environmental analysis; pesticides; synthetic carbonaceous resins.

Monitoring the food supply of the American consumer for pesticide residues is a continuous task. This is necessary because there are legal limits (tolerances) to the amount of residue that is permitted in the raw commodity.

The general environment should also be monitored to detect any variances in the types of residues found in wildlife, non-agricultural plants and water sources.

In order to establish a statistical trend for residue background, many thousands of samples should be analyzed over a period of several years. Conventional approaches make this task extremely difficult. Automation could simplify the procedure by allowing more samples to be analyzed at less cost, and enhance the reproducibility and accuracy by minimizing human error.

One approach to automation is to review the manual methods that are in current use and evaluate their adaptability. Once this is done, the availability of commercial instrumentation has to be ascertained. A review of the current methods reveals a basic approach applied for pesticide residue analysis.

1. The solid sample is prepared by grinding it to a small particle size and weighing out representative sub-samples. The sub-sample size is in the range of 25–100 g.

2. The residue is extracted from the sample by homogenization with a suitable solvent and the homogenate is filtered to remove solid particles.

3. The filtrate is evaporated down to a small volume or to dryness so that it can be dissolved in a different solvent for further treatment.

4. The dissolved extract is treated further to remove as much of the non-pesticide extractives as possible. This is accomplished by liquid/liquid partitioning, liquid/solid chromatography or both.

5. The final cleaned up solution is concentrated and analyzed for residue content by whatever technique the analyst wishes to use.

Step 4 was implemented by the discovery of a recyclable, macroporous silica gel [1] and its extension to analysis by combining it with a traveling wire transport system and selective detection systems [2,3].

Suitable modules are available from the AutoAnalyzer concept for clinical analysis by a continuous flow system. The homogenizing unit and pump were combined with the silica gel column and a gradient solvent for extracting and cleaning up [4].

The availability of a new controlled pore synthetic carbonaceous resin simplified the approach by allowing the extracting solvent to be used as the eluting solvent and a second solvent used for recycling the adsorbent.

I. Procedure and Instrumentation

The preparation of the sample is still done manually, with a smaller sub-sample of 2 g being used for analysis. The extraction is performed by a commercially available module called the SOLIDprep® II Sampler (Fig. 1).

This sampler consists of a turntable with polypropylene sample cups, a metering pump for dispensing solvent into the homogenizer, a connection to a vacuum system for draining out excess sample or solvent, and an optical programmer for controlling the functions.

The sample tray contains 20 cups, but only the odd number cups are filled with sample. The even number cups are kept empty so that only solvent will run through the analysis scheme. This is done to eliminate cross contamination of samples.

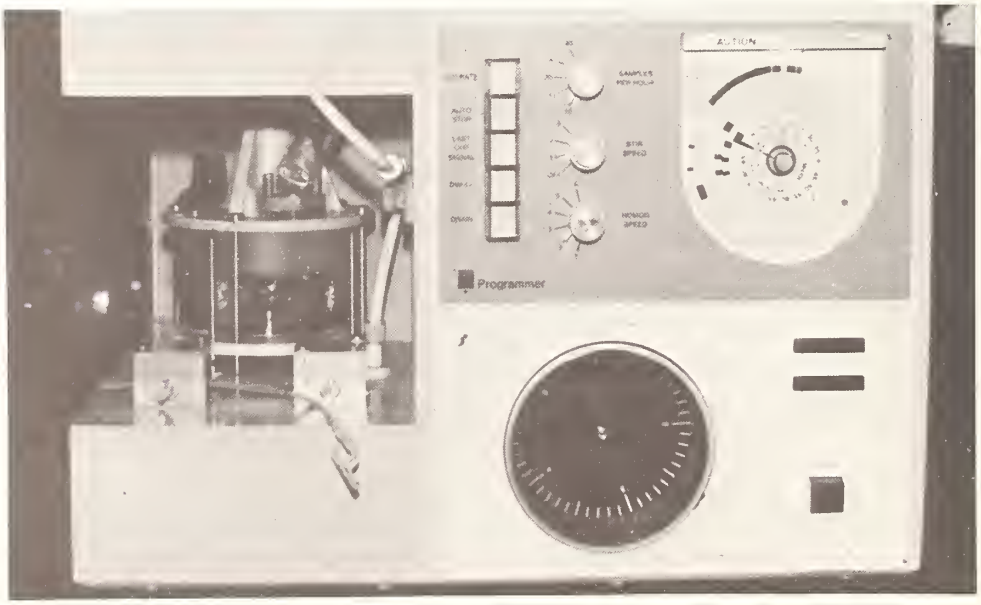

Figure 1. Photograph of SOLIDprep® II sampler.

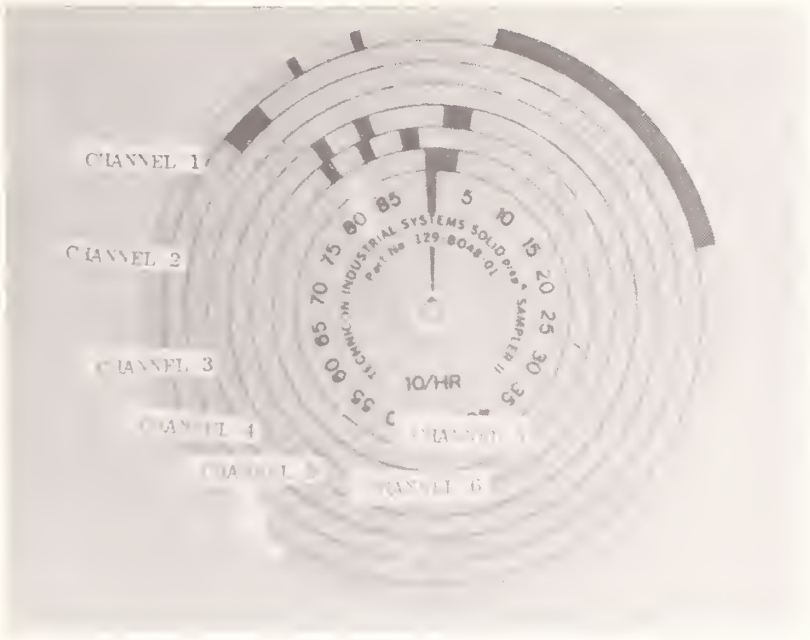

Figure 2. Optical programmer for SOLIDprep® II sampler.

The optical programmer is a circular plastic disc with 7 tracks and triggers the different steps by interruption of a light beam at time intervals (Fig. 2). The method being described uses 5 of the 7 tracks and goes through 10 steps.

Step 1—Track 7 is used which turns the sample tray allowing the cup to swing upside down and dispense the sample into the homogenizer.

Step 2—Track 5 is used which pumps the solvent into the homogenizer.

Step 3—Track 1 is used, and activates the homogenizer for the desired time interval.

Step 4—Track 2 is used, which opens the sample valve and allows the sample to be aspirated out by the peristaltic pump.

Step 5—Track 6 is used to activate a poppet valve on the bottom of the homogenizer and the vacuum sucks out any leftover sample.

Step 6—Track 5 is used to pump in rinse solvent to the homogenizer.

Step 7—Track 6 activates poppet valve and rinse solvent is removed.

Step 8—Track 5 again pumps in rinse solvent.

Step 9—Track 6 activates drain action.

Step 10—Track 7 starts the cycle all over again.

When step 4 is reached the sample is pumped out of the container by a multichannel peristaltic pump as shown in Figure 3. Flexible tubing of fixed length and controlled diameters are used for determining the volumes that are dispensed by the pump. The type of tubing used depends on the solvent flowing through it. This procedure has been developed so that only the two solvents CH_3CN and $CHCl_3$ are used.

Silicone rubber tubing is used with CH_3CN and has the capacity of surviving for several weeks before it has to be replaced. A fluoroelastomer called Acidflex® is compatible with $CHCl_3$ and also can be used for several weeks before it has to be replaced.

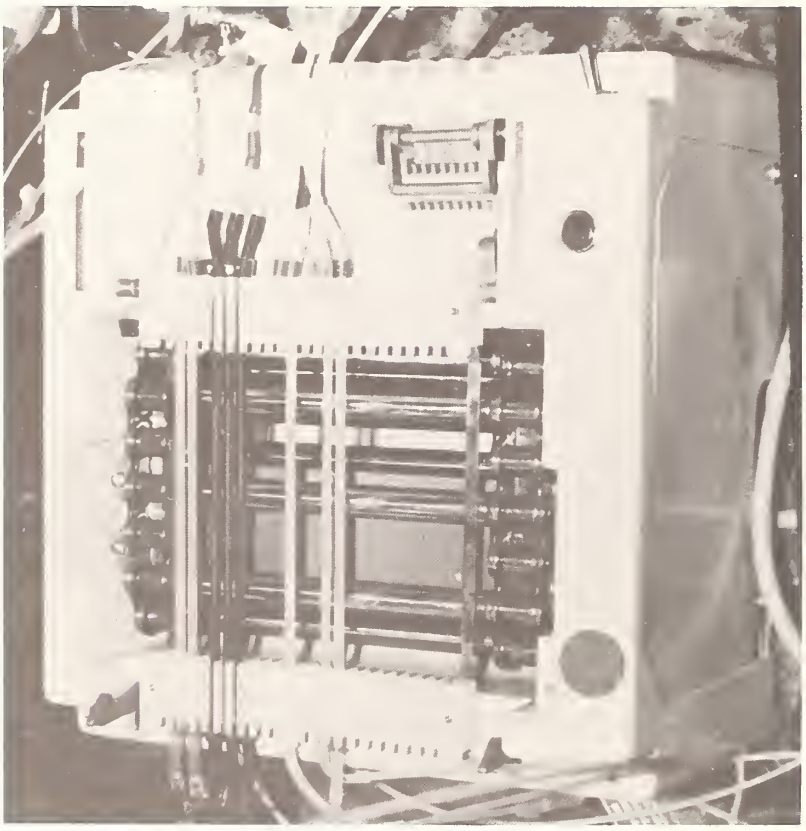

Figure 3. Multichannel peristaltic pump.

The pump meters the sample onto a continuous filter (Fig. 4). The filter consists of a roll of 1″ Whatman #1 filter paper continuously drawn over a Teflon platen. The sample flows onto the filter paper that is in contact with the platen. On the underside there is a connector and hole which goes back to the pump and keeps the filtrate moving. As the filtrate comes out of the pump it is debubbled to remove the air before arriving at the head of the cleanup column.

The cleanup column is a low pressure glass type having adjustable steel plungers with Teflon beds on each end. The column size is 10 mm i.d.×300 mm long and the plungers are adjusted so that the adsorbent fills 150 mm of the column. The column is packed with a carbonaceous resin called Ambersorb XE-340™ (Rohm and Haas). It is a synthetic activated carbon in beaded form having a controlled distribution of pores. Those in the range of 10–30 nm constitute 69% of the material, 4–10 nm pores contribute 13% and the rest lie in the 0.6–4 nm range.

This adsorbent has the property of allowing acetonitrile to elute many organophosphorus and carbamate pesticides with a minimal amount of coextractives. It is recycled by displacing the adsorbed interferences with chloroform and resaturating the column with CH_3CN.

Figure 5 is a flow diagram of the automated system. The SOLIDprep® sampler is designated by A; B indicates the peristaltic pump; C is the continuous filter; D is the CH_3CN solvent reservoir; E is the $CHCl_3$ reservoir; F is the cleanup column and G is the fraction collector.

There are several 3-way valves in the system [1–3] for two purposes. The cost of pesticide grade solvents is high and it is not economical to dump them to waste. A peristaltic pump is being used and you cannot close off the flow from any tube, because the back pressure would pop off the

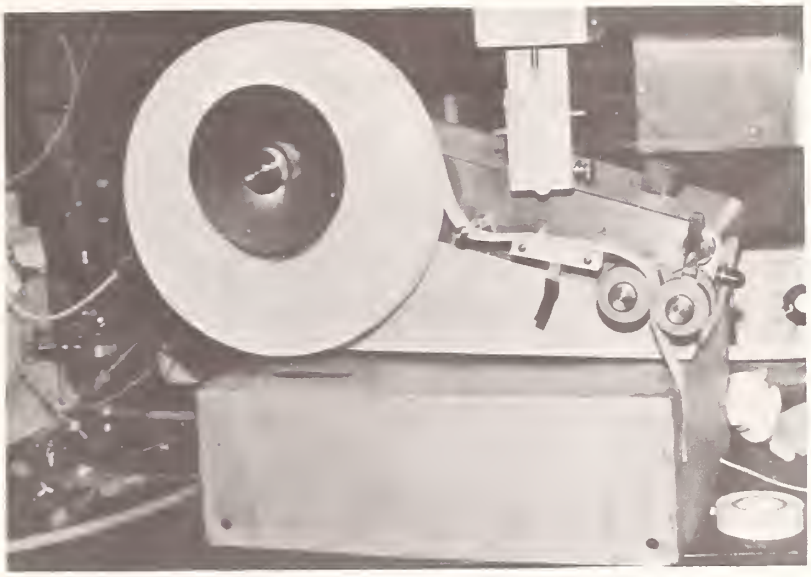

Figure 4. Continuous filter assembly.

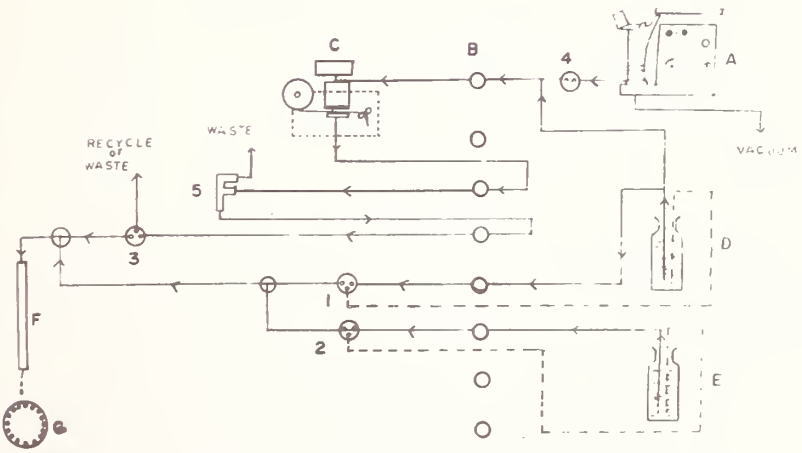

Figure 5. Flow diagram of the automated system. The SOLIDprep® sampler is designated by A; B indicates the peristaltic pump; C is the continuous filter; D and E are the solvent reservoirs; F is the cleanup column and G is the fraction collector.

tube connections. Three-way valves are used to recycle the solvents back to the reservoirs when they are not needed in the system.

A debubbler [5] is used before the sample goes onto the adsorbent column to eliminate channeling which interferes with cleanup efficiency.

The following sequence of steps are followed for the current method:

1. Apply power to SOLIDprep® sampler.
   a. Activates solenoids and allows vacuum to drop to usable level.
2. Check positions of all valves.
   a. $CHCl_3$ should be in recycle position.
   b. CH_3CN should be in column position.
   c. After filter flow should be in column position.
   d. Sample valve should be closed.
3. Start pump.
   a. CH_3CN will not pump through system onto the column.
   b. $CHCl_3$ will be recycled to its reservoir.
   c. While column is being wetted, load cups with samples.
4. Start cleanup.
   a. Push operate button.
   b. Cup empty sample into homogenizer.
   c. 25 mL of CH_3CN is metered into the homogenizer.
   d. The sample is homogenized for 3 min.
   e. After homogenization, sample valve opens.
   f. The sample is pumped out of the homogenizer onto the continuous filter.
   g. The filtrate is pumped through a debubbler.
   h. When the sample arrives at the column the fraction collector collects a total of 40 mL.
5. The second sample cup arrives in position.
   a. The whole cycle is repeated with just solvent.
6. The third sample cup contains sample. Every odd number cup contains sample and every even number cup is empty for the flush cycle.
7. After the 10th sample has been cleaned up, the pump is stopped.
   a. The $CHCl_3$ valve is opened to the column.
   b. The CH_3CN valve is opened to recycle.
   c. The after filter valve is opened to recycle or waste.
   d. Sample valve is closed.
8. Start the pump.
   a. Pump through 1 liter of $CHCl_3$.
9. Stop the pump.
   a. Divert $CHCl_3$ to recycle.
   b. Divert all CH_3CN valves to column.
10. Start the pump.
    a. Wash the column with 250 mL of CH_3CN.
11. System is now ready for next series of cleanup.

II. Results

Alfalfa and string beans were fortified with malathion, diazinon, methyl parathion, carbaryl, and carbofuran at the 1 μg/g level. Two grams of sample were placed in the cups for analysis and 5 samples containing each pesticide were run through the cleanup system.

Malathion, diazinon, and methyl parathion were analyzed by GLC and FPD detection. Carbaryl and carbofuran were analyzed by quantitative TLC. The initial recovery studies gave ranges from a low of 67% to a high of 87%.

The homogenizer as contained in the sampler used a ceramic rotor and stator. It became fractured when a sample of alfalfa containing a twig or pebble became jammed between the two components. This assembly has now been replaced with a stainless steel one (Fig. 6).

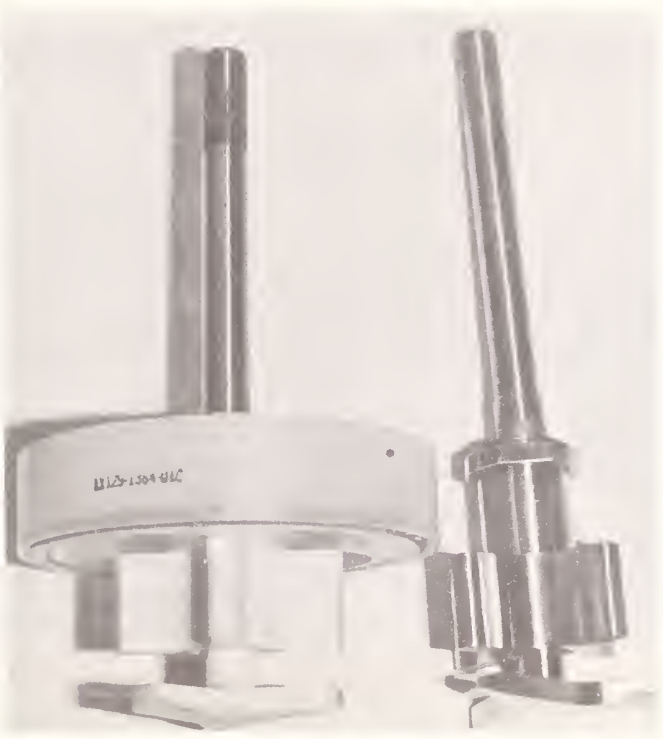

Figure 6. Ceramic rotor and stator (left) and stainless steel replacement (right).

III. Discussion

The preliminary work has shown that the concept of automating the extraction and cleanup procedure is viable and practical. The manual manipulations of stopping the pump and opening valves will be automated by use of solenoid valves in conjunction with an outboard programmer or by utilization of tracks 3 and 4 of the optical programmer. These 2 tracks will emit 28 volts pulses which are suitable for operating solenoid valves.

The ultimate goal is to develop a completely automated system for extraction, cleanup, and high performance LLC coupled with the traveling wire and selective detection [2,3].

This would be done by using the Sampler II (Fig. 7) as the fraction collector for the cleanup procedure. Then it would be used as the sampler for the analytical determination by having eluates pumped onto a continuous evaporation module [5] (Fig. 8). The basic operation is performed by having the sample drip slowly onto one end of a continuous Teflon tube and the solvent is evaporated off by blowing warm nitrogen across the tube. On the other end of the moving tube the desired volume of solvent is used to redissolve the sample and then pumped onto an analytical column whose eluate would be captured by the moving wire and detection system.

More matrices have to be analyzed including soil containing many representative pesticides and their metabolites to determine recoveries through the cleanup scheme.

Finally, samples would have to be analyzed with an accepted multi-residue manual method and the automated method for statistical comparison.

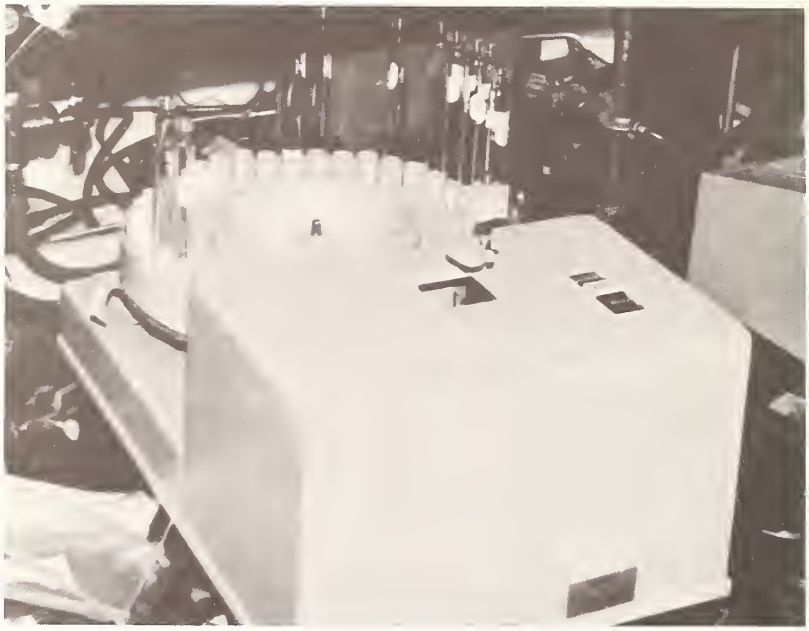

Figure 7. Fraction collector for cleanup procedure.

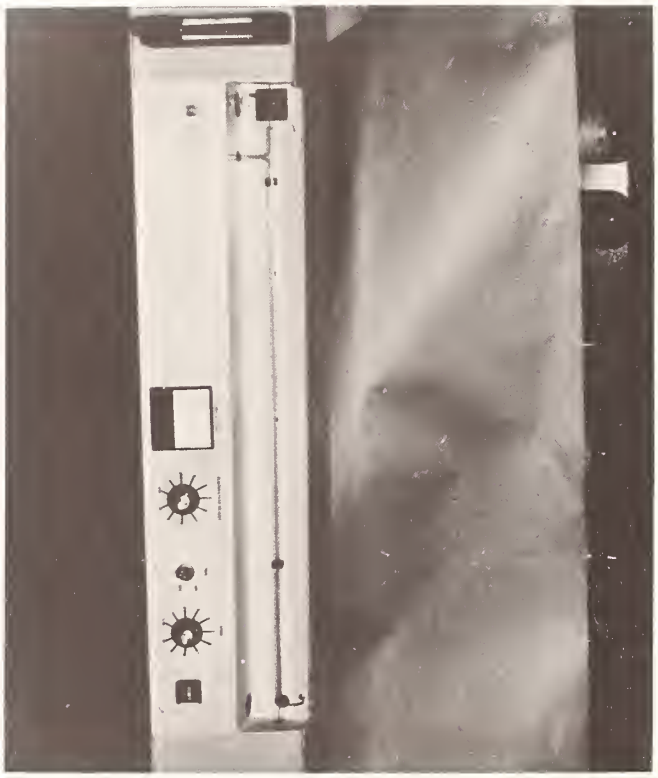

Figure 8. Continuous evaporation module.

IV. Acknowledgments

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METHOD OF AFLATOXIN ANALYSIS

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A "State of the Art" summary of methods for the analysis of foods and feeds for aflatoxins is presented. Included are: preparation, distribution and handling of standards, sample preparation, technique and solvents for extraction, toxin separation, detection and quantitation by techniques including two dimensional thin layer chromatography and high performance liquid chromatography, and a discussion of method limitations, problems, precision and accuracy. Also included is a bibliography of aflatoxin methodology covering the last 5 years.

Key words: Aflatoxin analysis; food and feed analysis; methods; preparation; sampling.

This paper presents general background information on the chemical methods of analysis of foods and feeds for aflatoxins. The analytical techniques, their application and limitations, and some of the problem areas are discussed. A reference framework is provided for understanding the *numerous* specific methods, assistance is given for solving some of the analytical problems which arise, and the meaning of the analytical results is explained.

I. Preparation of Sample

The relationship between an analytical result and the actual aflatoxin content of a lot is meaningless unless the analytical sample is representative of the lot. The current state of knowledge on obtaining a representative sample has been presented by other participants in the symposium. Suffice it to say, the best lot sample is obtained by continuous sampling of the lot as it is being moved, for example, on a conveyor belt during storage transfer. Where this is not possible, the lot sample should be obtained from as many random points within the lot as possible and should be as large as possible within practical limits. The lot sample is then coarse ground to pass a No. 8 or 10 sieve, blended, and a 1–2 kg subsample (5% of the original sample) is taken for fine grinding (i.e., to pass a No. 20 sieve). This is blended and the analytical sample (50–100 g) is taken. Alternatively, the entire sample is finely ground and the analytical sample of 50–100 g is removed.

Most soft samples such as peanuts and even samples such as in-shell Brazil nuts can be ground to a homogeneous mass in 3 min in a Hobart Vertical Cutter/Mixer. Dried figs can also be ground but a known weight of water, up to 10%, must be added. Green coffee beans are also handled this way but Dry Ice is added for better grinding. The Hobart mixer is considered especially convenient because of the ease and speed in handling large samples as well as the ease of cleaning the mixer between samples. In the peanut trade a hammer mill, the Dickens-Satterwhite mill, especially designed for grinding peanuts, is widely used. This mill grinds the sample and continuously splits out 5% of the sample as a discrete subsample. This mill produces a relatively coarse grind. Small grains and corn are best ground in rotary cutter (Wiley) or disk (Straub, Bauer) mills. Cottonseed can be ground by several passes through a rotary cutter.

Samples which are not analyzed immediately must be preserved to prevent changes. Storage at low temperature can be used to prevent insect damage in grains and similar dry materials or

microbiological changes in high moisture products. Grinding of natural materials promotes oxidative, hydrolytic, and enzymatic changes which often make the materials more difficult to analyze. The aflatoxin content of ground samples may change. For example, we have found that aflatoxins are stable for 5–10 years in refrigerated peanut butter and ground Brazil nuts; but they disappear completely from ground pistachios in a few months. Obviously, cheese, meat, milk, and eggs must be analyzed as soon as possible; the best means of preservation of these products is probably lyophilization.

II. Selecting the Analytical Method

The analyst must select the most appropriate method for analysis of the sample. For the regulatory chemist the method may be dictated by law (i.e., he may be required to use an official method). Even so, he may use a more rapid or economical, but reliable, method for screening purposes in order to eliminate negative samples, and then apply the official method to the positive samples.

The selection of an appropriate method is not always easy. Over the last 10 years the number and types of methods for the analysis of foodstuffs for aflatoxins have proliferated. As of April 1978, the FDA computerized reference file contained about 460 entries under the heading "aflatoxin methodology." In general, biological methods other than radioimmunoassay methods are less sensitive and have higher limits of detection than chemical types. They will not be discussed here. Methods are available from many sources; these include the collaboratively studies methods of analysis of the Associaton of Official Analytical Chemists (AOAC), the American Oil Chemists' Society and the American Association of Cereal Chemists, the International Union of Pure and Applied Chemistry, and the European Economic Community. The AOAC methods are updated annually by the AOAC General Referee (currently Leonard Stoloff), and his referee report is published in the March issue of the Journal of the AOAC. This report also includes a list of current Associate Referee topics. The Associate Referee responsible for aflatoxin analytical methods may be consulted for the latest information on his assigned topic. Scanning reviews of aflatoxin methodology may also be helpful.

Most methods have been validated for application to only one or, at most, a few commodities. Those methods using the least effectual extracting solvent and little or no extract purification generally are of limited applicability. Conversely, those methods applicable to the more complex materials which generally give more interferences in the extract, i.e., coffee, spices, milk, and cottonseed, can usually be used with the less complex materials. The simplest method is usually the most economical and efficient method.

Aflatoxins are found in nearly every food or feed which can be attacked by Aspergillus flavus or A. parasiticus and in tissues of animals which have ingested aflatoxins. Products in which aflatoxins have been found include: peanuts, almonds, filberts, walnuts, pecans, Brazil nuts, pistachio nuts, pine nuts; the non-edible oilseeds cottonseed and copra; corn, sorghum, millet, rice, wheat, oats, barley, rye, and beans; and miscellaneous commodities such as dried figs, meat, milk, cheese, and eggs. Although methods have been validated for analysis of only a few of these commodities, a few basic procedures have been found applicable to most commodities through general use. Details and data on applicability, recovery, and accuracy of the many other methods which have not been subjected to collaborative studies can be found in the original literature describing these methods or their use in occurrence of aflatoxin studies.

Commodities can be classed according to composition complexity: presence and amount of fat, protein, water, pigments, alkaloids, aromatics, ultraviolet (UV) absorbing or fluorescing materials, and other interferences. Products which are similar in composition can often be analyzed by the same or slightly modified methods. Based on the class of the product, past experience, and the literature record of method application, the most appropriate method can be selected.

In choosing a method for the analysis of a large number of samples, economics is an important factor. For example, the cost in time and reagents of AOAC Method I (the so-called CB method) for peanut butter is about twice that of AOAC Method II (BF method). In actual practice, commercial laboratories have been able to analyze as many as 70 samples per man per day using Method II. Method I suffers because additional time is required to remove interferences from the extract.

If validated methods are not available, the method chosen must be validated by the analyst himself on known, practice or spiked, check samples. Even in routine analysis with familiar methods and products, laboratory and analyst performance should be quality controlled. Check samples for this purpose are available from several sources: the International Agency for Research on Cancer, Lyons, France; the American Oil Chemists' Society; and the Association of American Feed Control Officials.

III. Factors Affecting the Analytical Results

Many factors can affect the final results. Among those which often do not receive enough attention are: purity of solvents, cleanliness of glassware; and calibration and care of standards. Most AOAC methods use solvents which conform to ACS specifications and which are distilled and stored in glass. These requirements are designed to avoid contamination of extracts with interfering, non-volatile fluorescent materials, originating from seam caulking, enamel liner, or closure gasket materials in metal cans or from bottle cap glues.

Laboratory plastic ware can be a source of interference. Solvents which have been stored for a long time in plastic wash bottles should be discarded. Significant amounts of aflatoxin are absorbed from solution by various plastics, including polyethylene. For analysis at the 0.1 ng/g level, such as in the analysis of meat, milk, and eggs, it is prudent to use the purest solvents and reagents available. The glassware must be scrupulously clean and free from soap or other residues.

The quality of the aflatoxin standard is important to the accuracy of the analysis. The analyst is responsible for checking concentration by UV analysis and purity by thin layer chromatography (TLC) as described in the AOAC methods. Once calibrated, standards must be protected from light, contamination, and evaporation. Standard solutions, when not in use, should be stored at low temperature in containers with Teflon, glass, or metal closures and sealed with Parafilm to reduce evaporation. Aflatoxins for use as standards are available from many sources (see Table 1). These purchased materials are not always pure, and consequently, it may be necessary to purify the aflatoxins by silica gel column or high pressure liquid chromatography (HPLC).

Crystalline aflatoxins are highly electrostatic, and must be handled in a glove box to protect personnel and prevent contamination of the laboratory. Aflatoxins B_1, B_2, G_1, and G_2 are dissolved in acetonitrilebenzene (2+98) rather than chloroform, according to AOAC procedures, to minimize evaporation and to avoid possible degradation by chloroform or its decomposition products. However, for the less soluble aflatoxins, such as M_1, M_2, P, or Q, chloroform or an equally strong solvent must be used.

IV. Extraction

No single solvent can quantitatively extract aflatoxins from all commodities. In early methods of analysis, products were exhaustively extracted with methanol or other organic solvents. This produced extracts which were very difficult to purify for later examination for the presence and amount of aflatoxin by TLC. Newer, rapid methods, chloroform, methanol, acetonitrile, or acetone in combination with water at neutral or slightly acid pH is used for extraction. Assuming a uniform distribution of the extracted toxin throughout the total solvent, only a measured aliquot of the extract is taken; this aliquot is carried through the rest of the method. The majority of conditions and techniques used for extraction (Waring blender for 1–3 min at full speed or

mechanical shaker for 10–30 min at room temperature) were originally established by trial and error; they are now accepted based on overall method performance.

V. Purification of Extract

The extract solution is next clarified by centrifugation or filtration through cellulose or glass fiber filter paper, often aided by diatomaceous earth filter aids such as Celite. Only recommended types of filter aid should be used, because the aflatoxins are not completely recoverable from some types of diatomaceous earth or from asbestos. Acid-washed diatomaceous earth is preferred and is commercially available. Extracts from heavily contaminated samples can be quantitated directly or after concentration. All others must be purified by one or more of the many available techniques. These include the removal of fat from aqueous methanol, acetonitrile, or acetone extracts by partition with hydrocarbon solvents such as hexane. The aflatoxins are then further purified by partition from the aqueous phase into chloroform, methylene chloride, or benzene. Pigments and other materials less water-soluble than aflatoxin may be removed by precipitation with salts such as $AgNO_3$, $(NH_4)_2SO_4$, or $Pb(OAc)_2$ and by increasing the water content of water miscible organic solvents. This technique is especially effective with products such as cottonseed, green coffee, corn, eggs, cocoa, milk, cheese, and alfalfa.

Column filtration or chromatography with various adsorbents is the most commonly used purification technique. Silica gel is most often used; occasionally it is used in combination with other adsorbents such as alumina or Florisil ($MgSiO_3$). Many adsorbents irreversibly adsorb some aflatoxins. The least active of the available commercial silica gels, further deactivated with water, are therefore used.

Most of the AOAC methods recommend the use of a slurry technique for packing silica gel columns in order to minimize the trapping of air bubbles. Some analysts prefer to let the silica gel settle by gravity with no solvent flow (closed column stopcock), believing this gives the best column reproducibility. Once the sample is on the column, the aflatoxins should be eluted as rapidly as possible using fast flow rates without interruption, i.e., in less than 1 hr for the typical 10 g silica gel column. A slow column flow before sample application may result from using too fine a sodium sulfate as the base and cap of the column, or from packing the cotton or glass wool plug too tightly. Sometimes the flow rate slows after application of the sample to the column. This may be due to fine sample particles in the extract which are blocking the column, or to excessive water in the extract causing caking of the sodium sulfate cap. This problem can sometimes be remedied by carefully stirring the top sodium sulfate layer without disturbing the silica gel.

Alumina is another effective adsorbent. Only acid or neutral alumina should be used since aflatoxins decompose on basic alumina. Florisil treated with acetic acid is used in the AOAC method for green coffee. The aflatoxins attach very strongly to this adsorbent and only B_1 is recovered. Charcoal mixed with magnesia (SeaSorb) and diatomaceous earth to improve the flow rate are used for extract cleanup in a minicolumn method for aflatoxins M_1 and M_2. Aflatoxins B_1, B_2, G_1, and G_2 are not recoverable from this mixture.

In addition, there are many techniques for extract purification which currently find only limited use, but which may become more important as the need for improved methodology increases. These techniques include preparatory silica gel TLC. In this technique the entire partially purified extract from 10 to 1000 g product is chromatographed on 1 or 2 standard TLC plates. The aflatoxin spots are scraped off the plate and the aflatoxin is eluted from the silica gel. The best recovery expected by using this technique is about 50%. However, aflatoxins have been quantitatively recovered from the silica gel plate using a commercially available apparatus for *in situ* elution. TLC is a versatile, powerful technique for extract purification. It offers great selectivity for aflatoxins relative to interferences by using a variety of solvents. TLC also offers the possibilities of 2-dimensional multiple solvent and controlled atmosphere development. This is an

essential part of a method, recently adopted by the AOAC, for the detection of aflatoxin B_1 in eggs at levels as low as 0.1 ng/g.

The aflatoxins have also been purified by dialysis; reverse phase chromatography on methanol-water impregnated cellelose; formamide impregnated diatomaceous earth; reverse phase chromatography on octadecyl groups chemically bonded to small particles of silica gel; and cleanup by steric exclusion or gel permeation chromatography.

VI. Detection and Quantitation

A variety of techniques are sensitive enough for detecting or quantitating the aflatoxins at the nanogram and picogram level. Among these are UV adsorption, fluorescence, polarography, mass spectrometry, and radioimmunoassay. Most of these are still in the developmental stages. Only fluorescence and UV adsorption have found general practical routine use. UV spectrometry is used for determining the concentration of reference standards. Using standard 1 cm light path cells, 3 mL solution containing 4 μg/mL is needed to obtain an adequate response. With the more sensitive UV detectors employed in HPLC, adequate response is obtained with 2–10 ng per injection of each of the 4 common aflatoxins. More recently this has been improved through the use of fluorescence detectors. Thus the same 4 aflatoxins have been quantitated by HPLC by injecting as little as 0.2 ng B_2 and G_2 directly, and B_1 and G_1 after conversion to B_{2a} and G_{2a}. When a silica gel filled cell is used for detection 50 pg of each toxin can be quantitated. HPLC has been used for the preparation of pure aflatoxins and has been successfully applied to the analysis of cottonseed, corn, and pistachio nuts. Published results show a within-laboratory precision of + 7.3–7.4% in analysis of cottonseed meal. A collaborative study of 3 HPLC methods for aflatoxin is presently being planned.

Analysis by visual or densitometric comparison of the fluorescent intensities of sample and reference compounds on TLC adsorbents was the earliest, and is still the most widely used, determinative procedure. As little as 0.5 ng aflatoxin can be seen on a TLC plate under longwave UV light.

Detailed directions for conducting the TLC detection and quantitation of aflatoxins are given in the AOAC methods. These probably are ignored by many analysts. Successful TLC is neither guaranteed by slavish adherence to these directions, nor is it precluded by deviation. The control and reproducibility of TLC separations is a problem which greatly influences the final results. The analyst needs to verify the quality of his TLC technique for himself, both as to resolution of the 4 aflatoxins, B_1, B_2, G_1, and G_2, and as to their stability as evidenced by the absence of fading. Acetone-chloroform is the recommended developer for such testing, because it is less affected by TLC parameters than developing solvents containing formic or acetic acid, or 3-component mixtures such as benzene-ethanol-water or ether-methanol-water. The parameters having the greatest effect on TLC quality include: adsorbent properties, layer thickness, presence or absence of $CaSO_4$ binder, moisture content, presence of air contaminants, and vapor phase composition in the developing chamber. The size and shape of the chamber and whether or not the chamber is equilibrated are critical. Development is faster but resolution is poorer in an equilibrated chamber when volatile solvents are used.

Only a few commercial silica gel adsorbents are suitable for good TLC of aflatoxins; these include Adsorbosil-1 or -5 (Applied Science Laboratories), MN GHR (Macherey, Nagel & Co., Duren, West Germany), SilicAR 4G or 7G (Mallinckrodt Chemical Works), silica gel D-5 (Camag), and Bio-Sil A (Bio-Rad Laboratories). Most of these adsorbents contain $CaSO_4$ as a binder. Commercial precoated plates, suitable for aflatoxin analysis, are presently available from several sources. They are more expensive than those prepared in the laboratory but have the advantages of saving labor and having greater physical hardness and thickness uniformity. Plates prepared on clean glass with a pure water-silica slurry 0.25–0.5 mm thick are partially dried at room temperature for 30 min and then fully dried at 80–100 °C for 1–2 hr; they may also be dried at room

temperature if the relative humidity is less than 60%. A silica gel layer moisture content of 15–25% is desirable. The plates must be protected during drying and storage from contamination with lint, dust, and volatile chemicals, particularly acidic or basic vapors.

For application to the TLC plate, the extract is dissolved in the least polar, volatile solvent in which it is soluble. The standard should be dissolved in the same solvent as the sample extract, and each TLC plate must be spotted with its own standards (aflatoxin spots on different TLC plates are not comparable). Although some procedures allow for the dissolution of extracts in small volumes, down to a few μL, dispensed with a microsyringe, this is not desirable and can cause difficulty. Small vessels must be used; great care must be taken to avoid evaporation. The indeterminate dilution error contributed by improper control over extract volume can become a significant source of error.

The extract is most conveniently applied to the TLC plate with a microsyringe; however, Drumond disposable Microcaps, Mitchell pipets or disposable tip pipeters may also be used. The solution should be applied without damaging the silica layer. For colorless solutions, the plate may be scored with channels and a template used to mark the location of the spots.

When volatile organic spotting solvents are used, 2 μL should be spotted for good reproducibility. The spotting should be done under incandescent light; the standards are spotted last to minimize decomposition. After development, the plate is dried in the dark at room temperature for about 5 min; estimates are then made by visual or densitometric comparison of the fluorescent spots. The visual technique is widely practiced because it is rapid and requires no expensive equipment. AOAC methods direct application of a series of standards and sample extract spots differing in quantity by increments of about 20% (for example, 3.5, 5, and 6.5 ng). This was determined to be the smallest difference visually detectable, and is therefore the sensitivity limit of the method. About 0.5 ng aflatoxin B_1 or G_1 is the minimum amount of pure standard that is clearly visible by fluorescence on a TLC plate; larger amounts are needed for visualization in the presence of sample extract background.

In general practice a standard solution consists of a mixture of aflatoxins B_1, B_2, G_1, and G_2 in the ratio 1:0.2:1:0.2 μg/mL. This compensates for the difference in fluorescence among the compounds and approximates the ratio often encountered in natural products. Individual analysts develop their own preferred standard series. For screening, time is saved by spotting the sample extract in duplicate, one spot with and the other without internal standard, and by spotting the standards to cover a range of 2–15 ng/spot. If only a single sample is to be spotted, a wide range of sample spots can also be spotted so that several comparisons of standard and sample spots can be made.

The reproducibility of results from visual comparisons approaches a minimum of 20%. Published coefficients of variation from collaborative studies vary between 15 and 70%, with a pooled average of 30%. Fluorodensitometric quantitation of thin layer chromatograms is more objective, more precise (coefficients of variation of ±5–15%), and more sensitive (differences approaching 2% are detectable). The accuracy and limit of detection are the same as for visual comparison. Most commercially available filter and spectrofluorodensitometers can be used. A few of the slit type instruments are listed in the AOAC *Official Methods of Analysis*. There are reports from Europe that the so-called flying spot TLC scanner is also suitable for aflatoxin quantitation.

For densitometric measurements the following are required: 365 nm excitation light and a filter in front of the detector to screen out light with wavelengths less than 400 nm. The emitted light can be measured as reflected from the top of the plate or transmitted through the silica layer. The plate is often divided into 1 cm channels by scoring the silica gel layer. The instrument is calibrated with standards in the range of interest (1–100 μg/spot) and a standard curve passing through the origin is constructed. The highest concentration standard can be scanned first and again after all the other spots have been scanned to detect possible changes which may have resulted from fading or evaporation of residual solvent. (The fluorescent intensity depends on adsorbent, moisture content, and amount and nature of the solvent.) If fading of the spots is

observed, the plate should be dried at 50 °C immediately after development and the silica gel covered with clean glass while still hot, using masking tape along the edges between the plates as protective spacers.

Once linearity and stability are established, extracts and standard are spotted in duplicate as described in the AOAC cottonseed procedure. The aflatoxin concentrations of standards and sample are related to peak areas, integrated mechanically or electronically. Peak areas from duplicate spots should agree within 5% and are averaged. Peak heights can not be used for quantitation.

VII. Confirmation of Identity

There have been more false reports concerning the occurrence of aflatoxins in foodstuffs than most analysts would like to admit. The importance, therefore, of positive confirmation of identity of a suspect contaminant can hardly be overemphasized. This is particularly true for regulatory samples, and for commodities with a limited history of analysis or incidence of contamination.

The detection of aflatoxins in a sample on the basis of their absorbance, fluorescence, or chromatographic properties is not specific. Many UV-absorbing or fluorescent compounds have Rf values or retention times similar to the aflatoxins. Rechromatography with different solvents and adsorbents adds corroborative but not conclusive evidence of positive identity. This type of evidence can be conclusive only in a negative sense; i.e., it can show that an unknown spot is not aflatoxin.

The preparation of chemical derivatives with changed chromatographic properties is the simplest method for confirmation. A widely used test for aflatoxins B_1, G_1, or M_1 is the formation of a water addition product using trifluoroacetic acid as the catalyst. TLC or HPLC after derivative preparation gives different Rf values or retention times (for the water adducts) for comparison with those of authentic standards. In the first AOAC official method for confirmation of identity, the suspect aflatoxins were isolated from the sample extracts for derivatization, using preparatory TLC. The presently preferred method forms the derivative on the TLC plate by application of trifluoroacetic acid to the sample and standard origin spots. The plate containing both treated and untreated spots is developed and observed under UV light for formation of the expected derivatives.

For regulatory purposes the FDA for the past 10 years has required additional verification of identity of aflatoxin B_1 by testing for biological activity or toxicity in fertile chicken eggs. Enough toxin is isolated and purified to inject 10–20 eggs. In positive tests failure to hatch is observed after 20 days of incubation. If the proper doses are injected, failure of the embryo to develop can be seen after 4 days. Over the years this test has been applied to more than 400 samples with 100% correlation with chemical tests.

Spray reagents have also been used for confirmation. All of these spray tests are non specific and some require larger amounts of the toxin than is isolated by most analytical procedures. One of the widely used sprays is 50% sulfuric acid. This changes the fluorescence of aflatoxin from blue to yellow. Since a great many other compounds undergo the same change, the major value of this test is for negative confirmation.

The most specific technique for confirmation of identity is mass spectrometry. Recent technological improvements have resulted in greatly lowered limits of detection (down to fractions of a nanogram of aflatoxin B_1 from crude extracts). A major drawback is the very expensive and generally unavailable equipment that is needed.

VIII. Laboratory Safety

Aflatoxin B_1 is a potent carcinogen in some experimental animals. Therefore, the question of safety in the laboratory frequently arises. Dry, crystalline aflatoxin must be handled in a

confined space such as a glove box. Because it acquires a static charge, it is difficult to handle, and disperses into the environment and clings to nonconductive surfaces.

Vessels containing aflatoxin solutions should be kept in pans or beakers so that if containers are spilled or broken the solution is confined. Spilled toxin can be destroyed and cleaned up by application of sodium hypochlorite. TLC plates, ordinarily containing only ng quantities of aflatoxins, can be washed clean with alkaline soap and water.

The inhalation of organic solvent vapors such as benzene or chloroform, or silica dust, or unknown sample dust during grinding is a hazard and must be avoided. Work areas may be covered with disposable materials such as plastic backed paper which can be easily rolled and disposed of by burning at the end of the day or of the analysis.

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Whitaker, T. B., Whitten, M. E., and Monroe, R. J. (1976) Variability associated with testing cottonseed for aflatoxin, *J. Am. Oil Chem. Soc.* **53,** 502-505.

TABLE 1. *Sources of Mycotoxins Standards*

1. Aldrich Chemical Co., 940 St. Paul Avenue, W. Milwaukee, WI 53233
   - Aflatoxins, B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin, Patulin, Penicillic Acid, Diacetoxyscirpenol
2. Applied Science Laboratories, Inc., P.O. Box 440, State College, PA 16801
   - Aflatoxins B_1, B_2, G_1, G_2, M_1 (Reference standards)
3. Dr. M. Bachman, Commercial Solvents Corp., Terre Haute, IN 47808
   - Zearalenone
4. Dr. H. Burmeister, USDA/ARS, 1815 N. University St., Peoria, IL 61404
   - T-2 Toxin
5. Calbiochem, 10933 N. Torrey Pines, LaJolla, CA 92037
   - Aflatoxins B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin, Patulin, Rubratoxin B, Diacetoxyscirpenol
6. Eureka Labs., Inc., 401 N. 16th Street, Sacramento, CA 95184
   - Aflatoxin M_1
7. ICN-K&K Labs, 121 Express Street, Plainview, NY 11803
   - Aflatoxin B_1, B_2, G_1, G_2, Patulin, Diacetoxyscirpenol
8. Dr. L. Leistner, Federal Meat Research Institute, 865 Kulmbach, Germany, for European distribution only
   - Aflatoxin M (Reference standards only)
9. Makor Chemicals Ltd., Box 6570, Jerusalem, Israel
   - Aflatoxin B_1, B_2, G_1, G_2, ^{14}C-B_1, M_1, Ochratoxin A, Sterigmatocystin, Patulin, Penicillic Acid, Rubratoxin B, Diacetoxyscirpenol, Roridin A, T-2 Toxin, Verrucarin A, Zearalenone
10. Moravek Biochemicals, 15302 E. Proctor Avenue, City of Industry, CA 91745
    - ^{14}C and ^{3}H-labelled Aflatoxin, Patulin
11. Myco Lab Co., P.O. Box 321, Chesterfield, MO 63017, c/o Mr. Robert Sankey
    - Aflatoxins B_1, B_2, G_1, G_2, Zearalenone, T-2 Toxin
    - Diacetoxyscirpenol, Vomitoxin
12. Dr. A. E. Pohland, Food and Drug Adm., 200 C Street SW, Washington, D.C. 20204 (Reference standards only only)
    - Aflatoxins B_1, B_2, G_1, G_2, Ochratoxins A, B, B Ethyl ester, Sterigmatocystin, Patulin
    - Penicillic Acid, Zearalenone, T-2 Toxin, Citrinin
13. RFR Corp., 1 Main Street, Hope, RI 02831
    - Aflatoxins B_1, B_2, G_1, G_2, Patulin, Penicillic Acid, Sterigmatocystin
14. Rijksinstitut voor de Volksgezondheid, P.O. Box 1, Bilthoven, The Netherlands
    - Aflatoxins B_1, (Reference standards only)
15. C. Roth, Postfach 1387, 7500 Karlsruhe 1, W. Germany
    - Aflatoxins B_1, B_2, G_1, G_2
16. Senn Chemicals, CH-8157, Dieseldorf, Switzerland
    - Aflatoxins, B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin
17. Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178
    - Aflatoxins B_1, B_2, G_1, G_2, Diacetoxyscirpenol, Ochratoxin A, Phalloidin, Patulin, Penicillic Acid,Rubratoxin B, Sterigmatocystin
18. Mr. R. M. Stubblefield, USDA/ARS, 1815 N. University St., Peoria, IL 61604
    - Aflatoxin M_1 and M_2
19. Supelco, Inc., Supelco Park, Bellefonte, PA 16823
    - Aflatoxins, B_1, B_2, G_1, G_2
20. Wale Corp., P.O. Box 27174, 1975 Ocean Ave., San Francisco, CA 94127 c/o Dr. Wallen Lee
    - Radiolabeled aflatoxins B_1, B_2, G_1, G_2, Aflatoxins Q_1, B_{2a}, Sterigmatocystin, Ochratoxin A, Patulin, Zearalenone, Penicillic Acid, Rubratoxin B, Diacetoxyscirpenol

Section III. CLINICAL ANALYSIS

NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

TRACE ORGANIC SAMPLING IN THE CLINICAL AREA

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Since the blood volume of a newborn is only about 270 mL and the clinical state of a sick infant may have to be monitored several times a day it is essential that the quantity of blood used for clinical analysis be kept to a minimum (preferably <10 μL per test). The collection of blood by venipunctures is practical in older children, but heel or finger punctures are the most efficient and least traumatic for obtaining blood from infants. Heel pricks must be performed carefully and a free flow of blood is essential to avoid contamination with tissue fluid and to prevent hemolysis of the specimen. Variability attributable to collection factors may be minimized by reducing the number of individuals who are allowed to collect blood. At present, there is no mechanical device that can ensure reproducibility of specimen collection. Sample identification can also cause problems as it is impossible to attach a label directly to a capillary blood tube. A convenient way of manipulating, transporting, storing, and identifying blood samples from newborns is to spot the blood onto filter paper (Guthrie blotters). The dried blood spots can then be punched out to deliver a fixed volume (5.5 μL) for clinical analysis. It is possible to selectively remove metabolites such as the fatty acids, cholesterol, and the amino acids or drugs from the filter paper by a sequential solvent extraction. These extracts can then be analyzed by conventional means, or more conveniently by a fast and more sensitive direct mass spectrometric technique. The new method has already been applied successfully, to confirm abnormals from a genetically oriented screening program using the duplicate blood spots from Guthrie blotters.

Key words: Blood samples; clinical sampling of infants; clinical trace organic sampling; Guthrie blotters.

The first quantitative measurement of an organic constituent of blood plasma was done by Garrod in 1851, who showed that patients with gout had higher levels of uric acid than normal persons. It had to wait till the 20th century for blood analyses to really multiply. The delay is partly accountable by the large volumes of blood which were required for all but a few clinical methods and the difficulties which were experienced with the collection of such large samples. Before the hypodermic syringe became widely available the sampling of blood required a cut down to expose the vein, venisection and cupping. A major impetus to microchemical analyses came in the 1930's from the Linderstorm-Lang and Holter who developed equipment methods for the measurement of enzymes. Nowadays the use of new methodology, instrumentation and automation have drastically reduced the sample size and the time required for each analysis. However, it is important to continue these refinements until a drop or two of blood is sufficient for a complete chemical analysis. The increased precision and accuracy of clinical analyses has led to a growing recognition that variability in the collection and handling of blood specimens may lead to changes in test values. As a consequence the interpretation of analytical results may sometimes be difficult. The National Committee for Clinical Laboratory Standards has now published a standard procedure (PSH-3) which should be followed by anyone obtaining blood by venipuncture. The collection of blood specimens from children, especially newborns, is best done by skin punctures. Heel or finger punctures are the most efficient and least traumatic procedure for obtaining blood from infants. Other acceptable but less desirable sites for obtaining capillary blood are the great toe and the earlobe. Before the blood is collected the heel should be warmed to increase blood flow and the blood should be allowed to drip freely into a glass collection tube. During collection of the specimen the skin surface should not come into contact with the tube to avoid hemolysis. Variability may be minimized by following the standard method for skin puncture specimen collection (PSH-4) and by limiting the number of individuals who are allowed to collect blood.

Precautions should be taken to minimize evaporation of specimen and the samples should be stored on ice until centrifuged. Sample identification can also cause problems as it is impossible to attach a label directly to a capillary blood tube. The precision of pipetting a sample must be well controlled, less than 1% is desirable and the laboratory instruments must be capable of performing satisfactorily with a small volume of sample (as little as 10 μL). An alternative way of storing and transporting whole blood samples is to spot them onto filter paper cards (Fig. 1).

This is done by placing the paper blotter on the skin puncture and allowing the blood to soak through the filter paper to fill completely a marked circle of approximately 20 μL capacity. The dried blood can then be punched out to deliver a fixed volume for analysis. Such blood spot specimens should be stored in a dessicator, and under these conditions the sample will keep satisfactorily for many months. Filter paper is an ideal medium for transporting blood samples to a central analytical facility. Blood filter discs are already used for the screening of newborns for inborn errors of metabolism by the Guthrie microbial inhibition assays, but no chemical analysis of blood spots has been published. Our work has shown that dried filter paper samples are quite homogeneous, i.e., no chromatographic fractionation of the sample occurs on the paper, and that the blood proteins are denatured and firmly held on the cellulose. As a result we find that many blood constituents can be removed from the paper by a brief sonication of the sample with an organic solvent. By the use of a sequential extraction with different solvents it is possible to selectively remove many low molecular weight compounds from the paper. Although some of these extracts contain some higher molecular weight impurities it is possible to analyze for some blood constituents by published microtechniques. Since one of our interests is the screening of the newborn population for inborn errors of metabolism, we tried a metabolic profile analysis on the extracts. Our initial attempts to carry out a quantitative amino acid analysis by gas or ion exchange chromatography were not successful, but we have found that chemical ionization mass spectrometry without prior chromatographic separation is sufficiently sensitive for this purpose. This is done by converting the amino acids contained in an 80% ethanol extract to the volatile N-acetyl amino acid methyl ester derivatives by a 5 min treatment with acetic anhydride and methanol at 80 °C. The chemical ionization mass spectra of these derivatives are dominated by large protonated molecular ion peaks and unique m/e values are obtained for all amino acids with the exception of leucine and isoleucine, which can not be distinguished by this method (Table 1).

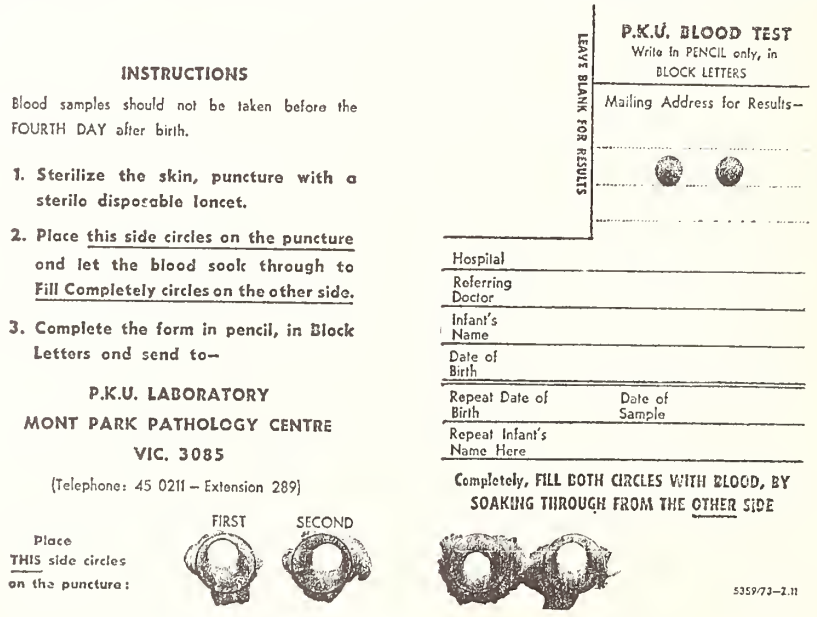

Figure 1. Guthrie microbial inhibition assay blotter.

TABLE 1. *Mass spectral data for N-acetyl amino acid methyl esters*

Amino acid	$(M+1)^+$
Glycine	132
Alanine	146
Serine	162
Proline	172
Valine	174
Threonine	176
Leucine	188
Isoleucine	188
Asparagine	189
Glutamine	203
Aspartic acid	204
Methionine	206
Histidine	212
Glutamic acid	218
Cysteine	220
Phenylalanine	222
Lysine	245
Tryptophan	261
Tyrosine	280

A quantitative analysis can be made by the addition of known amounts of isotopically labelled amino acid standards to the extract and comparing the intensities of the protonated molecular ions of the endogenous amino acids with that of the corresponding standards. In practice we have found it more convenient to prepare filter paper discs containing known amounts of the stable isotopically labelled standards and adding these prior to the solvent extraction step. Calibration curves for some selected amino acids have been determined (Fig. 2), and the slopes of the calibration lines were used to calculate the calibration factors which correct the results for the chemical and isotopic purity of the internal standards. Blood spot samples from patients suffering from inborn errors of amino acid metabolism have been analyzed by the new technique and the concentration of the amino acids have been determined. The mass spectral profiles obtained from a normal control and from patients suffering from phenylketonuria (PKU), maple syrup urine disease (MSU) and hyperglycinemia are shown in Figure 3.

The method has been checked by analyzing 20 PKU blood samples by both mass spectrometry and ion exchange chromatography (Table 2). The good reproducibility and the excellent agreement between the two analytical procedures suggests that the new technique accurately determines the free amino acid content. Under routine operating conditions it is possible to quantitise 25 ng of an amino acid, whilst still maintaining a signal-to-noise ratio better than 10:1.

Other low molecular weight blood constituent such as cholesterol and the fatty acids which are also present in the alcohol extracts, can be detected in the mass spectra, but do not interfere with the quantitative amino acid analyses. The new technique is being used as a follow-up test to a positive Guthrie screening result. An analysis takes about 15 min and requires a 4 mm diameter filter paper disc containing 5.5 μL of blood and an extraction with 200 μL of 80% ethanol.

Quantitative mass spectral analyses of free and esterified cholesterol and of fatty acids for use on microsamples of dried blood have also been developed. Again isotope determination was employed as the quantitating technique using deuterium labelled cholesterol and its palmitate ester as internal standards. The relative standard deviation of the method was shown to be less than 5%. Total blood cholesterol values obtained by the new method were compared with results obtained from the corresponding Calbiochem enzymatic assays (Table 3).

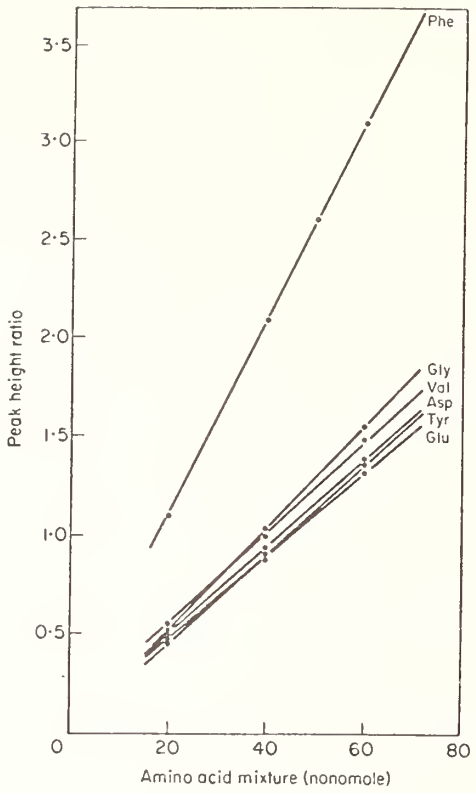

Figure 2. Calibration curves obtained from an amino acid mixture.

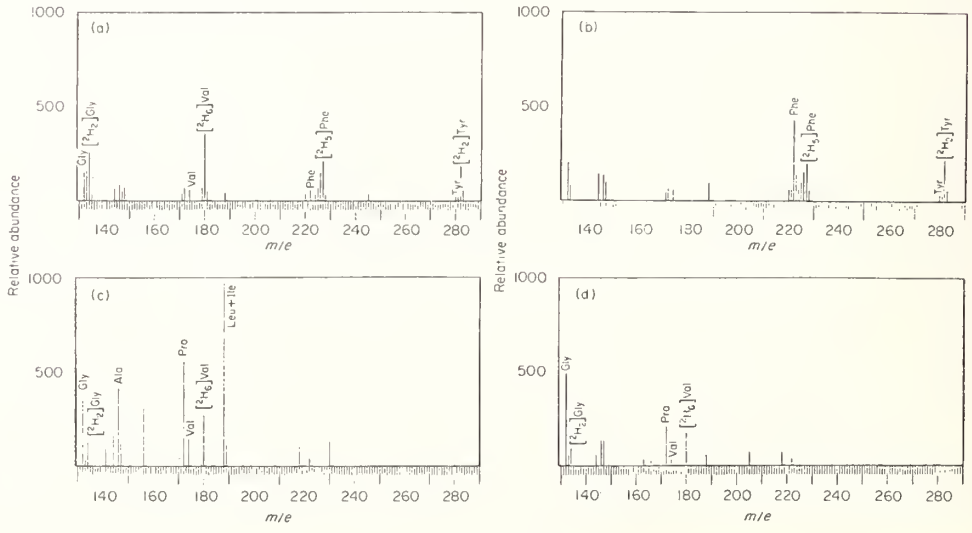

Figure 3. The c.i. (isobutane) mass spectral profiles of blood spots obtained from (a) normal control and (b), (c), (d) patients suffering from phenylketonuria, maple syrup urine disease and hyperglycinemia, respectively.

TABLE 2. *Phenylalanine analyses of phenylketonuric blood and plasma by mass spectrometry and ion exchange chromatography*

Patient	Mass spectrometry (blood spot) concentration mg%	Means mg%	Ion exchange (plasma) mean mg%
1	18.24		
	18.10		
	18.68	18.48±0.30	18.81
	18.53		
	18.83		
2	20.89		
	20.60		
	20.45	20.60±0.23	20.30
	20.74		
	20.30		
3	14.86		
	15.16		
	15.01	14.98±0.28	15.80
	14.57		
	15.30		
4	12.80		
	13.39		
	13.09	13.09±0.23	13.04
	12.95		
	13.24		

TABLE 3. *Comparison of the M.S. method and the Calbiochem enzymatic method for determination of total cholesterol*

Sample	Mass spectrometry (mg%)	Calbiochem enzymatic method (mg%)
1	219.2, 222.3	222.8
2	238.5, 236.1	242.1
3	240.5, 238.6	243.7
4	100.1, 101.7	99.9
5	224.1, 226.5	227.8
6	243.6, 246.3	252.5

The method has also been used successfully for the simultaneous quantitation of the five anticonvulsants diphenylhydantoin, mephobarbital, carbamazepine, phenobarbital and primidone in serum. Only a single extraction is necessary and chemical modification of the sample is not required. In all these applications data generated by the mass spectrometer is processed in the following manner. After each scan the ion intensity at each mass value is stored by the computer. At the completion of the run, the ratio of the intensities of the mass value of the compound and its internal standard are calculated for each spectrum where this compound is present in significant amounts. In practice this is determined as a series of ratios for spectra, where the ion intensities exceed a preset threshold value. From these results the average ratio is determined together with the standard deviation and the coefficient of variation (Fig. 4). It is also possible to incorporate the

Den. Int.	Num. Int.	Spect. No.	Ratio
489	942	5	+.1926380E+01
546	996	6	+.1824176E+01
634	1209	7	+.1906940E+01
786	1500	8	+.1908397E+01
1003	1951	9	+.1945164E+01
1156	2046	10	+.1769896E+01
1231	2349	11	+.1908205E+01
1215	2515	12	+.2069959E+01
1119	2243	13	+.2004468E+01
908	1802	14	+.1984581E+01
651	1280	15	+.1966206E+01
381	780	16	+.2047244E+01

Mean = +.1938468E+01
SD = +.8573669E-01
SD% = +4.4229

Figure 4. Aspartic acid assay.

Den. Int.	Num. Int.	Spect. No.	Ratio
244	922	10	+.3778688E+01
325	1232	11	+.3790769E+01
445	1581	12	+.3552809E+01
488	1836	13	+.3762295E+01
559	2080	14	+.3720930E+01
497	1910	15	+.3843058E+01
364	1395	16	+.3832417E+01
211	845	17	+.4004739E+01

Mean = +.385713E+01
SD = +.1267768E+00
CV% = +3.3488

Conc phenobarbital umoles/l = +120.2296

Figure 5. Serum phenobarbital assay.

slope and intercept values calculated from the standard curves into the software to enable calculations of the concentrations of the compounds present (Fig. 5). The accuracy and statistical reliability of the method could be further increased by employing a fast-scanning mass spectrometer in the selected-ion detection mode, since many more scans can be generated in the same period of time. For example, in a recent trial a 2 μL sample of a serum extract containing phenobarbital and its 1,3[^{15}N] labelled internal standard were injected into the ion source of a quadrupole mass spectrometer. The machine was operated in the selected ion detection mode and

the ion intensities at m/e 233 and 235 were recorded. Under these conditions it was possible to make 75 measurements in 5 seconds with a coefficient of variation of $<5\%$.

It is highly desirable that blood spot analyses should be automated as fully as possible so that they could be carried out on a rapid routine basis. The degree of simplification introduced by the use of direct mass spectroscopy facilitates this. We are working on an automatic injection system for the mass spectrometer and we are producing an interactive software package which will enable users to collect and completely process data for each specific application with a minimum of user intervention.

NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

SAMPLE PREPARATION FOR CLINICAL TRACE ORGANIC ANALYSIS

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An overview is presented of some common error sources in analytical methods and of knowledge which has contributed to the design of more reliable drug assays. Factors related to the initial state of the biological specimen, which can cause significant interferences and errors in measurements, are reviewed. The principle of the distribution law is utilized to explain the efficient extraction of acidic and basic drugs from media in which the drugs are largely in the ionic form. Extraction schemes which eliminate interferences by fatty acids in gas-liquid chromatographic (GLC) methods, are discussed. The internal standard method in GLC assays is reviewed. Ground rules for the selection of an internal standard and the need for selecting multiple, appropriate internal standards for the simultaneous determination of multiple drugs by GLC are also reviewed. Some factors important to the design and practice of *intra*-laboratory quality control of a drug assay are explained.

Key words: Clinical chemistry; drug analysis; gas chromatography; internal standards; methylation; sample preparation.

I. Introduction

An awareness of common error sources in analytical methodologies becomes germane in the challenge to develop more reliable drug assays. The objective of this discussion is to present an overview of the error sources and of present knowledge which has contributed to the design of more reliable drug assays. The discussion is directed towards the use of gas-liquid chromatography (GLC) and its applications to antiepileptic drug determinations, primarily because GLC is widely employed in drug determinations and because the art of monitoring the antiepileptic drugs by GLC has received considerable refinement during the past 15 years. Many of the analytical problems now recognized in antiepileptic drug determinations may be anticipated to appear in other drug assays, as the art of drug monitoring continues to develop as a guide in the practice of medicine.

The methodologies for routine laboratory monitoring of the antiepileptic drugs include spectrophotometry, thin-layer chromatography (TLC), gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC), radioimmunoassay (RIA), and the enzyme multiple immunoassay technique (EMIT). GLC remains as the most widely used methodology [1–3], presumably because of its potential for specificity, accuracy, rapidity, flexibility, and cost-effectiveness. However, it is abundantly clear, despite persuasive manufacturers' claims, that no fail-safe methodology exists for the routine monitoring of these drugs. All of the methodologies are subject to inherent instrumental and methodological error sources, amplifiable by inadvertent human errors [4].

II. Sample Condition

Lipids, bilirubin, hemoglobin, and drugs other than those being quantitated cause significant problems in spectrophotometric and chromatographic assays and immunoassays [4–6].

Plasma protein, if not held below the tolerable limit, can affect the RIA of phenytoin [7]. Even plasma specimens with normal appearances sometimes present difficulty in the EMIT system [8].

Extensive hemolysis of a blood specimen results in observation of a significantly low phenytoin level [9–11]. Phenytoin is extensively bound to plasma protein, resulting in a grossly unequal distribution between plasma and the erythrocytes [11–13].

Blood separating devices, ingenious commercial products that enable the manual or centrifugal compression of the formed elements or clot, are acceptable in the preparation of the blood specimen for some routine chemistry tests [14,15], but normally these devices are viewed as a plague to GLC-drug determinations. Gas chromatograms of blood samples prepared with these devices have a multitude of unidentifiable peaks, sufficient grounds for rejection of any sample.

A new issue is whether the collection of plasma or of serum be the appropriate specimen for drug assays. Differences between plasma and serum, which are of possible clinical significance, have been noted for some of the routine chemistry tests, notably calcium, glucose, inorganic phosphorus, potassium and total protein [16–19]. I am unaware of any studies of the quantitative differences between the use of serum and of plasma in drug assays, except for our own preliminary experiments. Our laboratory has studied the difference between phenytoin concentrations in plasma and serum samples from the same individual in a population of patients with epilepsy.

Figure 1 shows a comparison of two sets of calibration data for the determination of phenytoin by a gas chromatographic on-column methylation technique (OCMT) [20]. The calibration data were obtained in independent experiments with two series of calibration samples, one series prepared by addition of phenytoin to serum, the other series by addition of phenytoin to plasma [20,21]. Control serum and plasma were prepared from blood of the same individual (non-epileptic). Figure 1 supports the conclusion that the constituents of plasma and serum have no

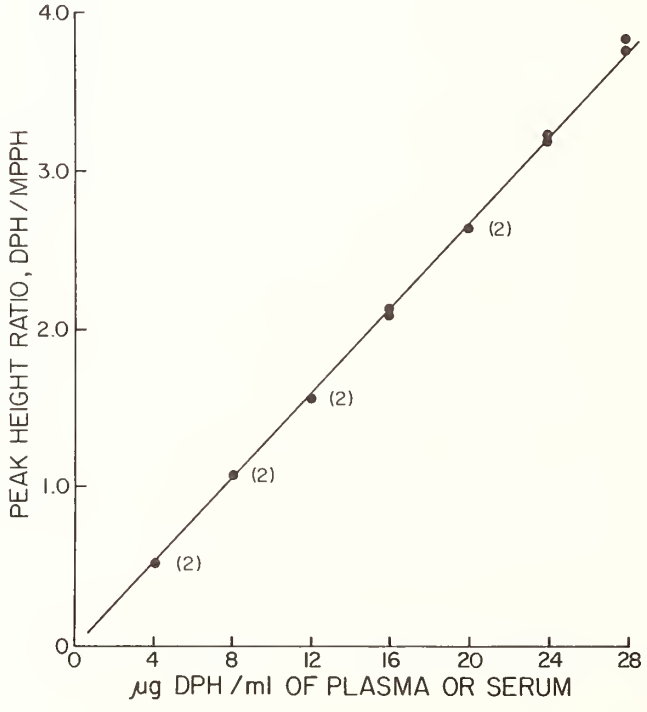

Figure 1. Calibration curve for the determination of phenytoin concentration (DPH) in serum or in plasma by use of a gas chromatographic on-column methylation technique (OCMT) [20]. (5-phenyl-5-(*p*-tolyl)hydantoin (MPPH) was the internal standard. Peak height ratios are plotted for both a plasma and serum series of calibration samples, the "(2)" indicating that the plasma and serum peak height ratio (DPH/MPPH) values were essentially the same at the concentration of DPH specified on the abscissa.)

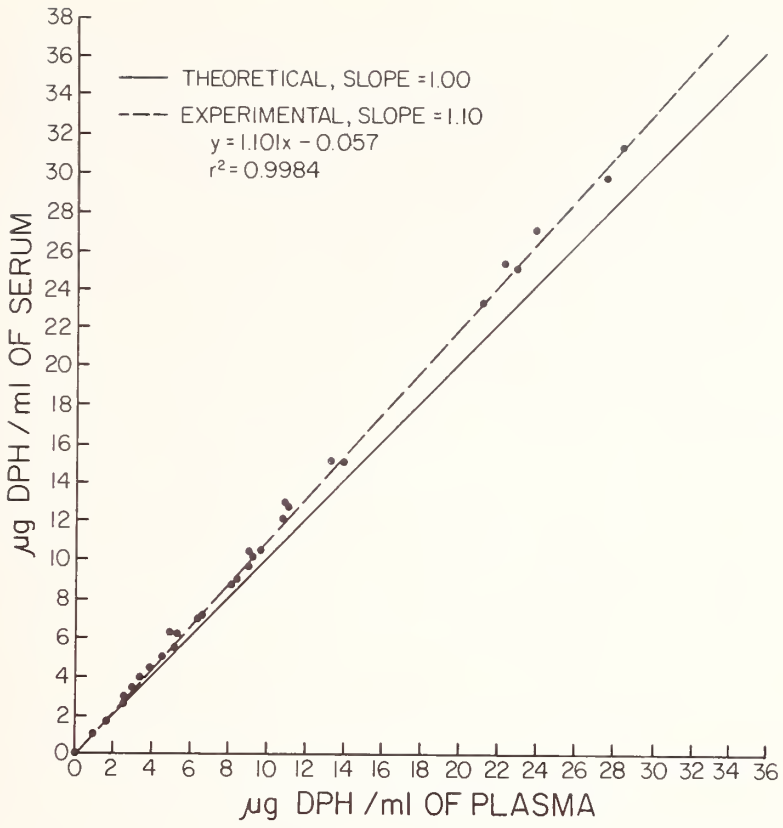

Figure 2. Comparison of plasma and serum concentrations of phenytoin in a population of patients with epilepsy. (Each point represents a different patient from which a plasma and serum sample were obtained simultaneously at the time the patient appeared in the clinic.)

effect on the determination of phenytoin by the OCMT [20] used in these studies. Figure 2 shows a comparison of the phenytoin levels in serum and in plasma, as obtained from 31 different epileptic patients, 19 of which appeared in the clinic on a given day and the remainder of which appeared in the clinic 4 weeks later. Double blood specimens were drawn from each patient, a "lavender top" EDTA vacutainer[1] tube being used for the plasma preparation and a "red top" vacutainer[2] being used for the serum preparation. Plasma and serum, respectively prepared by centrifugation and clotting, were transferred to clean tubes within 1 hour after collection. Phenytoin determinations were performed within 24 hours by a technician who randomly selected samples without knowledge of the objective of the experiment. The results in Figure 2 indicate that a serum phenytoin level exceeds a plasma phenytoin level to the extent of about 10%.

Essentially the same result has been found for phenobarbital, its level being 10% greater in serum than in plasma (24 patients).

Although one may argue that a 10% difference is clinically insignificant in the monitoring of antiepileptic agents, it may not be denied that a 10% difference, when coupled to analytical errors in the same direction, could have impact upon clinical judgement of a patient's drug management program [22]. If this relationship of a 10% difference between serum and plasma concentration should be found valid for drugs in general, it may be of interest to establish new criteria for the choice of the appropriate specimen in the monitoring of drugs with extremely narrow therapeutic ranges.

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[1] Vacutainer®, 7 mL lavender top, EDTA, liquid (K3), 0.07 mL 15% solution, #4675, Becton, Dickinson and Company.

[2] Vacutainer®, 7 mL red top, plain tube, silicone coated, #4736, Becton, Dickinson and Company.

III. The Extraction Scheme in GLC

The extraction scheme is designed for the primary purposes of recovery of drug(s) from the biological fluid and elimination of interfering materials. If the intention is to recover multiple drugs from a single specimen, as is often done in the determinations of antiepileptic drugs by GLC, sacrificial reduction in recovery yields of one or more drugs may be required in order to satisfy the conditions needed for elimination of interfering materials. The demand for recovery of a solute is not so great in GLC as in spectrophotometry. A 34% recovery of primidone is adequate for its accurate determination by an on-column methylation technique designed for multiple drugs, when care is taken to eliminate all interferences and to use an internal standard which presumably has similar partitioning behavior in the extraction scheme [20,23].

The partition ratio, K′, is a constant that defines the distribution of a solute between two immiscible phases. The partition ratio is a useful tool for designing an efficient extraction scheme for drug recovery [24]. Knowledge of a K′ value of the undissociated form of a weakly acidic or basic drug and of the pK'_a value of the drug permits an approximation of the percentage recovery of a drug as a function of pH. Some drugs can be recovered by organic solvent extraction from an aqueous medium in which the drug is largely ionized. For example, phenobarbital is ionized to the extent of about 98% in a buffer of pH 8.80. About 80% of the initial amount of phenobarbital in the pH 8.80 buffer can be recovered by extraction with a three-fold volume of ether [24].

It is now appreciated that the presence of lipids can be devastating to the reliability of GLC-methods for the antiepileptic drugs, unless preventive measures are taken [8]. A simple cleanup extraction step, which eliminates lipids, involves partitioning of a residue from a plasma extract between hexane and methanolic hydrochloric acid solution [25,26]. Any lipids which may be present in the residue distribute into the hexane phase and the drugs distribute into the methanolic hydrochloric acid phase. This latter phase can be separated and further processed for quantitation of the drugs. A hexane/aqueous methanol system has been used for the same purpose [27]. These methods appear well-suited to general applicability in most GLC methods in which lipids have not been removed prior to the quantitation step, provided that a drug and its internal standard have a low hexane/methanolic HCl, or hexane/aqueous methanol, partition ratio. An on-column methylation technique (OCMT), a derivatization technique in which drugs are partitioned between an organic solvent and a methanolic solution of an alkaline methylating agent [20,28], does not normally require special effort for removal of lipids. Apparently the partition ratios of endogenous lipids are of such nature that, even with the most severe lipemic plasma specimens, essentially all lipids remain in the organic phase in the final step of the OCMT.

IV. Internal Standard Method in GLC

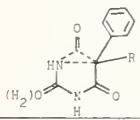
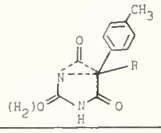
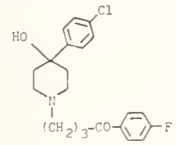
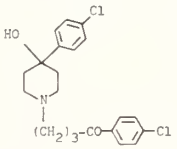
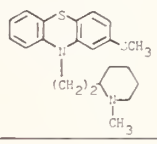
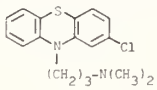
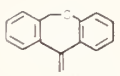
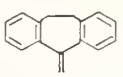
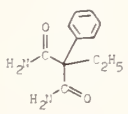
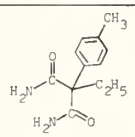
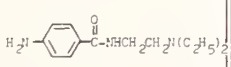
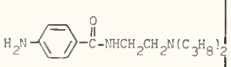
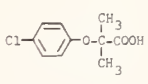
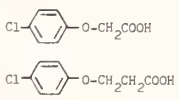
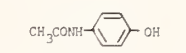
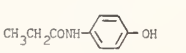
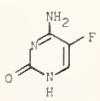
In the internal standard method [29–33] observation is made of the response parameters (peak heights or areas) of the internal standard and the solute. Quantitation of an unknown sample is made by calculation of the ratio of the peak heights (areas) observed for the component and internal standard, and this ratio is transformed into an amount, or concentration, of the unknown component by reference to a calibration curve. The calibration curve is constructed by plotting known ratios (y-axis) against known amounts, or concentrations, of the component (x-axis). The measurement of a ratio of response parameters is, in essence, a compensatory treatment by which the effects of common error sources in GLC are eliminated or, at least, minimized. The premise of the internal standard method is that both the solute and internal standard will experience the effect of an error source to essentially the same proportional extent.

The selection process of an internal standard should include consideration of all aspects of the gas chromatographic method, so as tc permit selection of an appropriate compound that mimics closely the properties and disposition of the solute itself. An appropriate internal standard may be viewed, therefore, as a substance which has essentially the same chemical structure as the solute, with the only modification being the removal or addition of a small, chemically

insignificant structural feature which provides the desirable gas chromatographic properties (slightly different retention time and same peak shape and response characteristics). The structural modification should be at a site in the molecule that affects minimally chemical properties (ionization, derivatization) and solute-solvent interactions (extraction).

Table 1 shows some internal standards with structures closely related to the drugs or metabolites for which their uses were designed. More comprehensive lists of such examples are available [34]. Because of the lack of appropriate non-drug candidates, a common practice has been to select as internal standards structurally related drugs from within a drug class and to

TABLE 1. *Summary of some internal standards with structures closely analogous to drugs and their metabolites*

Drugs		Internal Standards		Reference
Generic Name	Structure	Drug (Analog)	Structure	
Phenytoin Phenobarbital Primidone		para-Methyl analogs		20
Haloperidol		Chloro analog		42
Thioridazine		Chorpromazine		43
Dothiepin (and metabolites)		Amitriptyline		44
Phenylethyl- malonamide (primidone metabolite)		para-Methyl analog		45, 46
Procainamide		Bis(N-propyl) analog		47
CPIB (Clofibrate metabolite)		CPAA CPPA		48
Acetominophen		N-Propionyl analog		49
5-Fluorocytosine		Cytosine		50

manipulate internal standard solutions to suit the needs of the assay. In patient monitoring, however, this practice is valid only so long as the laboratory has access to complete patient history of drug therapy. An appreciation of the pharmacokinetic properties of the drugs and of the principles of drug elimination are also required, for it is an obvious error to use as an internal standard any drug which a patient had received and for which ample time had not been given for complete disappearance from the body. It is also an obvious error to select a drug analog as an internal standard if there is evidence or even suspicion that the analog is an *in vivo* metabolite of the drug to be quantitated. An example is the rejection of the *meta*-hydroxy derivative of phenytoin (*m*-HPPH) as an internal standard for the determination of *para*-hydroxy phenytoin (*p*-HPPH), the principal urinary product of phenytoin metabolism in man. *m*-HPPH is a minor urinary product of phenytoin metabolism in man and is also produced in small quantities from degradation of a "dihydrodiol metabolite" by the acid treatment required for deconjugation of the *p*-HPPH-glucuronide [35].

In Figure 3 are summarized the structures of primidone (PD), three candidate appropriate internal standards for the determination of primidone, and MPPH, a hydantoin derivative which has been employed as the internal standard for primidone [36]. Any one of these primidone analogs, because of their analogous structures, might be expected to exhibit solvent partitioning, chromatographic, and derivatization properties more closely aligned to those of primidone itself, than would MPPH, a compound with a dissimilar heterocyclic structure. The *para*-methyl primidone analog (MPD) has been adopted in some recent GLC methods as the internal standard for PD [20,37,38].

In a laboratory where a GLC assay is performed routinely on a daily basis, the evaluation of a new candidate internal standard is a relatively simple matter. The candidate may be added directly to an existing internal standard solution, this permitting a comparative evaluation of the candidate against the adopted compound without interruption of laboratory services. Some daily *intra*-laboratory tests of primidone (PD) determinations by an OCMT [20] are summarized in

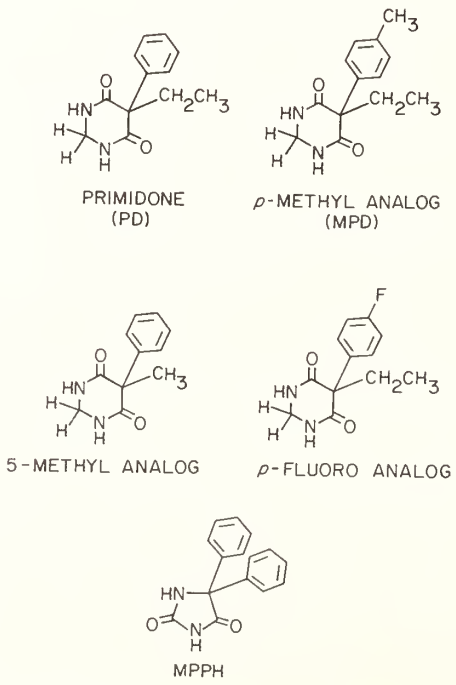


Figure 3. Structures of primidone (PD) and its *p*-methyl-, *p*-fluoro-, and 5-methyl analogs, and of 5-phenyl-5-(*p*-tolyl)hydantoin (MPPH).

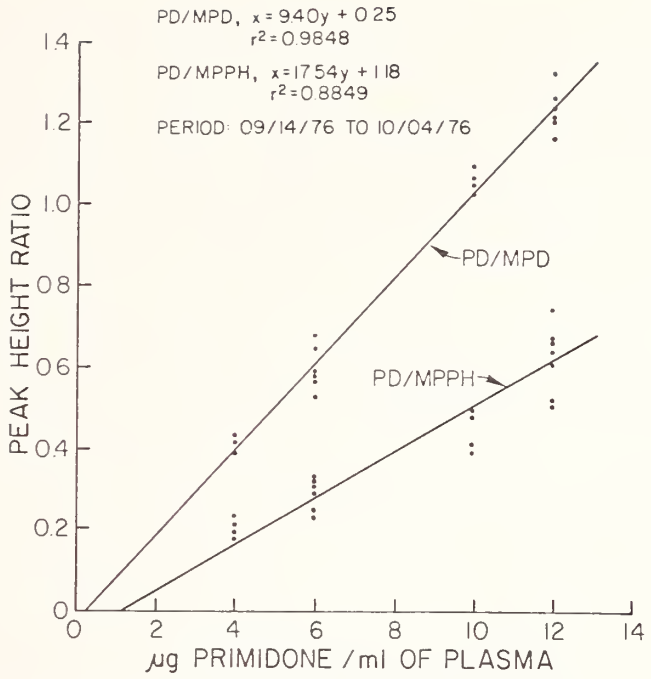


Figure 4. Variability of primidone (PD) determinations with *p*-methylprimidone (MPD) and 5-phenyl-5-(*p*-tolyl)hydantoin (MPPH) as internal standards in a gas chromatographic on-column methylation technique (OCMT) [20].

Figure 4. The variabilities of the PD values were assessed when MPD and MPPH were employed simultaneously as internal standards. The tests were performed daily over a period of about 3 weeks. Each point represents an *intra*-laboratory quality control sample, prepared freshly on a given day and worked up in exactly the same manner as a patient plasma sample [20]. It may be seen from a comparison of the correlation coefficients (r^2) of the two data sets that the use of MPD reduces the variability of the PD values. With MPPH as the internal standard, variability representing about 20% error in the accuracy of the PD determination was not unusual.

V. Intra-Laboratory Quality Control

Proficiency testing programs, like those available for the antiepileptic drugs in the United States [3,39] and in Europe [1,2], have pinpointed many of the causes responsible for the problem of interlaboratory variability and have contributed significantly to self-improvement of the performances of many laboratories. To demonstrate an adequate performance in a monthly proficiency program is, however, insufficient grounds for justification of a method, for the obvious reason that interest is normally biased toward external proficiency test samples. Rigorous *intra*-laboratory control, with the objective being a daily challenge of the analytical method under the actual conditions of its use, provides a more meaningful assessment of the reliability of a method.

Calibration and daily quality control tests of a drug assay should be performed by the same procedural steps as those used in the assay of unknown samples [33,39]. The recommendation has been made that calibration and quality control samples should also be prepared in the same biological matrix for which the assay has been designed [40]. This recommendation implies that solutions of drug standards in aqueous buffers should not replace solutions of drug standards prepared in blank plasma, or in blank urine, etc., when an assay is designed for one of these fluids. Such precaution eliminates any error sources that could arise when differences between the constituents of the biological matrix and of a non-biological matrix influence drug properties, two

hypothetical examples being drug recovery in a GLC-extraction scheme and drug binding behavior in a RIA or EMIT.

The preparation of standard solutions of a drug in a biological matrix may sometimes be thwarted by properties of the drug itself. Phenytoin, for instance, has a low solubility, to begin with, in an aqueous medium at physiological pH, and, when a sample of the thoroughly dried crystalline compound is placed in an aqueous medium, the crystals are difficultly "wetted" and show a very slow rate of dissolution. If a weighted sample of crystalline phenytoin is to be dissolved directly in plasma or other fluids, one cannot rest assured that the actual final concentration is equal to the theoretical concentration, unless other primary standard solutions are available for comparison.

Reliable antiepileptic drug calibration and quality control samples for a gas chromatographic assay [20,21] have been prepared by dissolving the agents first in a small volume of ethanol and diluting this alcohol solution with blank plasma to make a stock plasma solution. Different volumes of the stock plasma solution are further blended with known volumes of blank plasma to provide a series of calibration samples in which the alcohol content of any sample does not exceed 2%. Only a few selected samples from the series are necessarily required for the practice of daily *intra*-laboratory control. However, the assumption cannot be made that such additive-containing standard solutions are equally suitable for calibration and control of drug binding assays (RIA and EMIT). When ethanol was used as an additive in the preparation of calibration samples for a RIA designed for tetrahydrocannabinol (THC) in plasma, addition of ethanol to unknown THC-plasma samples was required in order to correct for the effect of ethanol [41]. Pooled serum of patients receiving phenytoin has been used in quality control tests of a phenytoin-RIA method. The concentration of phenytoin in the pooled serum was predetermined by gas chromatography-mass spectrometry [7].

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MEASUREMENT OF CATECHOLAMINES AND THEIR METABOLITES IN TISSUE AND PHYSIOLOGICAL FLUIDS USING REVERSE-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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Liquid chromatography with electrochemical detection (LCEC) has proven to be a very versatile tool in the neurosciences. A number of laboratories have now adopted this technique as the principal means of investigation of catecholamines and their acidic (DOPAC, HVA, VMA), neutral (MHPG, etc.), and basic (3-MT, NM, M) metabolites. The LCEC approach has also been used to assay the activity of enzymes responsible for the synthesis and catabolism of the catecholamines. Urine, blood, spinal fluid, tissue slices, and tissue homogenates are the usual sample matrices of interest.

Key words: Catecholamines; electrochemical detection; liquid chromatography; phenolic metabolites.

I. Introduction

The first practical electrochemical detector for liquid chromatography was developed for the purpose of monitoring the catecholamines in brain tissue [1]. Following this initial publication, liquid chromatography with electrochemical detection (LCEC) has been applied to a wide variety of problems involving the trace determination of easily oxidized or reduced substances. The principles and practice of the technique have been reviewed [2,3]. In the present report we briefly review applications of interest to neuropharmacologists and clinical chemists.

Techniques for the determination of catecholamines and their metabolites must be capable of quantitating picomole amounts in very complex mixtures (i.e., tissue homogenates and biological fluids). The methodology must also be sufficiently straightforward that large numbers of samples can be processed without need for a high level of technical expertise. Although many assays have appeared in the literature, few meet these criteria and progress in the neurosciences continues to be impeded in part by the lack of satisfactory procedures. It is, for example, nearly impossible for a given laboratory to measure all of known neurologically important compounds in a given specimen. It is therefore frequently necessary to compromise the value of key experiments by selecting a few substances. Often the choice is dictated by what can be done rather than by what should be done.

Classical coulometric or fluorimetric measurements for the biogenic amines are generally not satisfactory due to a lack of sensitivity and/or selectivity. Radioenzymatic methods have excellent sensitivity, but they are not widely applicable, can be very awkward, and suffer from expensive reagent costs. All of the catecholamines and their major metabolites have been determined by Gas Chromatography-Mass Spectrometry (GC/MS). The high resolution of gas chromatography coupled with selected ion detection provides a highly specific approach with excellent sensitivity and linearity. The principal problems with GC/MS results from the need to form volatile derivatives, the expense of the instrumentation, and the poor sample throughput. There have been a number of highly innovative and imaginative assay procedures published in all of the above areas.

We do not claim that our approach has overcome all (or even most) of the difficulties. Nevertheless, there are obvious advantages to liquid chromatography for the trace determination of polar organic substances. The instrumentation and reagents are quite inexpensive and the number of sample manipulations can often be reduced when compared to gas phase, fluorescence, or radioenzymatic methods. The primary disadvantages include (1) the fact that samples must be processed in series for the final quantitation and (2) that the reliability of the instrumentation (including columns) is not perfect. While the latter problem has been dramatically improved in the last few years there remains considerable room for further progress, particularly with respect to columns. Recent developments in electrochemical detector design are described elsewhere in this volume [3]. The first difficulty is made less troublesome by the improved speed of separation, the use of column switching techniques, and the fact that the LC instrument can be easily automated. The fact that a liquid chromatograph can be constructed or purchased very inexpensively makes it possible to utilize several instruments in parallel. For example, in our laboratory we normally work with individual instruments dedicated to the catecholamines (norepinephrine, epinephrine, dopamine), metanephrines (metanephrine, normetanephrine, 3-methoxytyramine), the acid metabolites (homovanillic and vanillmandelic acids), and tryptophan metabolites (tryptophan, 5-hydroxytryptamine, and 5-hydroxyindole acetic acid). The modular nature of these chromatographs permits great flexibility in tailoring an instrument for a given job.

II. Principles

Phenolic compounds are ideally suited for analysis by electrochemical techniques. A dilute solution (typ. <1 mmol/L) of a phenol in an aqueous buffer solution can readily be studied by oxidation at a carbon electrode (typ. carbon paste, glassy carbon, or pyrolytic graphite). It will be instructive for those not familiar with voltammetry to examine the current voltage curves (voltammograms) which are obtained when such a solution is pumped through a flow cell such as that illustrated elsewhere in this volume [3]. When the potential between the carbon electrode and the flowing stream is gradually (or stepwise) increased in a positive direction "voltammograms" are obtained such as those schematically illustrated in Figure 1 for several phenols. The voltammograms represent a steady-state situation in which mass transport to the surface is normally rate controlling. As long as the flow rate and concentration of reactant are constant, the current at every potential will be independent of time. The amperometric detector for liquid chromatography is most often operated at a fixed potential just beyond where the limiting current has been achieved (e.g., at $+0.4$ V for the trihydroxy compound). The current at this potential varies as a function of the concentration profile of metabolite eluting from the LC column and is thus a good measure of the amounts of material injected on the column. This approach to LC detection has several advantages compared to alternate approaches.

Amperometric detectors are selective, sensitive, and inexpensive. The selectivity and sensitivity are both dependent on the oxidation (or reduction) potential of the compound(s) of interest. Easily oxidized or reduced compounds can be detected at very low concentrations with great specificity. For example, in order to detect a monophenol (Fig. 1) a potential is selected which would be more than adequate to oxidize a hydroquinone or catechol derivative. The selectivity would therefore not be nearly as good as for a trihydroxy compound which could be selectively oxidized in the presence of the other phenols. Sensitivity is diminished for those molecules which require a high positive or negative potential because the background current (mobile phase only) and its accompanying noise are also very much more significant at high potentials ($>\pm 1$ volt).

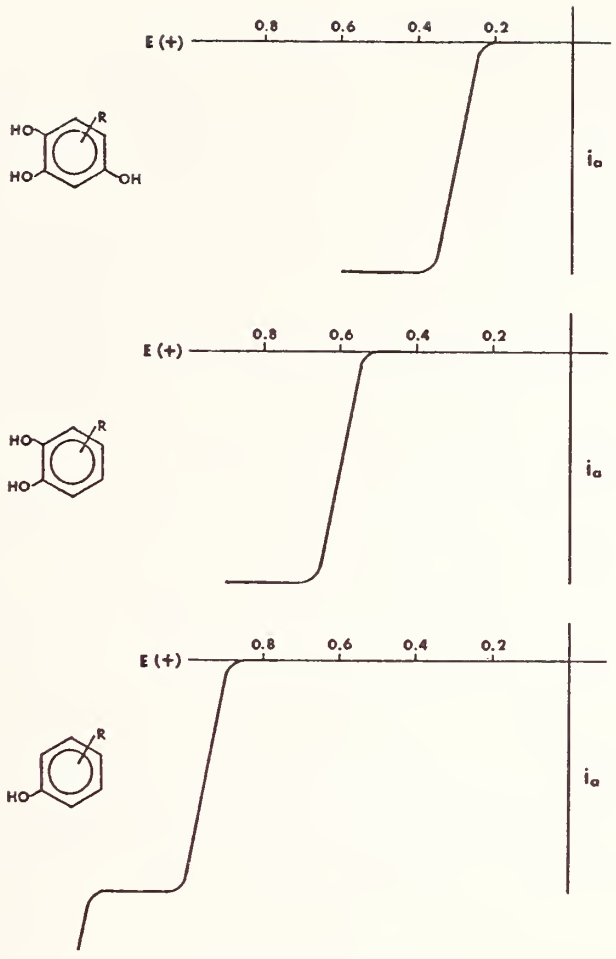
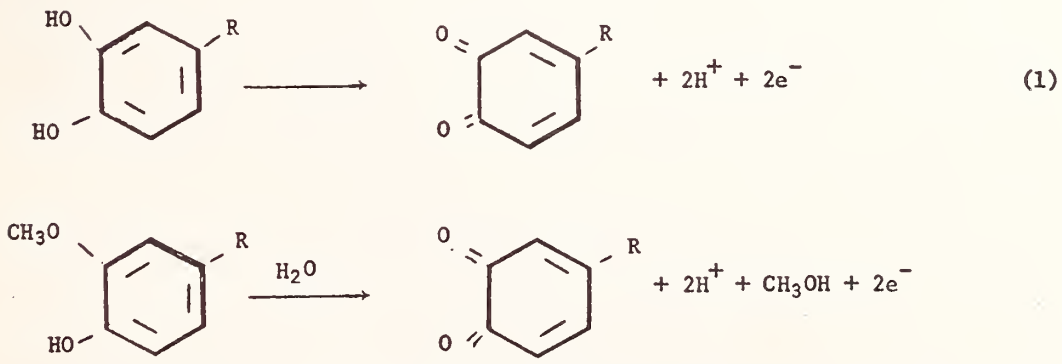


Figure 1. Hydrodynamic voltammograms for three classes of natural phenols (schematic representation).

The catecholamines and essentially all of their known unconjugated metabolites undergo one of the following two electrochemical reactions:



These reactions are quite facile at carbon electrodes and therefore have been the basis of a number of published assay procedures.

III. Applications Review

A. Catecholamines and Serotonin

The catecholamines (CA's) have been studied in a variety of biological samples using both pellicular cation exchange and reverse-phase chromatography with electrochemical detection. Pellicular cation exchange materials have been used for determination of CA's in whole brain [4], brain parts [5], and urine [6]. L-dopa in the serum of Parkinson's disease patients [7] and CA derived tetrahydroisoquinolines in tissue and biological fluids [8] have been measured using similar techniques. Most often the CA's are isolated prior to chromatography using the classical alumina adsorption technique. Recently Adams and co-workers used the LCEC technique to directly measure norepinephrine and dopamine release from brain slice preparations following both chemical and electrical stimulation [9]. This work is particularly noteworthy in that release of endogenous CA's could be studied. It was unnecessary to pretreat the slice with radio-labelled compounds as is frequently done in studies of this type. The new method avoids questions of interpretation related to distribution of exogenous labelled-CA's in the tissue slice.

Reverse-phase LC techniques have a number of important advantages for measurement of the CA's and their metabolites [10]. Methods have been reported for the metanephrines [11] and CA's in urine [12], and CA's in brain parts [13].

Serotonin and dopamine have been measured in mouse brain by Sasa and Blank using LCEC with a pellicular cation exchange resin [4]. We have recently been successful with determination of tryptophan, serotonin, and 5-hydroxyindole acetic acid using reverse-phase LCEC [15].

Liquid chromatography is sufficiently fast that it is practical to use the technique for enzyme activity assays. Blank and Pike have developed an excellent assay for tyrosine hydroxylase based on electrochemical detection of L-dopa formation [16]. A similar approach has been used to measure catechol-O-methyltransferase (COMT) and phenylethanolamine-N-methyltransferase (PNMT) activity [17]. Dopamine-B-hydroxylase activity has been successfully determined in human blood [10].

B. Acid and Neutral Metabolites

Determination of the acid metabolites of the catecholamines is complicated by the large number of phenolic acids of both endogenous and dietary origin. Although the principle acid metabolites (homovanillic acid (HVA) [18], dihydroxyphenylacetic acid (DOPAC) [19], and vanillmandelic acid (VMA) [20]) have all been measured using pellicular anion exchange and/or reverse-phase columns, these methods have already been made obsolete by the far superior performance of microparticle reverse-phase columns [10]. One problem with reverse-phase chromatography is the strong retention of "uninteresting" components which limit the rate at which samples can be applied to the column. We have solved this problem in several cases using (1) step-gradients, (2) column switching techniques [10], and/or sample preparation by thin-layer chromatography [18]. The latter approach is particularly useful for extremely complex mixtures as illustrated in Figure 2.

An ethylacetate extract of an acidified urine sample contains a great many phenolic acids which cannot be resolved by LC in a reasonable period of time (top chromatogram in Fig. 2). Resolution of weakly retained components can not be achieved without losing the strongly retained components in the baseline noise. In this example, we are specifically interested in two compounds, VMA and HVA, neither of which could be reliably quantitated in this urine pool. While some improvement could be achieved by means of a gradient or column switching, a dramatic change in overall selectivity is a better approach in this case because of the large number of dietary components of no particular interest.

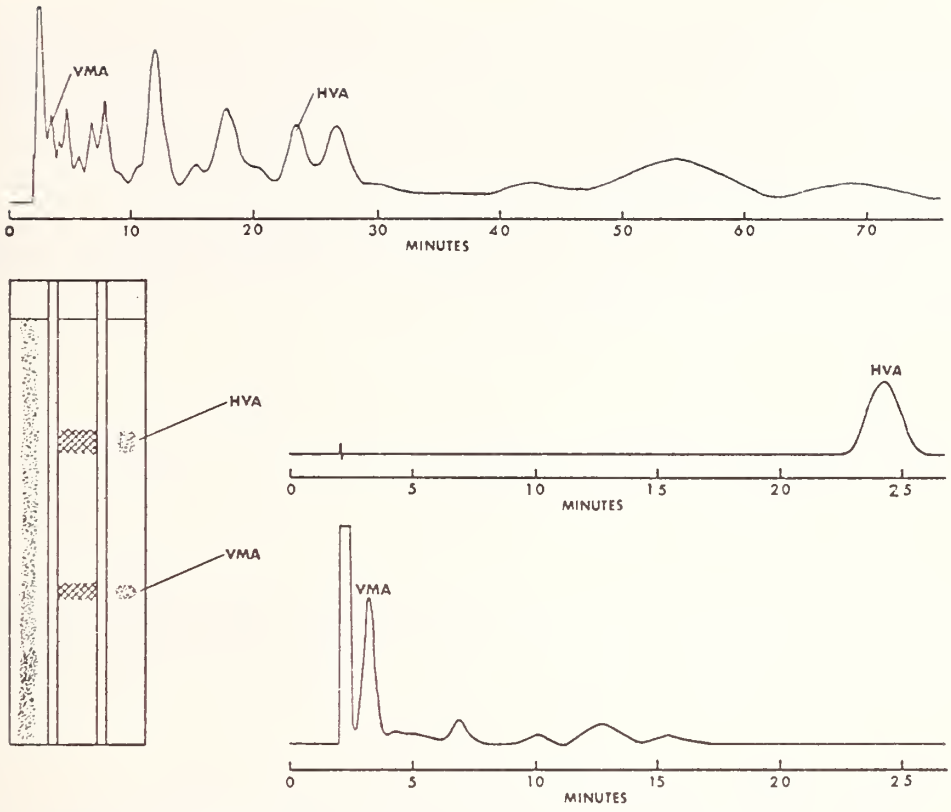


Figure 2. Determination of VMA and HVA in urine using a TLC sample preparation prior to LCEC.

Spotting the ethylacetate extract on a silica TLC plate with a preadsorbent layer is fast and convenient. For purposes of illustration three channels were used (Fig. 1). Two channels were spotted with sample and one with a standard mixture of HVA and VMA. One sample channel and the standard were sprayed with a phenol color reagent with the second sample channel masked. The two phenolic acids in the standard are easily resolved, however, the sample is too complex to permit determination of either acid by TLC alone.

If the R_f regions matching HVA and VMA are removed from the unsprayed sample channel and eluted with solvent, the contents of these regions on the plate can be studied using LCEC. As is clearly depicted in the figure, the resulting chromatograms are relatively simple and the compounds of interest can be quantitated with confidence. In order to shorten the analysis time the two TLC zones can be combined and both acids detected in a single chromatogram. The overall recovery and precision for an analysis of this type is often competitive with alternate sample workup procedures.

The advantages of TLC-LCEC can be summarized as follows:

(1) The fact that all components exist on the plate with $0 \leqslant R_f \leqslant 1$ means that nothing can be "lost" as is the case with column chromatography.

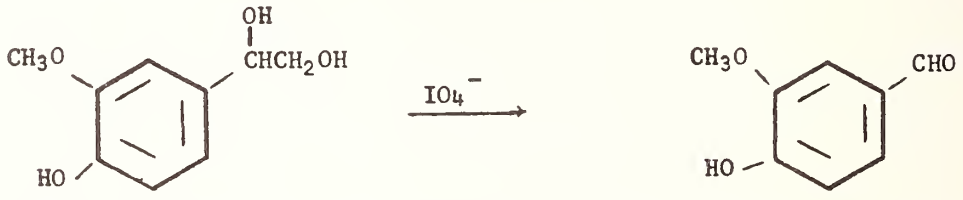
(2) TLC can save a great deal of time because many experiments can be carried out in parallel, whereas samples must be run in series on the column.

(3) The economy of time results from the fact that materials strongly retained on the column can be eliminated in the TLC step.

(4) If the basic mechanism of separation is different for thin-layer and column, then the overall selectivity can be greatly enhanced by using both techniques.

There are several obvious problems which may discourage some workers. Manipulation of the TLC plates does require some experimental skill and a knack for bookkeeping since samples are easily confused. Furthermore, fine particulate matter from the plate must never be injected on the column chromatograph. Injection valve faces can be scored and inlet frits clogged. Samples can be eluted from the TLC stationary phase and filtered using a 0.2 μm centrifugal filter device [Bioanalytical Systems, model (CF-1)].

Methoxyhydroxyphenylglycol (MHPG) has also been successfully determined in urine using the TLC-LCEC approach [21]. An earlier approach to this important norepinephrine metabolite required oxidation of MHPG to vanillin using periodate [10], a classical reaction for vanil alcohols such as MHPG, VMA, and metanephrine.



The vanillin was detected using LCEC. While this approach is feasible, the new TLC-LCEC method is less awkward, faster, and does not require measurement of a sample blank (a sample processed without the periodate oxidation).

IV. Conclusion

Although a great deal of work remains to be done, the advantages of LCEC for measurement of the neurologically important metabolites of tyrosine and tryptophan have been adequately demonstrated. A large number of laboratories are actively developing and routinely using methodology based on the LCEC technique. One novel application reported by Meek et al. involved the detection of [Leu^5]enkephalin, a pentapeptide (Tyr-gly-gly-phe-leu) [22]. The electroactive tyrosine residue provides an adequate handle for detection of nanogram amounts of this compound.

V. Acknowledgment

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QUANTITATIVE ANALYSIS OF MELATONIN IN HUMAN PLASMA BY NEGATIVE CHEMICAL IONIZATION MASS SPECTROMETRY

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A quantitative assay for melatonin in 1 mL of human plasma has been developed using gas chromatography-negative chemical ionization mass spectrometry. Extracted melatonin, along with a tetradeuterated internal standard can be measured from 1-100 pg/mL derivatized as the pentafluoropropionyl ester.

Key words: Human plasma; melatonin; negative chemical ionization mass spectrometry.

I. Introduction

Melatonin (5-methoxy-N-acetyltryptamine) is a neurohormone secreted by the pineal gland. Extensive physiological and biochemical studies in experimental animals have demonstrated that its synthesis is cued by light and darkness and follows a circadian rhythm. Reviews of the biochemistry [1] and pharmacology [2] of melatonin have recently appeared and there is a clinical interest to define its normal and its pathophysiological roles in man.

Most of the available human plasma data on melatonin has resulted from radioimmunoassay (RIA) [3–6] or bioassay [7] methods. Initial discrepancies and wildly fluctuating daytime and nighttime values led to criticism of the specificity of these methods and the resulting report of two GC-MS assays. Kennaway et al. [8] described an electron impact (EI) ionization analysis of the pentafluoropropionyl (PFP) derivative and CD_3-methoxy melatonin as an internal standard which was sensitive at the nanogram level. It was consequently only useful for validating an RIA method by measuring a 24 mL plasma sample containing 6 ng, but could not be employed for the measurement of individual plasma samples. Wilson and co-workers [9] described the analysis of the trimethylsilyl derivative of melatonin by GC-EI-MS using a hexanoyl ester analogue as an internal standard. They reported the addition of 50 ng of the internal standard to 2 mL of plasma, subsequent extraction, derivatization and detection of a minimum value 0.33 pg injected on-column. The reported normal values for plasma are approximately 40 pg/mL (daytime) and 125–450 pg/mL (darkness).

We became interested in the potential sensitivity of negative chemical ionization mass spectrometry following the demonstration by Hunt [10] of 10–100-fold sensitivity enhancement for electron capturing compounds. Appropriately derivatized organic compounds will undergo efficient ionization by resonance capture of a near thermal energy electron in an exothermic process. Excess energy is dissipated by fragmentation or collisional stabilization. Selection of a suitable electron capturing derivatizing reagent is a critical element. It is possible to efficiently capture electrons and then fragment to an anion which derives only from the reagent and does not contain any structurally specific elements. In our experience, cyclic derivatives or polycyclic compounds will generally yield structurally specific anions, whereas acyclic derivatives with good anion leaving groups (i.e., perfluroacyl derivatives of aliphatic alcohols) will generate only reagent specific ions.

II. Instrumentation

Mass spectra (positive and negative CI) were recorded with Extranuclear SpectrEl electronics and a Finnigan 3200-GC-CI analyzer. A single electron multiplier (ITT F4074) is employed after the method of Stafford [11] with the first dynode operating at +2 kV (negative ion mode) or −2.7 kV (positive ion mode) with the rest of the dynode string at −2.5 kV to ground (signal out). The multiplier is offset from the axis of the quadrupole rod assembly in order to obtain a lower noise signal. Consequently, it is not possible to directly measure multiplier gain factors. However, by using the same multiplier for recording positive and negative ion spectra, the sensitivity ratios for the two modes of operation can be readily determined.

III. Mass Spectra

Figure 1 shows the positive and negative CI (methane) mass spectra of melatonin-PFP. By comparing the relative intensity of MH^+ (m/z 361) in the positive spectrum with $M-2HF^-$ (m/z 320) in the negative spectrum, a ratio of 1/150 (P/N) was found. This ratio is somewhat variable, depending upon factors such as ion source temperature and state of cleanliness of the instrument.

The structure of the product obtained from reaction of pentafluropropionic anhydride (PFPA) with melatonin is a uniquely stable spirocyclic compound recently described by Blau et al. [12] and shown in Figure 2. It no longer has the polarity or poor chromatographic qualities of an indole, and has lost one hydrogen from the N-acetyl group. This structure accounts for the observation that deuteroacetylation of 5-methoxytryptamine, followed by formation of the PFP derivative yields a shift of 2 amu on EI and of 1 or 2 on positive CI.

A tetradeuterated melatonin (N-acetyl-5-methoxy-[$\alpha,\alpha,\beta,\beta$-$D_4$] tryptamine) was synthesized by Shaw [13] as an internal standard. On EI or positive CI mass spectral analyses, it showed the expected 4 amu shifts. However, negative CI revealed shifts of 3 amu (Fig. 3) for the ions at m/z 340 and 320 consistent with sequential losses of DF and HF from the molecular anion.

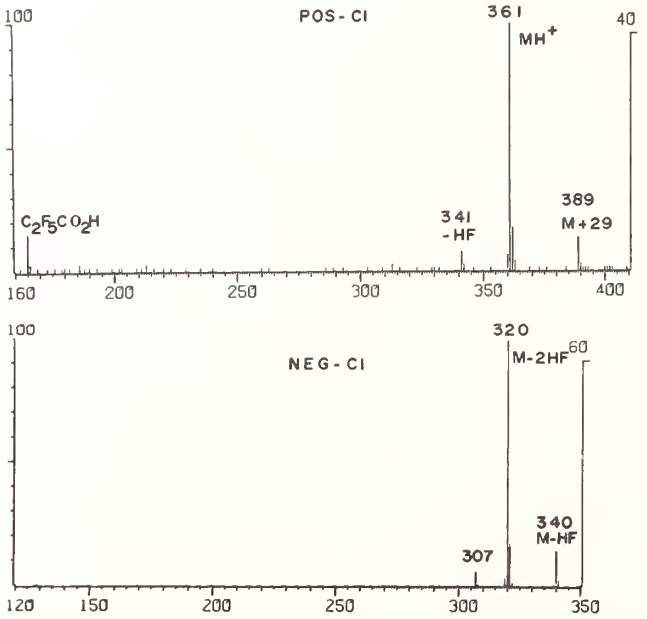

Figure 1. Positive and negative chemical ionization (CI) mass spectra of melatonin-PFP.

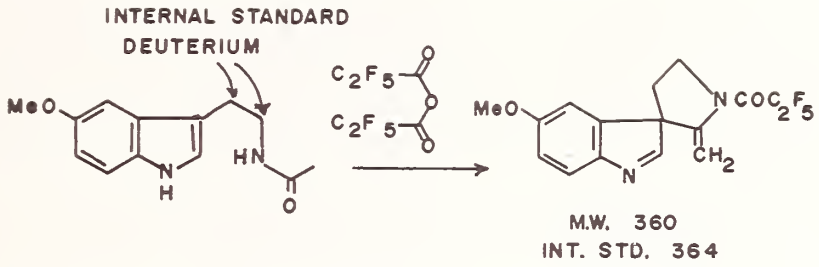

Figure 2. Structure of spirocyclic PFP derivative formed by reaction of melatonin with PFPA. Position of deuterium substitution on the internal standard is indicated.

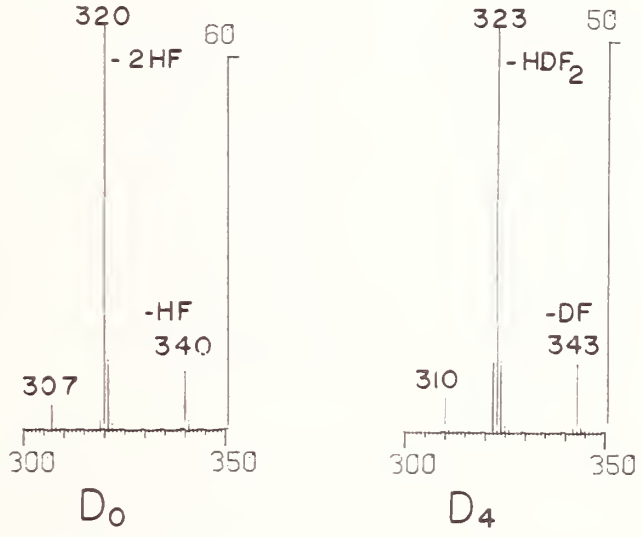

Figure 3. Partial negative CI mass spectra of non-deuterated (D_0) and tetradeuterated (D_4) melatonin-PFP.

IV. Plasma Assay

Plasma (1 mL) is mixed with an equal volume of 0.5 mol/L borate buffer (pH 10, saturated with KCl), and 30–60 pg of tetradeuterated melatonin is precisely added. A series of 6 aqueous standards covering the concentration range of interest (0–100 pg) is extracted and derivatized with each batch of samples. The aqueous phase is gently shaken with 10 volumes of petroleum ether to remove lipids and other substances. After aspiration of the petroleum ether, the aqueous phase is gently shaken with 10 volumes of chloroform. The chloroform extracts are dried under nitrogen, and a little benzene is added and re-dried to remove traces of water. The dried extract is dissolved in 0.4 mL dry acetonitrile and derivatization is performed using 25 μL PFPA plus 5% trimethylamine in dry benzene (0.5 mL) as catalyst, and heating at 75 °C for 15 minutes. The samples are washed with water (1 mL) and 5% NH_4OH (1 mL). Finally the benzene layer is blown dry, and the residue twice partitioned between hexane (1 mL) and acetonitrile (0.5 mL). This step is a useful means to reduce electron capturing impurities which would elute after the solvent and can cause a significant sloping baseline. The acetonitrile phase is blown dry and ethyl acetate (15 μL) added. Approximately one half of the sample is injected onto a 5 m×2 mm i.d. glass column packed with 1% OV-225 on 100–120 mesh Gas Chrom Q (Applied Science) operated at 235 °C. Melatonin elutes after 4–6 minutes; thus the column effluent is diverted from the ion source for the first 2–3 minutes following injections.

An example of typical daytime (2–10 pg/mL) and nighttime (25–85 pg/mL) selected ion recordings are shown in Figure 4. For the higher values, it is possible to monitor two fragment ions (m/z 320 and 340) in order to insure specificity. A typical standard curve is shown in Figure 5. The minimum detectable value is approximately 2 pg/mL which corresponds to an injected quantity of 200–400 femtograms depending upon the efficiency of the extraction and derivatization steps.

The results of a typical human circadian rhythm are shown in Figure 6. To date, we have not encountered instances of episodic bursts in the melatonin rhythm which characterize apparently less specific assays.

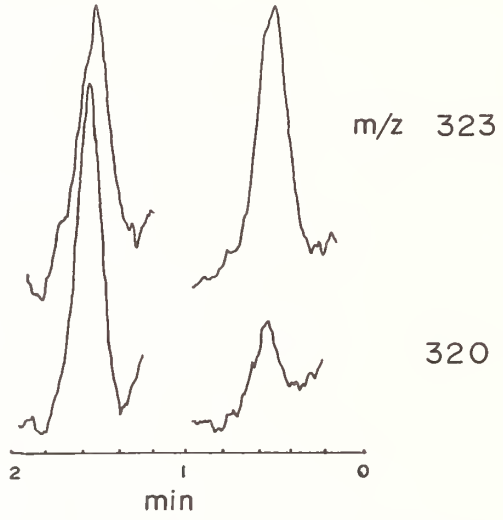

Figure 4. Selected ion recordings showing internal standard (37 pg/mL) at m/z 323 (upper traces), a daytime response (8 pg/mL lower right) and a nighttime response (50 pg/mL lower left). Peaks are approximately 25 s wide. Traces were made from strip chart recordings made with a programmable multiple ion monitor (PROM1M, Finnigan Corp.)

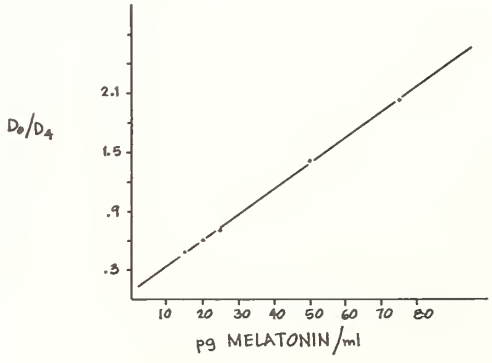

Figure 5. Typical standard curve obtained from 0–75 pg/mL of melatonin extracted from aqueous solutions described by equation $Y=0.0431+0.0267X$ ($R=0.999$).

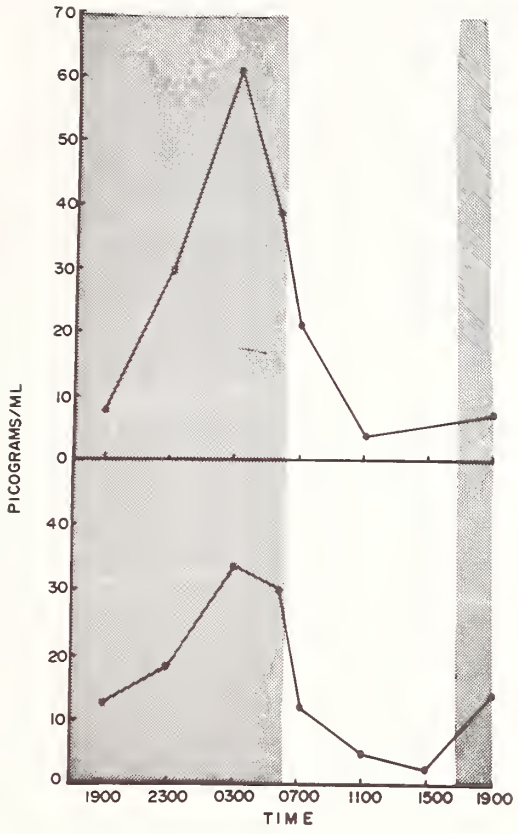

Figure 6. Plasma melatonin levels in two normal controls.

V. Instrumental and Assay Problems

Over the past year, several problems have arisen with regard to the maintenance of high sensitivity. All of these have derived from the ion source and/or quadrupole rods requiring stringent and frequent cleaning. In spite of the fact that this instrument is used exclusively for melatonin and thus never receives large samples, operation in the negative ion mode at high sensitivity does require additional precautions. Deterioration of ion peak shape and dramatic sensitivity decreases in negative ion mode precede deterioration of performance in positive ion mode.

Generally, the ion source and quadrupole assembly are cleaned every 2 weeks. The ion source is mechanically scrubbed with alumina and a wooden dowel followed by electropolishing and several solvent washes. Bake-out of the ion source at 300 °C overnight extends the useful lifetime of the ion source between assays. Optimal sensitivity is realized at an operating temperature of 110–150 °C, and the frequent cleanings are undoubtedly necessary due to buildup of organic contamination depleting the electron population in the ion source.

Tuning of the spectrometer for maximum sensitivity has remained an art. Approximate focus positions for ion energy and lens remain constant, but it is necessary to fine tune using column bleed ions rather than using a marker compound introduced via a solid probe inlet.

Contamination of glassware with interfering substances is another potential source of difficulty. During the development of this assay, it was found necessary to segregate melatonin assay glassware in the laboratory. It is acid-washed, rinsed, dried and silanized with hexamethyldisilazane in a heated vacuum dessicator [14]. Procedural blanks are routinely analyzed

to check for new sources of contamination. Pipets for deuterated and non-deuterated standards are reserved exclusively for their separate functions.

VI. Conclusions

A negative ion GC-MS assay suitable for quantitation of human plasma melatonin levels has been described which demonstrates the potential utility of this mode of ionization. Future developments in chromatography, ion source design, and quadrupole configuration are being sought in order to stabilize the high sensitivity achieved in this assay. Isolation, derivatization and analysis of compounds at or below the picogram level affords a new realm of opportunity for the biochemist and mass spectroscopist.

VII. Acknowledgment

The excellent technical assistance of Mr. Donald McKenzie, Ms. Pam Greenlaw and Melody Herbst has significantly advanced the pace of our efforts.

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A CANDIDATE DEFINITIVE METHOD FOR THE DETERMINATION OF TOTAL CHOLESTEROL IN SERUM

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An isotope dilution-mass spectrometry method for the determination of total cholesterol was developed. Cholesterol, isotopically labeled with deuterium at C-25 to C-27, was added to serum samples. After an alkaline treatment to hydrolyze esters of cholesterol, the cholesterol was isolated from the serum and converted into the trimethylsilyl derivative for analysis by GC/MS. The ratio of the unlabeled to labeled cholesterol TMS ether in the entire GC peak was measured in the mass spectrometer by focusing alternately on the molecular ions at m/e 458 and 465, and summing both signals. Duplicate GC/MS isotopic ratio measurements were bracketed by duplicate measurements of standard mixtures containing comparable proportions of SRM cholesterol and cholesterol-d_7. To ensure stability of measurement, the duplicate measurements of standard mixture or of sample had to agree within 1%, and these duplicate measurements of sample and standard had to be repeated again on another day. The weight ratio of the isotopic forms in a sample was obtained by linear interpolation between the bracketing standard mixtures whose compositions were accurately known. An overall relative standard deviation of 0.33 percent was obtained for the measurement of total cholesterol in five different serum pools that were analyzed as described on four different occasions.

Key words: Cholesterol; gas chromatography/mass spectrometry; isotope dilution; serum.

I. Introduction

Isotope dilution carried out in conjunction with mass spectrometry (ID/MS) has been shown to be a most accurate, compositional-analysis method for more than 20 elements in diverse matrices [1]. Analyses for these elements in homogeneous samples have typically been performed with a total error of 0.13% to 0.25% (stated as the approximate relative standard deviation). The accuracy and precision achieved can be attributed to the specificity that derives from the use of isotopic internal standards and from control over the sources of systematic and random error in both the wet-chemical processing and mass spectrometry of the samples [2]. In contrast, the accuracy reported for organic chemical analytes by ID/MS is significantly less good; inaccuracies estimated just from recovery experiments were from 3 to 5% and imprecisions (relative standard deviation based on duplicate serum determinations) have been only as small as 1.3% [3].

We have been interested in methods of analysis having high accuracy for application to the certification of Standard Reference Materials, and we thought that organic ID/MS should be capable of providing higher accuracy than had been thus far reported. We hoped ID/MS could also prove useful for a program being run under the Standards Committee of the American Association for Clinical Chemistry, in which we are cooperating with support of the Food and Drug Administration. The initial objective of that program is to establish the analytical accuracy of several reference methods used in clinical chemistry, so that, subsequently, accuracies of the routinely used clinical methods could be evaluated. Serum samples with "definitively known" analyte concentrations are needed for this program, and we are to provide these analyses.

In attempting to demonstrate the accuracy that could be attained by ID/MS, we therefore chose to work on one of the clinical chemistry determinations, i.e., total cholesterol in serum. In

the clinical laboratory assay, esterified cholesterol in the serum sample is hydrolyzed and then the freed cholesterol and the initially present cholesterol are measured ordinarily by spectrophotometry. Two ID/MS methods for the determination of total cholesterol have also been reported; their accuracy and precision were within the range cited above [4,5].

A. Materials and Methods

Serum Samples. Randomly selected vials of serum from several pools were provided by Dr. Gerald Cooper of the Center for Disease Control, Atlanta, GA. The specific gravity of each pool was determined by weighings in vessels that were calibrated for volume.

Cholesterol (cholesterol-d_0). SRM 911a, certified by NBS as 99.8±0.1% cholesterol, was used as the standard for natural cholesterol. An approximately 10-mg quantity (exact mass known to ±3 μg) was dissolved in about 7.9 g of absolute alcohol (exact mass known to ±0.1 mg) in a 10-mL volumetric flask, as a standard solution for use in preparing standard mixtures. The flask was stoppered with a thimble-shaped, polyethylene cap through which the needle of a hypodermic syringe could be inserted for withdrawing aliquots.

Cholesterol-d_7 [cholest-5-en-25,26,26,26,27,27,27-d_7-3-01(3β)]. This material was supplied by Applied Science Laboratories,[1] State College, PA [6]. It was purified by sublimation and recrystallization. After trimethylsilylation, GC/MS revealed 1–2% of a 24-carbon steroid impurity. Standard solutions in ethanol were prepared (as described above for the SRM cholesterol solution) in capped 10-mL volumetric flasks from 10-mg quantities of the purified cholesterol-d_7 for use in preparing calibration mixtures and for additions to serum samples.

Calibration Standards. A series of solutions of the cholesterol-d_7 and -d_0 were prepared in which their proportions as dry solids ranged from 0.8 to 1.2. The cholesterol-d_7 and -d_0 solutions were transferred by use of hypodermic syringes. The quantities were measured by weighing the filled and emptied syringes.

B. Sample Preparation (Wet-Chemistry)

Two aliquots of serum, each containing about 1 mg of total cholesterol, were taken by syringe from each vial (or from several vials after combining and carefully mixing them to ensure homogeneity). The mass of each was obtained by weighing the filled syringe and then reweighing it after delivering the serum aliquot into tubes in which weighed, 1-mL portions of the 1-mg/mL cholesterol-d_7 solution had been previously placed. Each mixture was warmed with alcoholic potassium hydroxide for 3 hours to hydrolyse cholesterol esters. Water and hexane were added and, after vigorous mixing, the hexane layer was separated and evaporated. The dried residue, consisting mainly of cholesterol-d_7 and -d_0, was dissolved in 2 mL of methanol. Thus, each aliquot provided a separate specimen for derivatization and GC/MS.

C. Derivatization of Cholesterol

An aliquot of a methanol solution of extracted cholesterol or an ethanol solution of a standard mixture (50 μL and 100 μL, respectively) was evaporated to dryness and treated with bis(trimethylsilyl)acetamide at room temperature for at least 0.5 hour before analysis by GC/MS.

D. Sample Measurement by GC/MS

A Varian 2740 gas chromatograph and a Varian MAT CH 7A mass spectrometer combination was used. The latter was equipped with a chemical ionization/electron impact ion source and a multi-ion selection device that controlled the switching of the magnetic field and the

[1] Identification of any commercial product does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

timing for sequential acquisitions of data which were collected in a multichannel scaler from which the data were then transferred to a Hewlett-Packard Model 9830 for calculation.

Injections representing about 3 μg of cholesterol were made onto a 125×0.3 cm (o.d.) stainless steel column packed with 1.5% OV-101 on Chromosorb G 100/120 mesh. The helium flow and temperatures were adjusted to maintain a retention time of about 6 minutes. Electron impact measurements were made at an ion source temperature of 250 °C and an ionization energy of 70 electron volts. For ammonia chemical ionization, the source temperature was 200 °C with an ion source gauge pressure of 4×10^{-5} torr. Two selected ions, one from the unlabeled cholesterol TMS ether and the other the corresponding ion from the labeled cholesterol TMS ether, were monitored alternately for 1-second intervals before the elution of the GC peak began to obtain background corrections, and then from the start of the peak until the peak intensity fell to 1% of the maximum. The ratio of corrected and integrated ion-intensities was then calculated.

II. Results

Some of the steps in our procedure were separately evaluated for their contribution to the overall uncertianty of measurement. Thus, for example, in the wet-chemical processing where the aliquoting of ethanol solutions was of particular concern, its precision was tested with a 10-mg/10-mL ethanol solution of cholesterol that contained cholesterol-4-^{14}C. By counting 10 weighed aliquots of approximately 1-mL each, a relative standard deviation of 0.16% which includes a 0.1% statistical error due to counting was found.

In order to ensure the reliability of the ion-intensity ratio measurements, several conditions were imposed: (1) the cholesterol-d_7 to -d_0 range of ratios measured would be restricted to be near 1:1; (2) two successive ion-intensity ratio measurements of a sample or a standard were to be made and have no more than a 1% maximum allowable difference between them to be acceptable. The average of these successive results would be considered a single valid measurement; (3) each valid sample measurement would be immediately preceded and succeeded by valid sample measurements of standard mixtures whose isotopic ratios closely bracket that of the sample; (4) the ratio measurements of the sample and the same standards would be repeated on a second day.

A test of the "within-day" precision of the wet-chemical steps of the method was carried out by performing all chemical manipulations in 1 day on 10, weighed, 1-mL aliquots of a serum pool. Valid measurements were obtained on 2 days. The relative standard deviation was 0.2% for this set of analyses.

Precision was further tested in an experiment involving both "within-day" and "between-day" measurement-components. Five serum pools were analyzed in duplicate simultaneously as a set, and then were reanalyzed similarly in three additional sets. For each set, the wet-chemical processing was initiated at weekly intervals. Valid measurements were completed for each set and then remeasured before the next set's measurements were begun. The timing of the mass spectrometric measurements was not otherwise restricted. The results are shown in Table 1. The relative standard deviation for this entire series was 0.33%.

The measurements just cited were obtained by electron impact using the molecular ions of cholesterol-d_7 and -d_0 TMS ethers. This method was verified by comparing the results with those obtained by other GC/MS methods on a subset of the same specimens using the same standard mixtures. Thus, as shown in Table 2, some of the specimens from set 2 were reanalyzed by using fragment ions generated by electron impact, by ammonia chemical ionization, and by the original method repeated with a different GC column about 8 months after the original measurements.

TABLE 1. *Concentrations of total cholesterol (in mmol/L) in five serum pools as determined by ID/MS*

		Serum Pools									
		I		II		III		IV		V	
		Aliquot		Aliquot		Aliquot		Aliquot		Aliquot	
Set	Day	1	2	1	2	1	2	1	2	1	2
1	1	3.425	3.439	4.713	4.728	6.154	6.130	7.434	7.450	8.781	8.786
	2	3.443	3.433	4.713	4.711	6.139	6.136	7.476	7.444	8.765	8.781
2	1	3.480	3.430	4.728	4.734	6.164	6.139	7.464	7.452	8.806	8.782
	2	3.420	3.434	4.726	4.718	6.164	6.145	7.462	7.455	8.799	8.787
3	1	3.412	3.413	4.696	4.699	6.135	6.103	7.436	7.437	8.773	8.755
	2	3.417	3.414	4.698	4.704	6.141	6.130	7.427	7.415	8.761	8.734
4	1	3.442	3.433	4.738	4.727	6.182	6.171	7.490	7.456	8.847	8.818
	2	3.448	3.443	4.738	4.732	6.175	6.183	7.508	7.482	8.819	8.817

TABLE 2. *Comparison of methods for the measurement of total cholesterol in serum (mmol/L) by ID/MS*

GC/MS Condition			Sera Analyzed				
Column	MS	Masses	I	II	III	IV	V
OV-101	E.I.	465/458	3.425	4.727	6.142	7.463	8.784
OV-101	E.I.	336/329	3.417	4.714	6.143	7.443	8.767
OV-17	E.I.	465/458	3.423	4.715	6.137	7.460	8.782
OV-101	C.I. (NH_3)	393/386	3.418	4.694	6.130	7.425	8.733

III. Discussion

The accuracy obtained by this ID/MS analysis depended in part on the wet-chemical processing steps, specifically on how well the quantities of the sample and isotopic internal standard were measured, on the completeness of the ester hydrolysis, and on the stability of cholesterol under the hydolytic conditions. Each pipeting was found to have a less than 0.16% relative standard deviation. The other wet-chemistry factors listed were tested by use of radioactivity methods. We found no evidence of decomposition of cholesterol after a 5-times-longer alkaline treatment than was required for complete hydrolysis of the cholesterol ester tested.

In addition, the accuracy of these results rested on the GC/MS measurement techniques. Thus, the TMS derivative of cholesterol was selected in order to avoid the dehydration that can occur on GC of cholesterol. Reliance on measurements at the higher mass of the TMS cholesterol molecular ion served to diminish the likelihood of interference arising from serum components. Ion-intensity measurements were run prior to the elution of the GC peak for each sample and standard analyzed to correct for instrument background, including interference from column bleed.

Bracketing the sample with standards of closely similar composition, prepared from the SRM cholesterol and the cholesterol-d_7 used as the isotopic internal standard, helped to minimize sources of error; for example, error that would have resulted from insufficient knowledge of the exact purity of the cholesterol-d_7 preparation as required for some ID/MS procedures. Note, however that knowledge of the purity of the cholesterol-d_0 is essential. Also the similarity of isotopic ratios of the bracketing standard mixtures and samples helped to minimize errors that would result from slow instrumental drift.

The close similarity of the results obtained under different GC/MS conditions showed that the measurements were not appreciably affected by interferences. The small differences found between these comparative measurements are at about the same level as was found for the between-day measurements shown in Table 1. We believe that statistical analysis of these data, and the quantitation and/or estimation of the magnitude of the various possible sources of systematic error we have discussed, which remains to be done, will show that the accuracy and precision of these ID/MS analyses are adequate for providing definitive analytical values for total cholesterol.

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THE DETERMINATION OF NEUROLOGICALLY IMPORTANT TRYPTOPHAN METABOLITES IN BRAIN CEREBROSPINAL FLUID

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The compounds tryptophan (TRP), 5-hydroxyindoleacetic acid (5-HIAA), indoleacetic acid (IAA), and indolepropionic acid (IPA) have been determined in human and rat CSF. Absolute detection limits of 5–25 pg have been obtained using a liquid chromatographic-fluorometric method. TRP and 5-HIAA are quantitated by the direct injection of 1–20 μL of CSF. IAA and IPA are determined by injection of 50 μL of CSF. IAA is determined in rat brain after homogenization and centrifugation by direct injection of supernatant. The fluorometric system is discussed in a signal/noise context, the limiting noise is seen to be photon shot noise. Several neurochemical studies are presented illustrating the utility of the method.

Key words: Brain; cerebrospinal fluid; fluorescence flow cell; liquid chromatography; tryptophan metabolites.

I. Introduction

The neurotransmitter serotonin (5-HT) and the behaviorly active trace amine tryptamine (TAM) are formed from tryptophan in the brain. The determination of these compounds and their acidic metabolites, 5-hydroxyindoleacetic acid (5-HIAA) and indoleacetic acid (IAA), in brain and cerebrospinal fluid (CSF) is of importance in many neurochemical studies.

Tryptophan (TRP) has most often been determined in human CSF by the fluorometric method of Denckla and Dewey [1], a detection limit of ~10 ng/mL is usually observed. The method has also been applied to the determination of TRP in single rat CSF samples (~100 μL sample volumes) [2]. An average TRP concentration of ~400 ng/mL is observed in rat and human CSF. 5-Hydroxyindoleacetic acid (5-HIAA) has been determined in CSF by fluorometric [3–5], liquid chromatographic-fluorometric [6], gas chromatographic-mass spectroscopic (GC/MS) [7–8], and amperometric [9] procedures. Typical concentrations of 5-HIAA in human and rat CSF are 30 ng/mL and 100 ng/mL respectively. The amperometric method, with an absolute detection limit of ~10 pg 5-HIAA, was the only method to be used for the determination of 5-HIAA in single rat CSF samples. The other methods having detection limits of 0.2–20 ng/mL require 1–2 mL of CSF and involve an extraction procedure. Recently [10] TRP and 5-HIAA have been determined simultaneously by a liquid chromatographic-fluorometric procedure, with absolute detection limits of 25 and 70 pg respectively. The method involved the direct injection of 1–20 μL of human or rat CSF.

Indoleacetic acid has been determined in human CSF by GC/MS [6,11]. Detection limits of ~0.2 and 2.0 ng/mL were obtained and an average value of 6.1 ng/mL reported [11] for human CSF. A liquid chromatographic-fluorometric procedure, involving a one-step extraction of 0.1 mL of rat CSF or 0.5 mL of human CSF, has been reported for determination of IAA and

indolepropionic acid (IPA) in CSF [10]. The compounds were determined with a detection limit of ~100 pg/mL.

Rat brain has been analyzed for IAA by a GC/MS procedure involving an extraction and derivatization step [12]. A detection limit of ~1 ng/g was obtained, a mean value of 11.6 ng/g was reported for IAA in whole rat brains.

II. Experimental

A. Apparatus

Liquid chromatography was performed with a Waters (Waters Assoc., Milford, Mass.) 6000-A pump, U6-K injector, and 25 cm×4 mm (i.d.) reversephase column of 10 μm "μ Bondapak C_{18}." An Aminco (American Instrument Co., Silver Spring, Md.) Fluoromonitor was modified so that a mercury penlamp (Model 11SC-1, U.V. Products, San Gabriel, Ca.) could be employed as the excitation source. A 90 mm diameter 254 nm interference filter (Waters Assoc.) with a transmissivity of 0.42 was employed as the excitation filter. A Corning 7-51 glass filter was used as the emission filter. A Supersil ~70 μL, 2 mm i.d. flow cell was used with a holder having 1.25 mm slits. The excitation filter was centrally positioned giving an effective (illuminated and collected) cell volume of ~14 μL. An RCA 931-B photomultiplier (S-4 response) was used with a supply voltage of −700 V. A diagram of the fluorometric detector is shown in Figure 1. Usually an RC low-pass filter with a time constant (τ) of 1.5 sec was employed in addition to a built-in damping circuit (τ=0.1 s at 0.3 sensitivity).

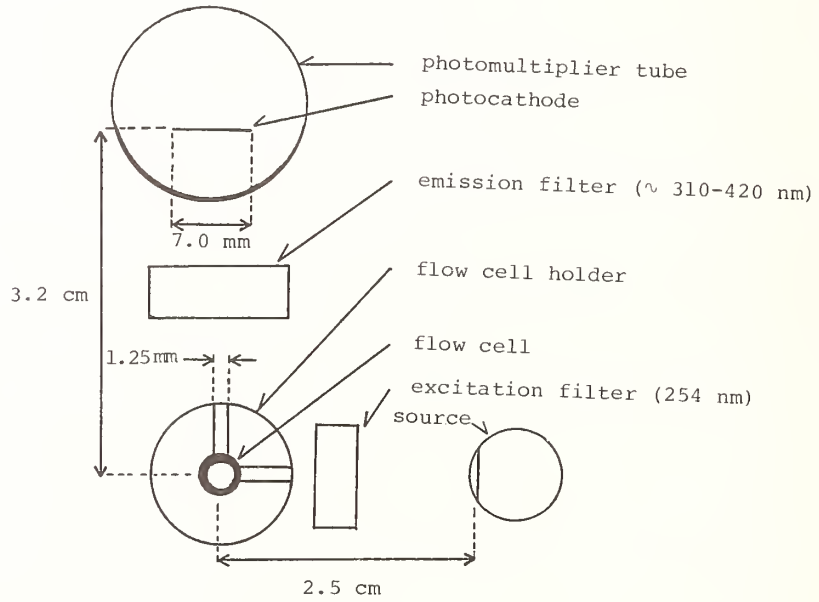

Figure 1. Top-view of the flow-through fluorometric detector. Dimensions not shown include an exposed source area of 0.5 cm×2.5 cm., flow-cell 0.2 mm×2.5 cm (0.9 cm illuminated length), and a photocathode length of 2.3 cm. The center of the source, flow-cell and photocathode are approximately coplanar.

B. Reagents

Standards were purchased from the Sigma Chemical Co. (St. Louis, Mo.). The acetonitrile used was "glass distilled" grade from BDH Chemicals (Montreal, Quebec). All other chemicals were reagent grade. Solvent systems were prepared by adjusting 0.01 mol/L sodium

acetate solution to the proper pH with glacial acetic acid. The proper proportion of acetonitrile was then added and the solution stirred for ~30 min before use.

C. METHODS

1. Determination of Indoleacetic Acid and Indolepropionic Acid in Human and Rat Cerebrospinal Fluid (CSF)

Human CSF was obtained via lumbar puncture from patients undergoing diagnostic pneumoencephalography (PEG). Rat CSF was obtained from the cisterna magna by cisternal puncture through the atlanta-occipital membrane. CSF samples were stored in acid washed or silylated tubes and were kept frozen at –70 °C until analysis. Human samples were centrifuged before freezing to remove debris. Chromatographic analysis was performed by direct injection of, typically, 50 μL of untreated human or rat CSF. The chromatographic conditions are given in Table 1.

2. Determination of Tryptophan and 5-Hydroxyindoleacetic Acid in Human and Rat CSF

Sampling and storage procedures were identical to those above. Analysis was accomplished by the direct injection of 1–20 μL (typically 10 μL) of human or rat CSF. Chromatographic conditions are listed in Table 1.

3. Determination of Indoleacetic Acid and Indolepropionic Acid in Rat Brain

Rat brains were removed intact, rinsed with 0.9% saline, blotted dry, and stored in Parafilm at –70 °C until analyzed. Whole rat brains (~2 g) were thawed at ~5° for 10 min in polycarbonate centrifuge tubes and then homogenized for 40 sec in 2.5 volumes of 0.1 mol/L $ZnSO_4$ containing 0.02% ascorbic acid. After the addition of 2.5 volumes of 0.1 mol/L $Ba(OH)_2$ the samples were stirred on a vortex mixer for 3 s and then centrifuged at 18,000 g for 15 min. Solutions were kept at, and operations performed at 0–5 °C. Approximately 1 mL of the supernatant was removed and stored at –20 °C in a polycarbonate tube. Chromatographic analysis (performed within 48 h) was by the direct injection of 50 μL of the supernatant. Chromatographic conditions are given in Table 1.

TABLE 1.

Compound	Retention time (min)	Chromatographic conditions*	Detection limit**
Tryptophan	3.50	1	20 pg
5-Hydroxyindoleacetic acid	4.60	1	25 pg
Indoleacetic acid	3.90	2	5 pg
Indolepropionic acid	6.00	2	7 pg

* Conditions:
1. 2.0 mL/min, 90% 0.01 mol/L sodium acetate (pH 4.3), 10% acetonitrile.
2. 2.0 mL/min, 70% 0.01 mol/L sodium acetate (pH 4.0), 30% acetonitrile.

** Injected quantity giving a signal/peak-to-peak noise ratio of 2, with series RC filters of $\tau = 1.5$ s and 0.3 s.

III. Results and Discussion

A. DETERMINATIONS

The retention times and detection limits observed for the compounds of interest are given in Table 1. The chromatographic conditions are those used for their analysis in physiological samples.

The determination of TRP and 5-HIAA in two different rat CSF samples is shown in Figure 2. The recovery of added TRP and 5-HIAA has been observed to be complete, and single point standards are used when quantitating the compounds by peak height. The ease, speed, and sensitivity of the method are evident. The amperometric method [9] has a comparable sensitivity for 5-HIAA but it is restricted to the determination of the hydroxylated metabolites.

In Figure 3 the determination of IAA and IPA in 50 μL rat CSF samples is shown. The method is an improvement on that previously reported [10], which required an extraction and employed an internal standard. IAA (and IPA) had not previously been determined in rat CSF, an average value of ~5 ng/mL has been observed. The origin of IAA and IPA observed in CSF will be discussed in a later section.

The determination of IAA and IPA in rat brain is shown in Figure 4. The recovery of added IAA is 98±2%, while 49±7% of the added IPA is recovered. The method is much simpler, and slightly more sensitive, than the GC/MS method [12]. A value of 8.3±2.0 ng IAA/g (mean±1 standard deviation) was observed for seven whole rat brains. The method is sensitive enough to allow the determination of IAA in single rat brain area samples. Any IPA observed can be attributed to IPA in blood, the brain containing ~3% blood by weight. A small amount (~1 ng/g) of the brain IAA also arises from this source.

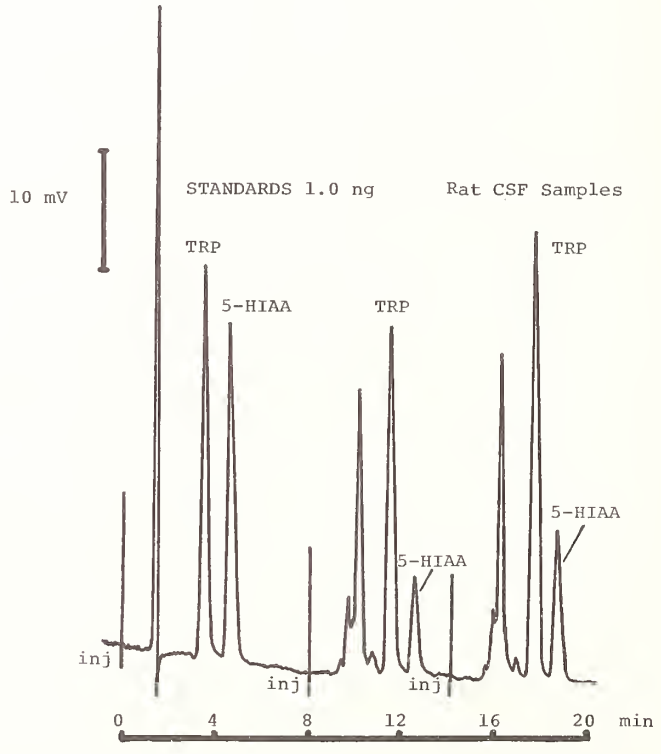

Figure 2. Chromatogram of the determination of TRP and 5-HIAA in 10 μL samples of untreated rat CSF, 1.0 sensitivity (standards 0.3 sensitivity). See Table 1 for chromatographic conditions.

Figure 3. Chromatogram of the determination of IAA and IPA in two 50 μL rat CSF samples, 0.3 sensitivity. See Table 1 for chromatographic conditions.

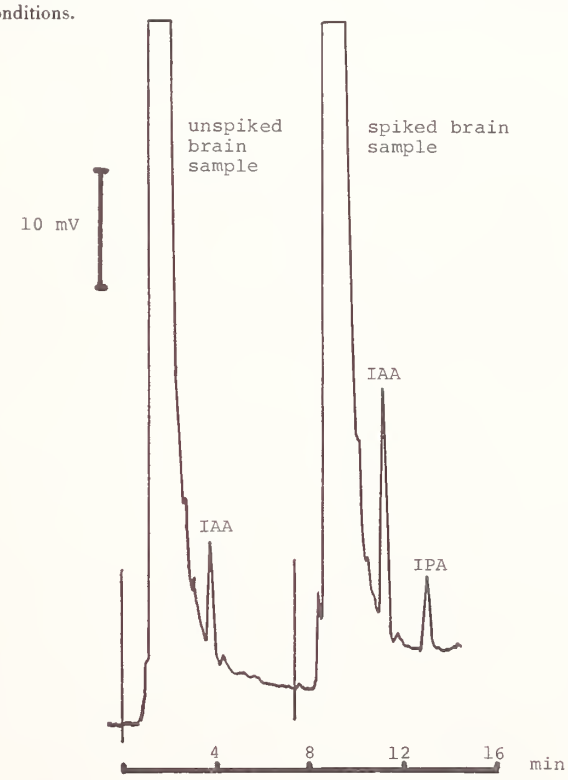

Figure 4. Determination of IAA and IPA in rat brain samples, 10 ng/g of IAA and IPA were added to the second sample. See text for experimental details.

B. Neurochemical Studies

1. The Origin of CSF IAA and IPA

While IAA is a usual oxidative metabolite of TAM in humans, IPA is considered a bacterial metabolite of TRP. The IPA observed in rat and human CSF was therefore suspected of arising from bacterial metabolism in the intestine. After sterilization of the gut with oral sulfasuxidine and neomycin the level of IPA in rat CSF dropped markedly, while the IAA level remained constant, as seen in Table 2. This and other related studies confirm the bacterial origin of IPA in CSF, and indicate that the majority of the IAA in CSF arises from the brain. The possible peripheral (extra-central nervous system) origin of metabolites measured in CSF has often not been thoroughly investigated.

Table 2. *Study of the origin of IAA and IPA in rat CSF*

Treatment	IAA ng/mL	IPA ng/mL
Control	3.3±0.4 (7)	5.1±2.0 (7)
IAA, IPA injection	14.3±1.5 (4)	24.9±9.9 (4)
Sulfasuxidine, neomycin	2.9±0.3 (9)	0.9±0.3 (8)

Sulfasuxidine (200 mg/kg) was given orally 24 hours before death. Neomycin (2 g/L) was put in the drinking water for 24 hours before death. IAA and IPA (both 1 mg/kg) were injected intraperitoneally 1 hour before death. Values given are mean±SEM, with number of samples in parenthesis.

2. Relationship of CSF IAA to CSF TRP in Humans

The influence of brain TRP concentrations on brain tryptamine levels is of interest in neurochemistry. Data for cisternal CSF obtained from a population of neurological patients undergoing pneumoencephalography are presented in Figure 5. IAA levels are seen to be positively correlated with the TRP levels. This indicates that, in human brain, tryptamine levels and/or turnover tend to increase with greater brain levels of the metabolic precursor TRP.

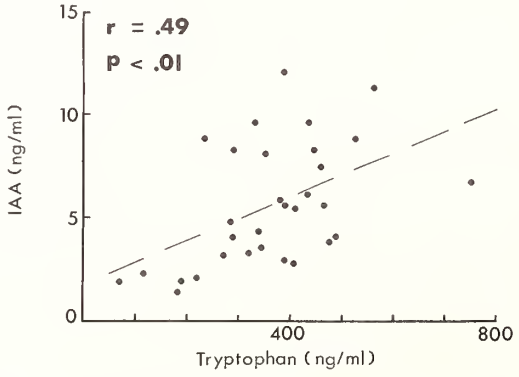

Figure 5. Relationship of CSF IAA to CSF TRP in human cisternal CSF.

3. The Effect of TRP on Indolic Metabolites in Rat CSF

It is of interest to determine how the brain metabolism of TRP is altered when the concentration of TRP in the brain is increased. As seen in Table 3 an increase in brain TRP (as indicated by increased CSF levels) causes a relatively larger rise in IAA levels than in 5-HIAA levels. This indicates that brain tryptamine synthesis becomes a more important pathway, relative to serotonin synthesis, at higher TRP concentrations. As expected no significant variation in the IPA levels was observed.

TABLE 3. *Study of the effect of tryptophan on indoles in rat CSF*

	Treatment			
Compound	Control		Tryptophan injected	
Tryptophan	247±9	(6)	3302±203	(8)
5-HIAA	117±13	(6)	251±23	(8)
IAA	5.0±0.7	(5)	32.5±2.4	(8)
IPA	7.6±1.2	(4)	7.9±0.5	(8)

Tryptophan (100 mg/kg) injected 1 hour before death.
Values are in ng/mL CSF±SEM, with number of samples in parenthesis.

C. SIGNAL-TO-NOISE STUDIES

The peak photomultiplier current (i) expected for a particular fluorophor in the system described can be estimated by the following equation:

$$i=2.3\ \epsilon\ b\ c_p\ I_{sx}\ A_c T_\lambda\ \phi_f\ \Lambda\ \tau_\lambda\ \Omega\ \rho\ \pi_\lambda\ G \qquad (1)$$

For the injection of 1.0 ng of tryptophan the terms have the following values; ϵ (absorptivity)$=2.5\times10^3$, b (path length)$=0.125$ cm, c_p (peak concentration)$=1.0\times10^{-8}$M, I_{sx} (source illuminance at cell)$=2.3$ mW cm^{-2}, A_c (illuminated cell area)$=0.11$ cm^2, T_λ (excitation filter transmissivity)$=0.42$, ϕ_F (quantum efficiency)$=0.14$, Λ (excitation/emission wavelength)$=0.71$, τ_λ (emission filter transmissivity)$=0.63$, Ω (collection efficiency)$=0.014$, ρ (reflective loss term)$=0.92$, π_λ (photocathode response)$=0.023$ A/W, and G (pmt gain)$=3.5\times10^5$. Substituting these values in Equation 1 gives an expected signal current value of 5.0×10^{-9} A. The observed peak values for 1.0 ng of tryptophan ranged from $4.2\text{–}6.2\times10^{-9}$ A. The predicted linear dependence of the signal on the terms of Equation 1 was observed when several (ϵ,b,C_p,I_{sx},A_c,T_λ,ϕ_f,G) of the terms were varied. The system appears to be well described, from a signal standpoint, by Equation 1.

The peak-peak system noise (i_{pp}) or background fluctuation, was observed to be 8.1×10^{-11} A ($\tau=1$ sec), the absolute level of the background (i_b) was 9.0×10^{-9} A. At this background level a noise level of 8.0×10^{-11} A is expected solely from photon shot-noise, ($i_{pp}=(2Q_e\ \Delta f\ G\ i_b)^{1/2}$). Electronic noise ($\sim10^{-14}$ A) and dark current shot–noise ($\sim10^{-12}$ A) levels were negligible contributors to the system noise. Source noise was also negligible, the lamp having a signal/noise ratio of $\sim10^3$ compared to the signal/noise ratio of the background of 1.1×10^2.

The system is $\sim$1–3 orders of magnitude more sensitive than other conventional fluorometric flow-cell systems. When the relative absorptivities and quantum efficiencies of the compounds determined are taken into account the system compares favorably with very sensitive laser fluorometric methods which have been recently developed [13–16].

IV. Conclusions

We believe the method to offer significant advantages in cost, speed and sensitivity over most previous methods for determining tryptophan metabolites in physiological samples. Determinations in plasma and urine confirm its wide utility. The ability of the system to detect nearly all indolic metabolites has allowed previously unreported and/or unexpected metabolites (e.g., IPA, among others) to be discovered.

V. Acknowledgments

We wish to thank S. Gauthier of the Department of Psychiatry, McGill for supplying the human CSF samples. The authors acknowledge the National and Medical Research Councils of Canada for partial support of this work.

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APPLICATIONS OF THE NITROGEN DETECTOR TO THE ANALYSIS OF STEROID HORMONES AND RELATED COMPOUNDS BY GAS CHROMATOGRAPHY

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The linearity and sensitivity of the nitrogen-phosphorus (N/P) detector was assessd with quinoline as a model compound. It was found that the selectivity ratio, hydrocarbon to quinoline, was approximately 1 to 5000. Direct comparison of a flame ionization with a N/P detector was made possible by splitting the effluent stream at the end of a capillary column. The N/P detector was found only 4 times more sensitive than the flame ionization detector for steroid derivatives O-methyloxime tri-methylsilyl (MO-TMS). Methods for removing the reagents are described as well as other means to prepare nitrogen containing-derivatives (enamines). The possibility of using phosphorus containing derivatives is also discussed.

Key words: Gas chromatography; nitrogen-phosphorus; selective detection; steroid hormones.

I. Introduction

Alkali-flame detectors have been known for some time [1], but they were plagued with instability and a short life span. In the last few years these detectors have been improved; the alkali bead is heated electrically which permits a better control of the temperature, hence a better stability. Although many different kinds of alkali detectors have been designed, those specific to nitrogen and phosphorus are most common. They find their applications in the analysis of drugs and pesticides [2–5].

The performance of this type of detector was assessed with quinoline, a compound which does not absorb on the chromatographic column, and was used as an internal standard in the determination of nicotine in biological fluids [6]. Steroids are compounds containing ketones and hydroxy groups; the polarity of the free compounds does not allow their quantitation by gas chromatographic methods. Many derivatization schemes have been proposed for reducing the polarity of poly-functional steroids. The most widely used procedure is the formation of O-methyl oximes (MO) for the protection of ketones followed by the trimethylsilylation of the hydroxy groups [7]. Several refinements have been reported in the literature [8,9]. Since a nitrogen function is introduced into the ketosteroid molecule, the latter can be specifically detected in the presence of a complex steroid mixture such as a urinary extract. Other derivatives may be formed selectively with conjugated ketones; it is also possible to derivatize hydroxy steroids with phosphorus containing reagents as shown by Vogt and his collaborators [10]. The present report is aimed at reviewing the derivatives that can be prepared for the analysis of steroids by gas chromatography with a N/P detector.

II. Experimental

A. Chemicals and Reagents

Steroids were purchased from Steraloids, Inc. (Wilton, NH). O-Methylhydroxylamine hydrochloride (Eastman Organic Chemicals, Rochester, NY) and the silylating agents, bis-

(trimethylsilyl)acetamide and trimethylsilylimidazole (Pierce Chemical Co., Rockford, IL) were used without purification. All the solvents were of analytical grade.

B. Gas Chromatography

A Hewlett-Packard Model 5710A gas chromatograph with a Hewlett-Packard Model 18789A nitrogen-phosphorus detector was used for the analysis with packed columns (2% OV 101, 4 ft×2 mm i.d.). When samples were run on glass capillary columns, a Hewlett-Packard Model 5701 gas chromatograph equipped with a Hewlett-Packard Model 18740A glass capillary inlet system was used. The effluent at the end of the capillary column was split equally, after adding helium (45 mL/min), to a flame ionization and a nitrogen-phosphorus detector. The glass capillary columns coated with SE 30 were prepared according to the procedure developed in this laboratory [11].

C. Steroid Analysis

The extraction of urinary steroids and the formation of MO-TMS derivatives were those used in this laboratory [8,9].

Enamines were prepared in alcoholic solution after addition of a small excess of base, according to the procedure described by Gardi and Ercoli [16].

III. Results

A. Detector Performance

Quinoline was chosen as a model compound to assess the performance of the N/P detector since it was used as an internal standard for the determination of nicotine in biological fluids by Feyerabend et al. [6]. Dilutions of quinoline in hexane were prepared, and 1 μL was injected in order to measure the limit of sensitivity and the linearity of the detector. As shown in Figure 1, 20 pg of quinoline could be detected, the response was linear up to 2.5 ng. No correction was made for error in the dilution or injection which explains why the response corresponding to 750 pg is slightly above the curve.

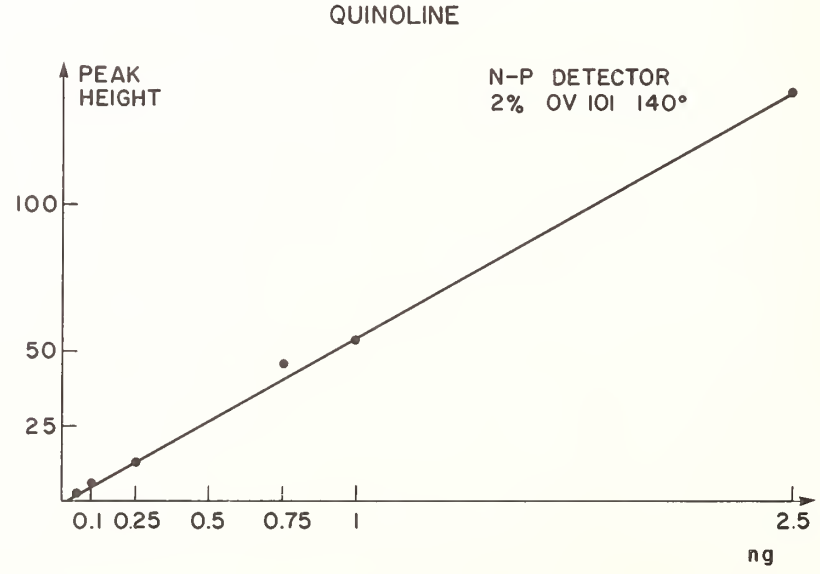


Figure 1. Linearity response of the nitrogen-phosphorus detector to quinoline. Quinoline (50 pg to 2.5 ng) was injected in 1 μL of hexane; no correction was made for errors in dilution or injection.

In the course of our study, we found that the results obtained with nicotine were not nearly as reproducible as for quinoline. Even when quinoline was used as an internal standard to compensate for error in injection and dilution, erratic response was sometimes observed for nicotine, but the detector was not the cause of the problem. Since nicotine is a strong base, loss may occur on contact with acidic surfaces. An example is shown in Figure 2, the left panel was obtained by injecting 1 ng of both quinoline and nicotine with a syringe used for other purposes; while the same sample drawn with a clean new syringe produced the chromatogram shown in the right panel.

Although quinoline has a retention time similar to the one of nicotine, it is not suitable as an internal standard for the determination of nicotine due to a difference of adsorption properties. A more appropriate standard, N-propyl nornicotine, was synthesized from nornicotine by reaction with propyl iodide in basic medium. The separation of nicotine and its propyl analog is shown in Figure 3. With this new internal standard, a linear relationship was obtained for nicotine, and the limit of sensitivity was similar to the one obtained for quinoline (20 pg). For samples in the subnanogram range, adsorption onto the support is the limiting factor in the sensitivity of the method.

The work with nicotine clearly demonstrated that a linear response could be obtained with the nitrogen detector. In our hands a selectivity ratio of 5000 was observed between nicotine and a straight chain hydrocarbon, which is comparable to the value reported by Sevcik [12]. (See fig. 4.)

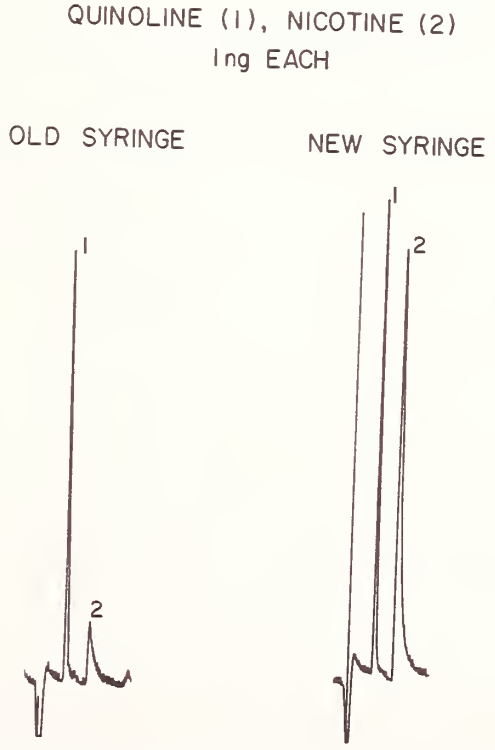


Figure 2. Difference of adsorption between quinoline and nicotine observed when these 2 compounds (1 ng each) were injected in 1 μL of hexane under the same conditions (packed column 2% OV 101, 140 °C with N/P detection) with a syringe used for injecting other types of samples (left panel) and with a new syringe (right panel).

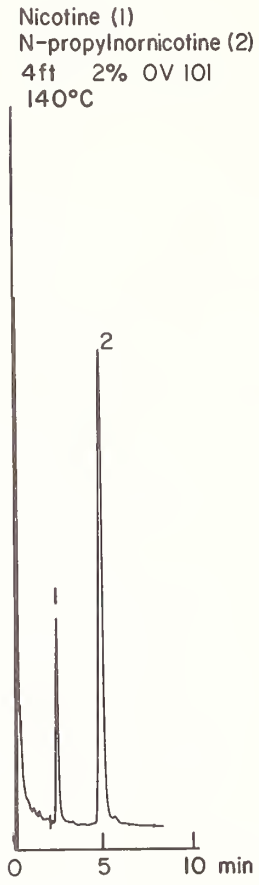


Figure 3. Separation of nicotine (1) and N-propylnornicotine (2) on a packed column (4 ft. 2% OV 101) at 140 °C (N/P detection).

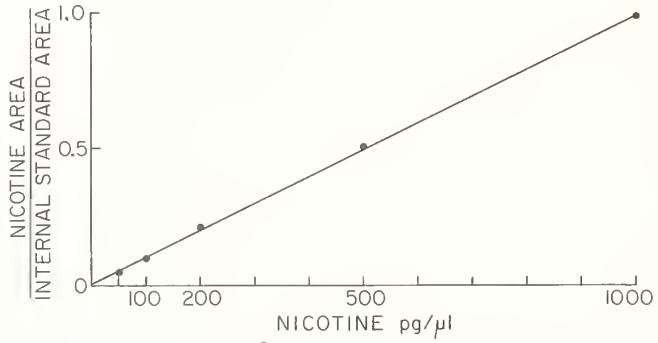


Figure 4. Linear response of the nitrogen-phosphorus detector to nicotine; n-propylnornicotine was used as an internal standard.

B. Steroid Analysis

The analysis of steroids by gas chromatography is well established since 1960 due to the pioneer efforts of Horning and his collaborators [13]. It was recognized early in their work that in order to avoid degradation or loss on the column it was necessary to reduce the polarity of polyfunctional steroids by derivatization. O-Methyloximes introduced by Fales and Luukkainen [14] are now the most widely used derivatives for the protection of ketones.

Oxime formation is similar in mechanism to the addition of a primary amine to a carbonyl function. In both cases the reaction proceeds through a tetrahedral intermediate, the difference occurs in the elimination of water from this intermediate; the formation of the oxime being irreversible while the Schiff base may be converted back to the starting materials upon addition of water. For this particular reason, oximes are preferred to Schiff bases in the analysis of keto steroids.

In order to compare the sensitivity and selectivity of the N/P detector to those of a standard flame ionization detector, the effluent stream off a capillary column was split equally to a N/P and a FID detector. With this design the sensitivity for both detectors could be compared with the same sample. MO-TMS derivatives of steroids in the androstan series were prepared according to the procedure developed in this laboratory [8]. The left panel of Figure 5 shows the chromatogram obtained with the FID detector; every steroid gave a response, while the right panel shows the selective detection of keto steroids. Androstan 3-ol-TMS does not give a response with the N/P detector, and only one peak is observed after derivatization of androstone 17-one, due to the steric hindrance of the C_{18} methyl group, while androstane 3-one gave the syn and anti isomers. It is of interest to compare the ratio between androsterone/etiocholanolone MO-TMS with the two detectors. From the flame ionization response one would assume that etiocholanolone is present in higher amount than androsterone; however an impurity, androstan 3,17-diol, was present in the etiocholanolone sample and although a capillary column was used the two compounds could not be separated on SE 30. The N/P detector gave the correct ratio between androsterone and etiocholanone since the diol TMS did not produce a signal with the N/P detector.

From Figure 5, it can be seen that both detectors have similar sensitivity (the N/P detector being only 4 times more sensitive). The application of the N/P detector lies in its selectivity rather than in its sensitivity.

Steroid hormones, after being metabolized, are conjugated and excreted in urine. It is possible to analyze all the neutral steroids in a single run by gas chromatography with capillary

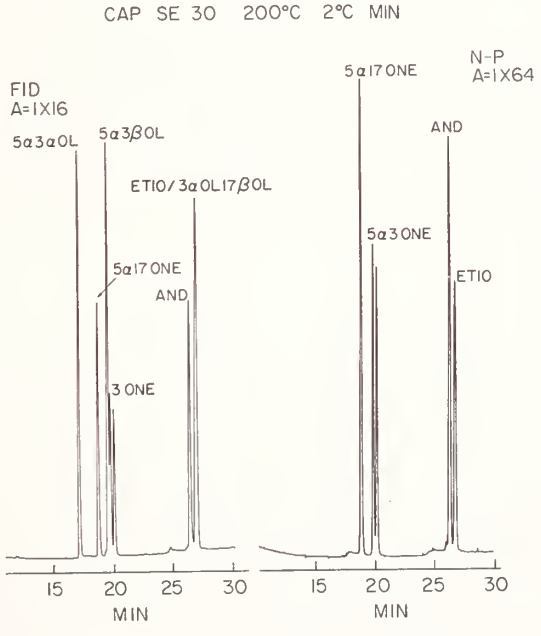


Figure 5. Separation of steroids MO-TMS on a SE 30 glass capillary column. The effluent stream was split equally to a flame ionization detector (left panel) and to a nitrogen phosphorus detector (right panel); the respective attenuations were 1×16 and 1×64. The following abbreviations were used: 5α3αol=5α-androstan-3-ol-TMS; 5α3βol=5α-androstan-3β-ol-TMS; 5α17one=5α-androstan-17-MO; 3one=5α-androstan-3-MO; AND=androsterone MO-TMS; ETIO=etiocholanolone MO-TMS; 3αo117βol=3α-androstand-3α,17β-diol-diTMS.

columns [11]. Figure 6 represents a profile of the baboon neutral fraction, the top panel was obtained with the FID and the arrows indicate hydrocarbons added to measure the methylene

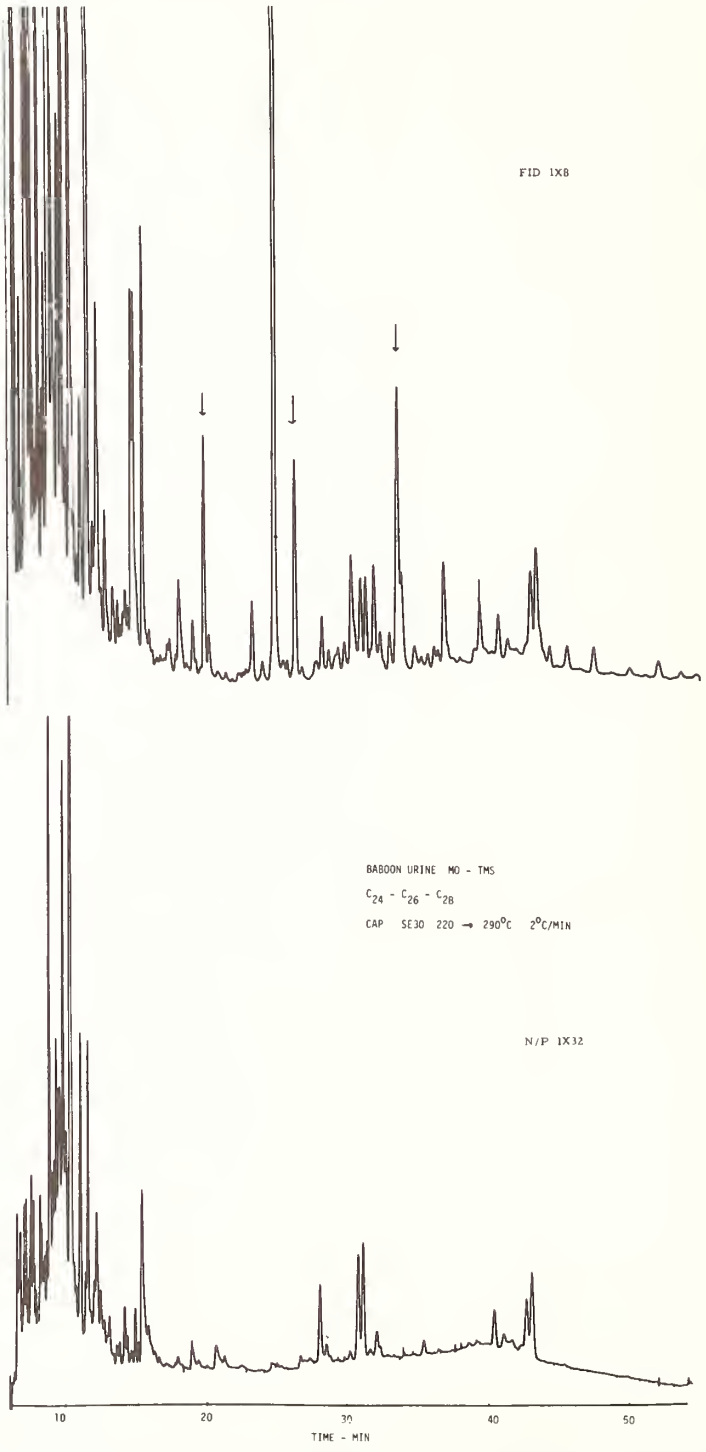


Figure 6. Separation of baboon urinary steroids MO-TMS on a SE 30 glass capillary column (220 °C to 290 °C at 2 °C/min). The signal obtained with the flame ionization detector is shown on the top panel (attenuation 1×8). The arrows indicate the 3 hydrocarbons C_{24}-C_{26}-C_{28} added for the measurement of the methylene units values. The lower panel was obtained under the same conditions with the N/P detector (attenuation 1×32).

units. The bottom panel gives the response obtained with the N/P detector. Again a similar sensitivity was achieved with both detectors.

C. CLEAN-UP PROCEDURES

Since the group responsible for the detection is introduced through derivatization, it is necessary to remove the excess of reagents after the reaction in order to avoid the saturation of the detector. The problem is similar to the one encountered with the electron capture detector.

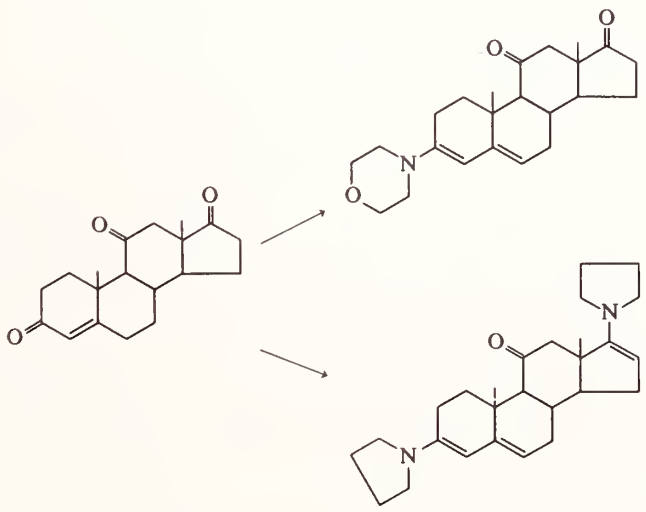
MO derivatives, usually prepared in pyridine, are not purified before the silylation step since it was observed that the O-Methylhydroxylamine hydrochloride was a powerful silylation catalyst [9]. Since the silylating agents bis(trimethylsilyl)acetamide and trimethylsilylimidazole contain a nitrogen atom as part of their molecule, purification of the derivative is advisable. If trimethylsilylimidazole is used as the silylating agent the derivatized steroids can be extracted with hexane. A few percent of dimethoxypropane may be added as a water scavenger but the sample must be kept dry as much as possible since methanol, the reaction product between dimethoxypropane and water, can also hydrolyse TMS derivatives.

Axelson and Sjövall developed a more general procedure to remove the excess of reagents. The derivatized steroids are adsorbed onto a small Lipidex 5000 column (or similar material) while the reagents are not retained. The steroids are then eluted with a mixture of hexane-pyridine-hexamethyldisilazane-dimethoxypropane (97-1-2-10 v/v). Pyridine may be omitted if tailing presents a problem.

D. OTHER NITROGEN-CONTAINING DERIVATIVES

It has been observed that MO-TMS derivatives may lower the response of the N/P detector. This is particularly true when larger amounts are analyzed with packed columns. MO-Derivatives are formed with all keto groups (with the exception of the C_{11}) due to steric hindrance [9]. It would be valuable to obtain a derivative specific for conjugated ketones since the 4-ene-3-one group is a common entity of all steroid hormones (at the exception of estrogens).

It is known that cyclic secondary amines (such as pyrrolidine, morpholine, piperidine in alcoholic solutions) react extremely fast with ketones to yield enamines [16]. Although the reaction is possible with simple ketones, it is much faster with conjugated ketones: the rate being also dependent upon the nature of the secondary amine. The following scheme shows the possible derivatization of 4-androstene-3,11,17-trione with pyrrolidine and morpholine. The 11-ketone does not react due to the steric hindrance in the formation of the tetrahedral intermediate; morpholine



reacts only with the conjugated ketone while pyrrolidine is able to form the enamine both in C_3 and C_{17}. We have prepared the enamine of testosterone, and this derivative is amenable to gas chromatography.

It should be remembered that enamine is easily cleaved under acidic conditions to give the parent ketone. The enamine can also be stabilized by reduction with sodium borohydride [16]. Benzylamine would also give stable derivatives with conjugated ketones [12].

E. Phosphorus-Containing Derivatives

Since the detector is also sensitive to phosphorus (in most instances the sensitivity is even greater for phosphorus than for nitrogen), a logical step would be to introduce phosphorus into the steroids. This route has been developed in the last few years by Vogt and his collaborators [10,18]. Here the site of attack is the hydroxyl rather than the keto group.

Dimethylphosphinic esters were prepared first by reaction of dimethylphosphine in presence of oxygen [10]. However the stability of these derivatives was limited and recently the same authors reported the formation of thiophosphinic ester by condensation of dimethylthiophosphinic chloride with hydroxysteroids [18]. The method looks promising although some elimination of phosphorus containing-groups was reported. With that procedure estrone, as the dimethylthiophosphinic ester, was measured in the picogram range.

IV. Acknowledgments

This work was supported by Grants GM-13901 and HL-17269 from the National Institutes of Health.

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

ANALYSIS OF NEUTRAL LIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND NEPHELOMETRIC DETECTION

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A nephelometric detector, developed for high-performance liquid chromatography, has been applied to the sensitive detection of nonpolar lipids. By utilizing reversed-phase chromatography for the separation of cholesterol, cholesterol esters, and triglycerides, the distribution of these lipids in physiological fluids can be determined. Procedures have also been developed for the determination of the fatty acid composition of individual triglycerides in plant oils. The current detection limit is 0.5 μg for lipid solutes, and the response linearity of the system has been extended to approximately 100 μg.

Key words: Cerebrospinal fluid; detector; high-performance liquid chromatography; light-scattering; lipids; nephelometry; oils; reversed-phase; serum.

I. Introduction

An important class of biologic compounds, neutral lipids, has been a subject of numerous chromatographic studies [1,2]. Although column-chromatographic and thin-layer separations may still be predominant in most laboratories, high-performance liquid chromatography (HPLC) has many desirable features to be employed in the lipid analysis; pertinent analytical aspects are discussed in a review article by Aitzetmüller [3].

Proper column selection is of utmost importance in establishing routine chromatographic procedures. Whereas the experience gained with lipids in the classical adsorbents and solvent systems is basically transferrable to HPLC work, the recently available nonpolar bonded phases deserve additional attention. It has been a common experience that such phases, when used in a reversed-phase chromatographic mode, have a significant advantage of retention reproducibility. In the reversed-phase HPLC, the neutral lipids will elute in order of increasing carbon number and decreasing saturation.

Among the neutral lipids, cholesterol, cholesterol esters, and triglycerides are of major clinical importance. The methods for their determination make use of sequential chemical reactions (including enzymatic treatment) coupled with spectrophotometric or fluorimetric detection of the reaction products. Analytical accuracy is potentially impaired by the presence of interfering compounds [4]. Furthermore, these methods give no indication of fatty acid distribution within the triglycerides or cholesterol esters, but only total triglyceride or total cholesterol ester content. While no clinical significance has been attributed at this time to such distributions, an availability of chromatographic profile procedures for biomedical investigations appears desirable.

Advantages of HPLC in the lipid analysis could hardly be exploited without a suitable detector. Whereas the previously applied refractive index and moving-wire detectors [3,5] have not found wide acceptance, a use of the variable-wavelength spectroscopic detector at values close to 200 nm is not free of potential interferences. Several detection problems in the lipid analysis are largely overcome by the light-scattering (nephelometric) detector developed recently [6] in our laboratory.

This communication describes the application of the reversed-phase HPLC and nephelometric detection to the analysis of lipid extracts of physiological fluids and oil materials. Following the chromatography of lipids in a water-miscible organic mobile phase, the column effluent is mixed with a dilute aqueous salt solution, causing the "precipitation" of lipid solutes. The precipitate is then detected in a low-dead-volume nephelometric cell. The detector is of a non-destructive type and solutes can be easily recovered following their detection.

II. Detection and Column System

As shown in Figure 1, the effluent from a liquid chromatograph is combined in a mixing tee with a reagent which causes selected solutes to precipitate. The mixture then flows through a small (approximately 17 μL) glass cell that is probed by a helium-neon laser beam. The scattered light is detected at the right angle from the incident beam. The details of the experimental design are described elsewhere [6].

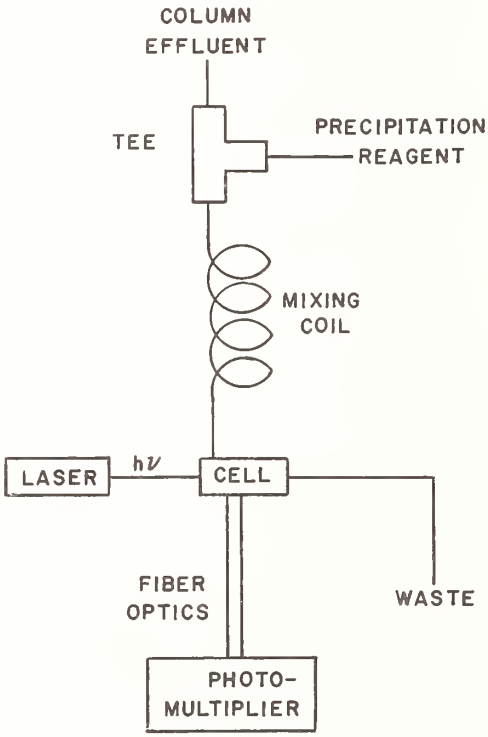

Figure 1. Schematic diagram of nephelometric detection system.

An earlier version [6] of the light-scattering detection cell was recently modified to extend the response linearity. Figure 2 illustrates the current cell arrangement. The mixture containing a precipitated solute enters the stainless-steel block and flows through a narrow slit in a 0.75 mm wide Teflon disc (sandwiched between a Pyrex glass window and a second Teflon disc) into the glass cell. The volume between the window and the glass cell is minimized together with the volume of solution and precipitate through which the incident beam passes before reaching the analytical cell.

All lipid separations were performed on a 30 cm×3.9 mm, i.d., μ-Bondapak C_{18} reversed-phase column (Waters Associates, Milford, Mass.). Optimum separation was achieved using a 2:1 (v/v) mixture of acetone and methanol as the mobile phase.

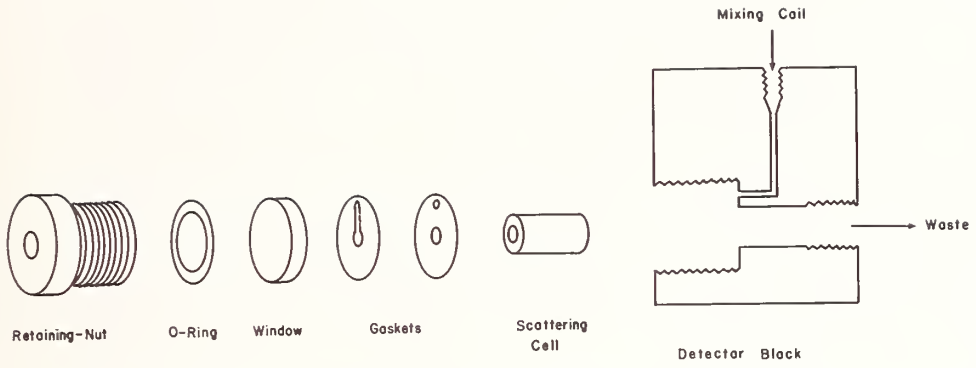

Figure 2. Simplified schematic of light-scattering detection cell.

As it was established in a previous study [6], precipitation of the lipid material is best accomplished by mixing the column effluent with an aqueous solution of ammonium sulfate. In addition, the previously established optimum values of the precipitant concentration and the flow-rates of both the mobile phase and the precipitation agent were found fully compatible with the improved detector system.

III. Analytical Performance

While the used mobile phase provides for an efficient separation of lipids, it has an adequate miscibility with the aqueous precipitation agent. Other solvents of a similar polarity range were found compatible with the nephelometric detection.

The detector response is directly proportional to the square of the sample weight for sufficiently small sample sizes. Figure 3 shows that for cholesterol and cholesteryl linoleate, the response is linear to approximately 100 μg, above which a negative deviation is observed. The departure from linearity with larger samples is primarily due to greater amounts of precipitate in the cell and the already discussed [6] "end-absorption" effect. However, the newer cell design described here has significantly extended the detector linearity. The squared response is also observed when two or more solutes are simultaneously detected as in the case of chromatographically unresolved peaks. In such a case, the deviation from linearity occurs above a combined weight of approximately 100 μg.

Figure 4 illustrates a high-sensitivity chromatogram of four standard lipids. Typical detection limits are around 0.5 μg. Quite importantly, similar response factors are exhibited by each of the standard solutes as indicated by the relative peak heights.

IV. Applications

The chromatogram of standard lipids (Fig. 4) provides already an indication that the column and detector operating conditions are fully compatible. The detection sensitivity appears adequate for lipid analyses in typical samples. The reversed-phase HPLC offers a fast, convenient method for the separations within the lipid classes. Such separations should find immediate application in biomedical investigations. Some examples related to the analysis of plant oils, human plasma, and the cerebrospinal fluid are given.

The triglyceride profiles of four different vegetable oils are shown in Figure 5. The separation on the reversed-phase column is determined by the carbon number and the degree of unsaturation in the fatty acid chains. The lipids elute in the order of increasing carbon number and saturation, while the addition of one double bond is equivalent to the "loss" of two carbon atoms [7].

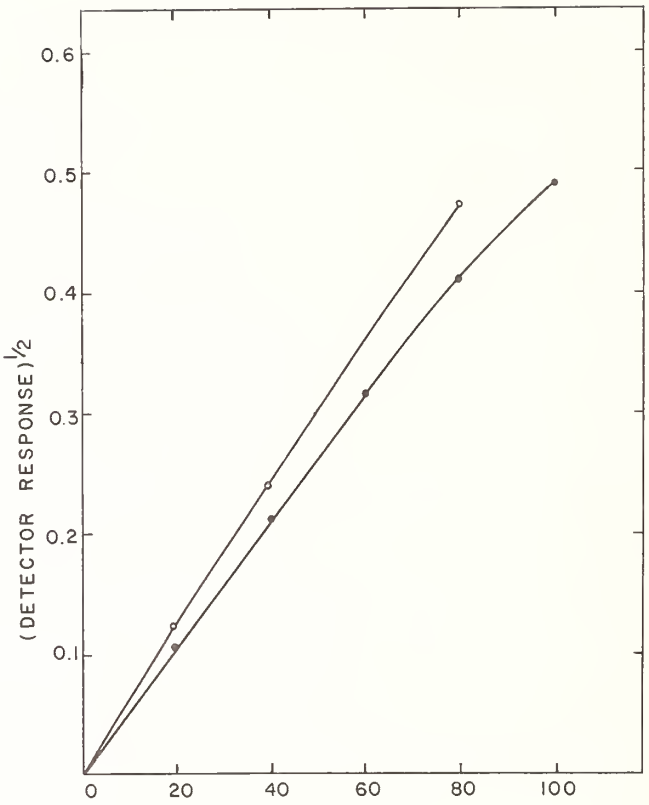

Figure 3. Dependence of detection response on sample size. Detector responses were measured as relative peak heights of the solutes chromatographed. Conditions: column flow-rate, 1.0 mL/min; mobile phase, acetone: methanol, 2:1 (v/v); precipitation agent concentration, 0.01 mol/L ammonium sulfate; precipitation agent flow-rate, 2.0 mL/min 0=cholesterol; ●=cholesteryl linoleate

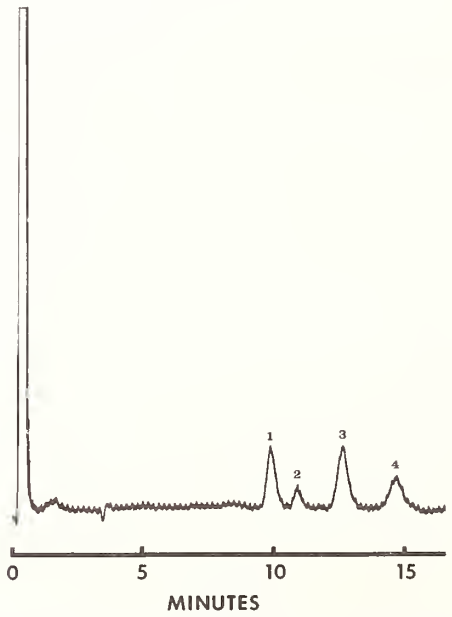

Figure 4. Chromatogram of standard lipids at high sensitivity. Conditions: column flow-rate, 1.0 mL/min; precipitation agent concentration, 0.025 F ammonium sulfate; precipitation agent flow-rate, 0.5 mL/min. 1=triolein (2 μg); 2=cholesteryl linoleate (1 μg); 3=cholesteryl palmitate (2 μg); 4=tristearin (2 μg).

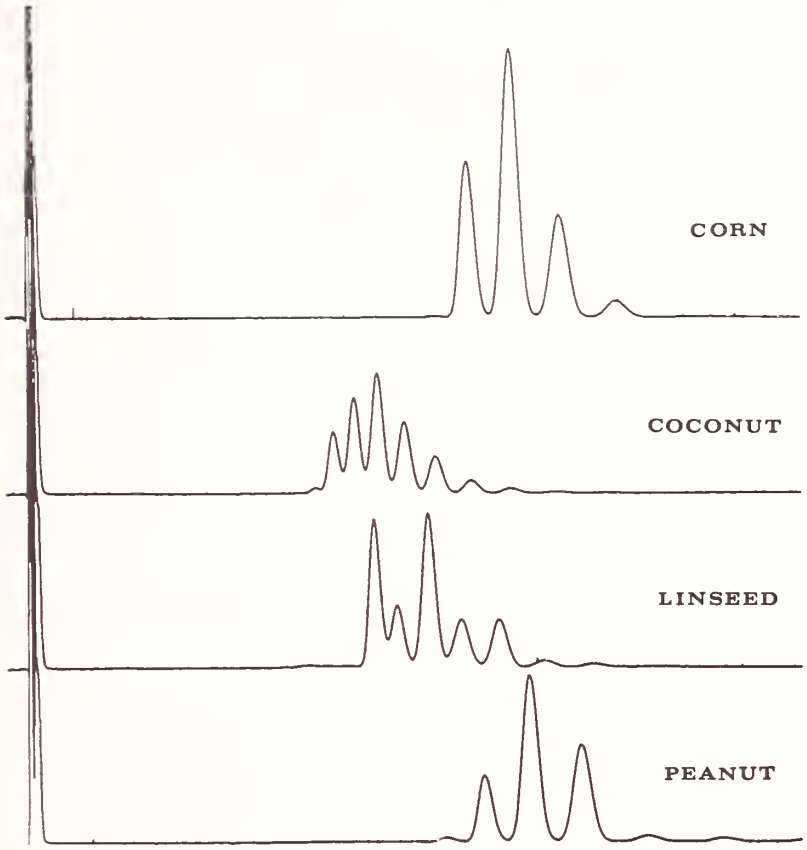

Figure 5. Chromatograms of four vegetable oils (100 μg each). Conditions: same as in Figure 3 except that precipitation agent concentration, 0.025 mol/L ammonium sulfate. Detected peaks are various triglycerides, which elute within 15 min of injection.

Whereas the triglyceride chromatographic profiles provide a means of "fingerprinting" an oil, a more complete analysis is feasible using the described methods. As shown in Figure 6, the determination of the fatty acid content in individual triglycerides is feasible. Chromatogram A shows the separation of cottonseed oil triglycerides by the reversed-phase chromatography. Since the nephelometric detection is non-destructive, the individual fractions can be collected for further analysis. Thus, fraction 2 was collected and rechromatographed (B) on the same column, but with a different mobile phase (2:3 (v/v) mixture of acetone:methanol, containing 0.4% $AgNO_3$). Since the mobile-phase silver ions cause complexation with the double bonds of triglycerides, the elution order is now primarily determined by a degree of unsaturation. Consequently, an additional separation dimension is available.

Further analysis may eventually lead to additional structural information. Fraction B of chromatogram B was collected, and the sample was hydrolyzed with alcoholic potassium hydroxide. Methyl esters of the liberated fatty acids were prepared using the hydrochloric acid/methanol procedure [7]. Finally, the resulting mixture can be analyzed by gas chromatography and a combined GC/MS. Chromatogram C of Figure 6 shows the separation of fatty acid methyl esters obtained with a 30 meter OV-101 glass capillary column. The ratio of methyl oleate to methyl linoleate is 1:2, indicating that this arbitrarily chosen triglyceride consists of one residue of oleic acid and two residues of linoleic acid.

While chromatography of human blood lipids resulted in a number of peaks [6], mass-spectrometric investigations indicated occurrence of some overlaps between cholesterol esters and

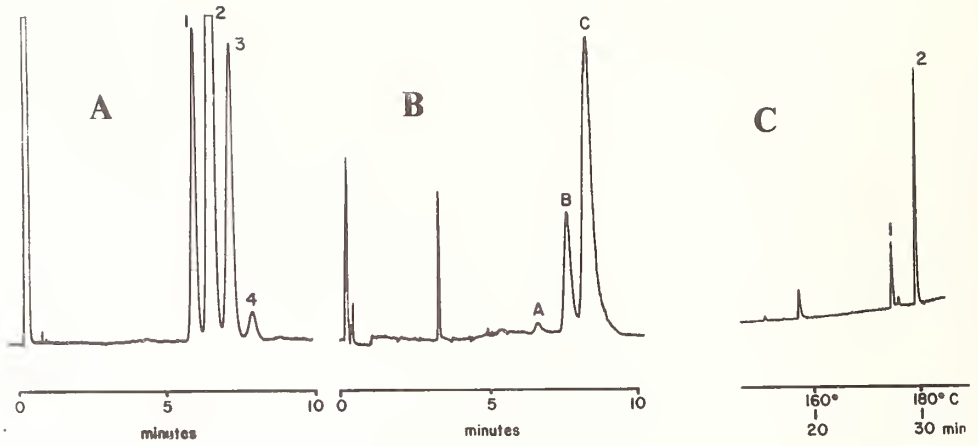

Figure 6. Analysis scheme for the determination of fatty acid distribution within vegetable oil triglycerides. (A) Chromatogram (HPLC) of cottonseed oil. Conditions: same as in Figure 3. (B) Chromatogram (HPLC) of fraction 2 of chromatogram A. Conditions: same as in Figure 3 except mobile phase composition, acetone: methanol, 2:3 (v/v), containing 0.4% $AgNO_3$. (C) Gas chromatographic separation of the hydrolysis products of fraction B of chromatogram B, chromatographed as fatty acid methyl esters. Chromatographic conditions: 30 m×0.2 mm, i.d., OV-101 glass capillary column, temperature programmed from 120 °C at 2 deg/min 1 = methyl oleate; 2 = methyl linoleate.

triglycerides. This difficulty can be overcome through an enzymatic sample treatment. Chromatogram A in Figure 7 demonstrates the separation of a Folch extract [8] of human serum, where peaks 2–7 are partially mixed cholesterol esters and triglycerides. Chromatogram B is the extract of the same serum sample after incubation with pancreatic lipase (obtained from Sigma Chemical Company, St. Louis, Missouri). The hydrolysis products are not precipitated under the used conditions and do not appear in the chromatogram. The four remaining peaks correspond to cholesterol and cholesterol esters.

High sensitivity of the nephelometric detection was utilized in obtaining a lipid profile of human cerebrospinal fluid (Fig. 8). The lipids were extracted from 9.5 mL of a cerebrospinal fluid with a 2:1 (v/v) mixture of cholorform and methanol, and an aliquot was used for sample injection. The retention time of the largest peak (3 minutes) corresponds to that of cholesterol, and the last 2 peaks (retention times around 10 minutes) appear to be cholesterol esters. However, only tentative identifications have been made at this time.

V. Conclusions

A novel detector based on the nephelometric principle has been described. The detector appears to be a valuable addition into the family of HPLC spectroscopic detectors. In selected applications, it surpasses and complements the other existing detectors. This is exemplified by a successful detection of neutral lipids with the nephelometric detector; analytical problems with other detector types in this field have been widely experienced.

While the response-affecting basic factors were reported in a previous communication [6], an improved cell design reported in this work yields the advantages of adequate sensitivity and the response linearity up to approximately 100 μg.

The detection conditions are compatible with the use of reversed-phase HPLC. Several successful applications to natural lipids have been demonstrated. Sterols, sterol esters and triglycerides in samples of both plant and animal origin can be separated. In addition, similar approaches are now feasible to investigate other non-polar materials such as petroleum constituents, plant waxes, terpenes, etc. The non-destructive nature of this detector is of advantage in further structural investigations of the separated and detected sample components.

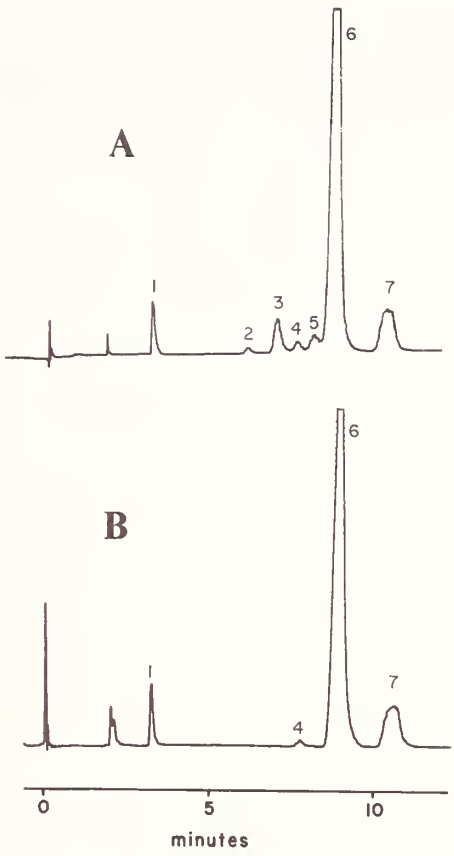

Figure 7. Chromatograms of the Folch extract of human serum. Conditions: same as in Figure 3. (A) Folch extract of lipids before enzymatic treatment. 1=cholesterol; 2–7=mixtures of cholesterol esters and tri-glycerides. (B) Folch extract of serum lipids after incubation with pancreatic lipase. 1=cholesterol; 4,6, and 7=cholesterol esters.

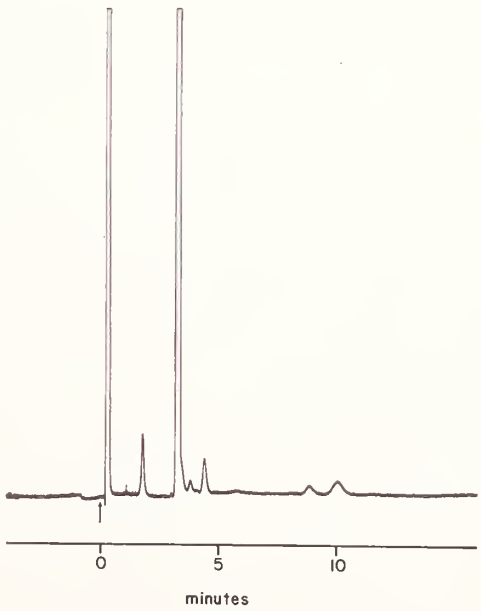

Figure 8. Chromatogram of lipids in 9.5 mL human cerebrospinal fluid. Conditions: same as in Figure 3 except precipitation agent concentration, 0.1 mol/L ammonium sulfate; precipitation agent flow-rate, 4.0 mL/min.

Additional future utilizations of the nephelometric detector will be strongly dependent on a design and proper conditions of post-column precipitations. These could include the solubility changes due to an altered environment of solute molecules, formation of insoluble complexes, or conventional chemical reactions.

VI. Acknowledgment

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THE DETERMINATION OF ANABOLIC STEROIDS IN HUMAN BODY FLUIDS

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For the determination and identification of synthetic anabolic steroids in human body fluids some simple and generally applicable screening procedures have been developed employing HPLC, GLC and capillary GC/MS as basic instrumental techniques.

This paper discusses steroids with a tertiary hydroxy group at C-17 and their detection in urine by improved GLC and capillary GC/MS methods.

For reasons of analytical accuracy and precision, a variety of important aspects and problems concerning clean-up and instrumentation have to be considered: (1) Sampling, sample stabilization and storage; (2) problems and sources of contamination; (3) problems of derivatization and their solution; (4) preparation of inert packed columns; and (5) GC/MS coupling of glass capillary columns.

Recent findings of pharmacokinetic and metabolic studies after oral administration may be summarized as follows:

(1) Steroids with a tertiary hydroxy group at C-17 and no other hydroxy group present in the molecule are normally excreted in their unconjugated form;

(2) For the same type of steroids the rate of metabolism is more rapid than their excretion. A drug is therefore only detectable via its metabolites;

(3) Without knowing the major metabolites of each anabolic compound a reliable drug administration regimen seems to be impossible; and

(4) The date of sampling is of great importance since all metabolites are totally excreted within a few days after the last drug application.

Key words: Anabolic steroids; clean-up; contamination; dianabol; gas chromatography-mass spectrometry (gc/ms); gas liquid chromatography (glc); gc/ms interface; high performance liquid chromatography (hplc); inert columns; metabolism; methandienone; urine.

I. Introduction

For many years [1] naturally occurring androgenes and synthetic anabolic steroids have been known to stimulate the biosynthesis of proteins. Although the mechanism of this biochemical activity has not been completely understood these compounds are frequently used to improve physical performance and to accelerate the production of muscular tissue. Because of these effects at least three separate areas for the application of anabolic steriods have to be considered:

- as therapeutic drugs and supporting adjuvants for various diseases with protein deficiencies [2–6],
- as food additives in animal feed to improve the nitrogen balance and to accelerate the rate of growth of slaughter cattle [7,8],
- as doping agents in several sports to achieve gains in muscular strength and size [9–14].

From their chemical structure (Fig. 1) it is evident that anabolic steroids may be classified as less masculinizing substitutes for the naturally occurring androgens. The search for compounds with a negligible androgenic component has swamped the market with some 100 or 200 different drugs [15]. However, these are all still afflicted with the undesirable androgenic effect, which is

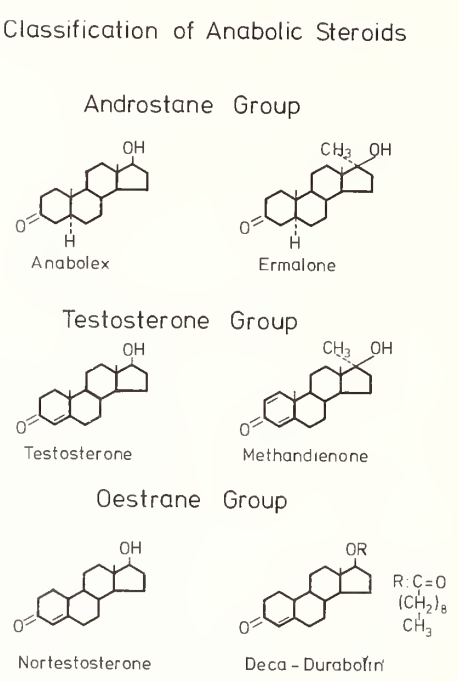

Figure 1. Classification of synthetic anabolic steroids and some typical representatives.

more or less pronounced depending on structural differences. Furthermore, cases of liver damage after the application of massive doses have been reported [16–18] and even small quantities administered over a long period of time may cause an irreversible change in voice pitch [19,20]. It is for these reasons that anabolic steroids are controlled drugs and that their application has to be monitored with a high degree of accuracy and reliability.

Nevertheless, up to now very little is known about their metabolism, their toxicity, their excretion rate and many other parameters of actual importance. Moreover, no generally applicable screening procedure for the control of their administration has been published so far [21–24].

To obtain a scientifically sound basis for legal regulations of the use of anabolic steroids, our institute has developed some generally applicable analytical methods employing hplc, glc and capillary gc/ms as instrumental methods [25–27]. A major part of this development has been dedicated to systematic basic studies necessary to bring the analytical procedure to a high degree of accuracy and precision. This was accomplished by comparing our results with those from other reliable institutions or by applying different and independent instrumental methods to the same sample in our laboratory to exclude sources of systematic errors. According to the principles of modern analytical chemistry, when the same specimen and sample are analyzed by at least two methods, these methods are expected to yield consistent and corresponding results. For amounts down to the low μg- or ng-level the analysis may require longer periods of time due to adsorption or contamination problems. Keeping these essential aspects and remarks in mind a generally applicable scheme for the determination of steroids in different specimens and different binding states is illustrated in Figures 2 and 3.

This paper is concerned with neutral steroids with a tertiary hydroxy group at C-17 and their determination in urine (double line in Fig. 2) by glc and capillary gc/ms. Moreover it is the aim of the paper to emphasize crucial points of the experimental performance rather than to repeat known and published general procedures of steroid analysis. Because of its particular significance

General Clean-up Procedure
for neutral steroid compounds

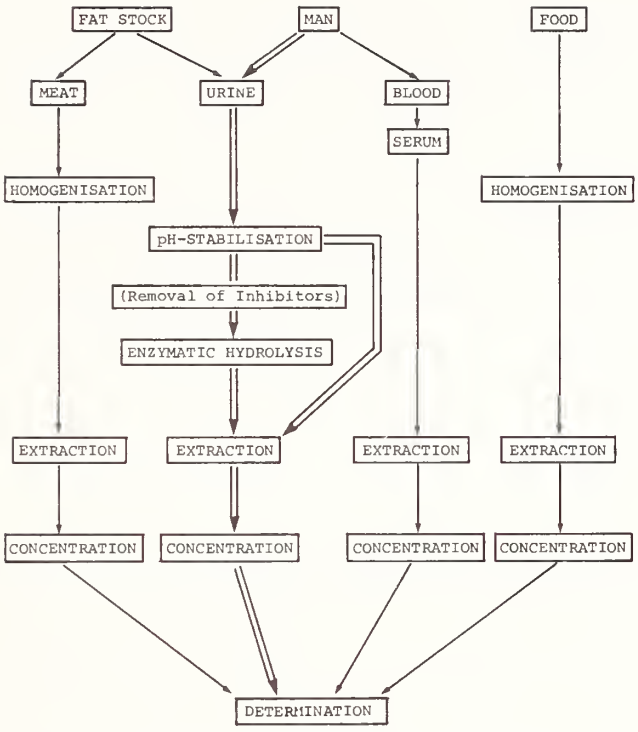

Figure 2. Schematic diagram for the steroid clean-up procedure.

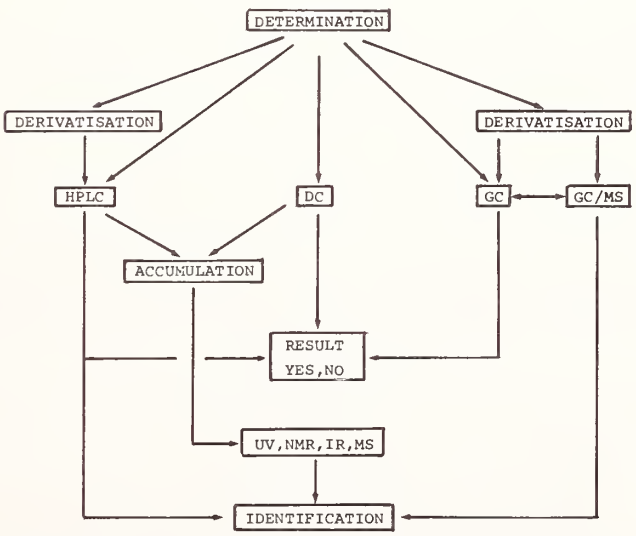

Figure 3. Schematic diagram for the determination of steroids in specimen extracts by different instrumental methods.

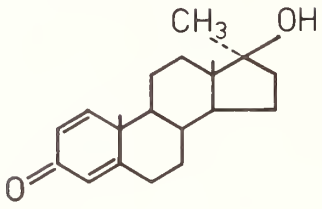

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Figure 4. Structure of methandienone.

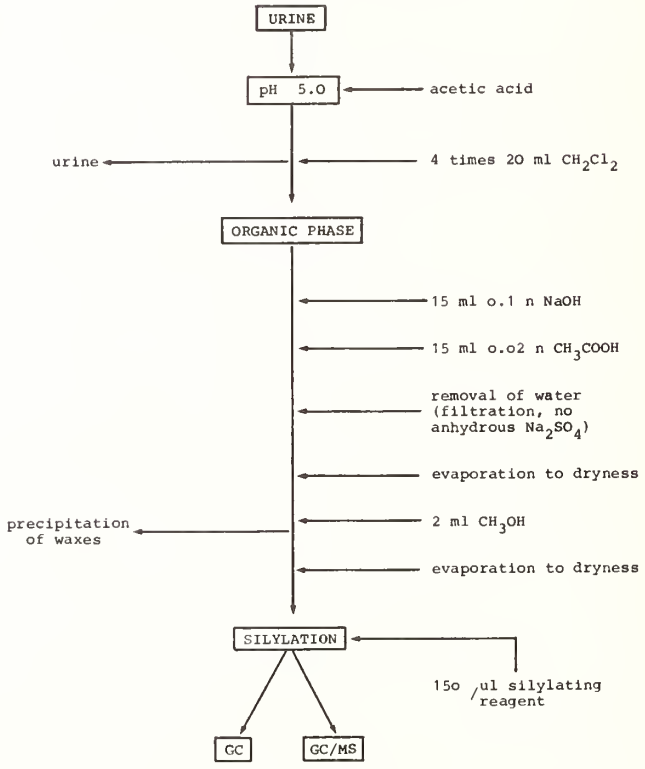

Figure 5. Detailed clean-up procedure for "free" neutral steroids in urine.

as one of the most applied synthetic anabolic steroids 17α-methyl-17β-hydroxy-androsta-1,4-dien-3-one (methandienone, methandrostenolone or dianabol) (Fig. 4) has been selected for discussion.

II. Experimental

In the clean-up procedure for unconjugated steroids (Fig. 5) some details of particular significance have to be mentioned.

A. Sampling, Sample Stabilization and Storage

The urine samples are collected in glass vessels and subsequently adjusted to pH 5.0+0.1 with concentrated acetic acid (quality: Merck suprapur). If determinations are to be carried out

immediately after sampling, the unconjugated steroids are removed from the sample by extraction with CH_2Cl_2 and cleaned up by the procedure given in Figure 5. Different methods of deep freezing and freeze-drying are being investigated for the storage of samples either for short, medium or long periods of time. Due to the very unstable character of steroids in aqueous solutions no method of storage has yet been found to be completely satisfactory. On the other hand, it is evident that steroids should be very suitable compounds for the monitoring of tissue specimen stability particularly in the context of specimen banking.

B. Contamination

All chemicals used during the clean-up procedure must be of the highest obtainable purity and should be checked for their suitability prior to use. In general this is indispensable for organic solvents which can be commonly contaminated with phthalates and silicones.

Moreover, in the drying procedure prior to derivatization, the normally used inorganic desiccants, such as anhydrous Na_2SO_4 or $MgSO_4$ are not suitable due to their high content of phthalates and other plastizisers. Thus, filtration through a soft paper filter is employed to remove adherent water droplets which is then followed by prolonged vacuum evaporation under strictly controlled temperature conditions.

C. Derivatization

The derivatization of the steroid extracts (silylation and/or methoximation, calls for the use of additional chemicals which may contaminate the samples. However, for steroids with polar substitutes, derivatization is required for glc or gc/ms analysis, since the resolving ability of the chromatographic columns and the sensitivity of the detecting systems is markedly increased.

Detailed preliminary experiments has shown the following procedure suitable for the complete silylation of sterically hindered hydroxy steroids. One hundred μL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and 10 μL of trimethylchlorosilane are dissolved in 1 mL of pyridine. One hundred or 150 μL of this reagent is then added to the dried urine extract and left at room temperature for approximately 30 min. Without further treatment this solution is then ready for gas chromatography.

D. Instrumental Points

The methods of hplc and glc (with packed or capillary columns) are employed predominantly for routine procedures, while capillary gc/ms is mainly used for the elucidation of unknown metabolite structures or in cases of uncertainty. Since details of these measurements as well as their operating conditions have been published elsewhere [25-27], only two points of general importance have to be emphasized here:

- The inertness of chromatographic columns and
- the gc/ms interface used with glass capillary columns.

An excellent method for testing the adsorptivity of chromatographic columns has been described in 1973 [28]. In this test the peak areas of some long chain fatty acids in form of their trimethylsilylesters are compared with the peak areas of suitable *n*-alkanes with similar retention indices. In the case of a totally inactive column, the quotient of these two peak areas (Q-values) normalized to equal amounts of sample injected has a value of 1. Adsorption of the fatty acids is indicated by Q-values <1. A successful deactivation of the columns may be achieved by proper selection of the solid support (particle size and surface) and by different precoating techniques with suitable liquid phases, such as Dexil 300 or Carbowax 20M [28].

In the combination of glass capillary chromatography and mass spectroscopy the quality of the chromatograms is mainly determined by the interface if an inert capillary column is used.

Ideally, the interface is expected to meet the following criteria:

- absolute inactivity to all compounds
- no deterioration of the chromatograms obtained with glass capillaries
- temperature stability
- long time stability
- suitable flow rates to meet the operating conditions of the gas chromatograph and the mass spectrometer.

According to our experience glass seems to be the only material to comply with these requirements. The necessary deactivation procedure is comparable to the techniques that have been described for the preparation of high performance glass capillaries. It consists of a gas phase reaction with hexamethyldisilazane (HMDS) followed by a coating with silicon polymers (UCCW 982) [29,30]. Glass interfaces treated in this manner exhibit Q-values of 1 which indicates complete inactivity of the interface.

III. Results

A typical gc/ms-chromatogram of the free fraction of a urine sample (collected 7 hours after the administration of 10 mg methandienone to an apparently healthy man of 24 years) is presented in Figure 6. The peaks of interest are numbered from 1 to 6. Peaks 1, 3, and 5 are also present in the control blanks and therefore are suitable as reference compounds. From ms measurements they have been identified as phthalate, squalene, and cholesterol, respectively.

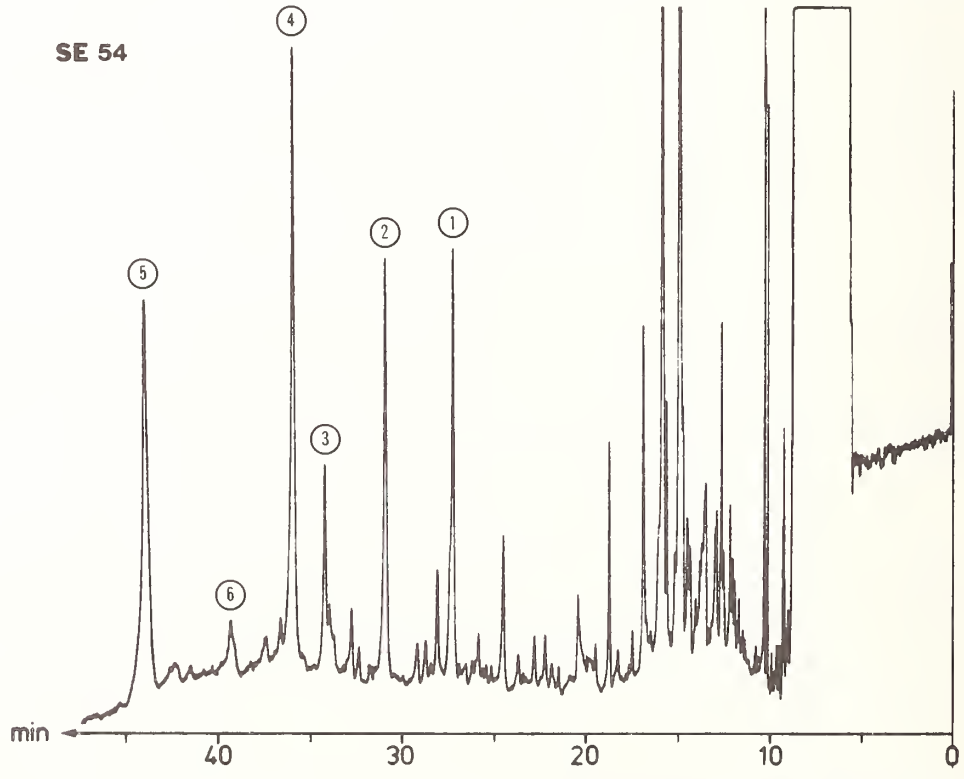

Figure 6. gc/ms-chromatogram of a urine sample after methandienone administration, order of compounds (1) phthalate, (2) 17-epi-methandienone, (3) squalene, (4) 6β-hydroxy-17-epi-methandienone, (5) cholesterol, (6) 6β-hydroxy-methandienone.

From retention index comparisons and extensive ms measurements it is evident that peaks 2, 4 and 6 are metabolites of the administered drug. The ms data indicate the structure of 17-epi-methandienone for peak 2 while peaks 4 and 6 have been identified as 6β-hydroxy-17-epi-methandienone and 6β-hydroxy-methandienone, respectively [31–33]. In all the samples investigated no measurable amount of the nonmetabolized drug could be detected [25].

Glc with packed columns of different polarity as well as hplc and capillary gc/ms are capable of detecting about 5 ng of either metabolite (RSD±8%) [25] which corresponds to a concentration of 10 μg/L in the urine samples examined. The sensitivity may be increased by at least a factor of 10 by gc/ms with single ion monitoring. Moreover, the determination by glc has been totally automated by applying autosampling and data processing devices thus yielding a simple and time saving screening procedure. The time for the total analysis including the clean-up procedure amounts to about 3 hours.

IV. Discussion

From the chromatogram presented (Fig. 6) and from the corresponding hplc and gc data it is evident that two major metabolites (peaks 2 and 4) and one minor metabolite (peak 6) are formed. Moreover, at least one other metabolite (androstan-1,4,16-trien-3-one) has been found. However, since it may be formed during the clean-up procedure it is not conclusively in the metabolic scheme of methandienone (Fig. 7).

In general the metabolic process proceeds without change of the total carbon skeleton, which is in agreement with the literature [33,34]. The stability of the dienone configuration in the steroid ring A seems to be responsible for the suppression of the normally occurring metabolic reactions: i.e., reduction of the double bond at C-4/5 and (or) the 3-keto group. Thus, the metabolic process is initialized by a rapid epimerisation at C-17, partially followed by the unusual formation of the 6β-hydroxy-derivative. Moreover, there seems to be evidence that the epimerisation is a more widespread metabolic process than has been supposed so far.

In addition, it is noteworthy that all metabolites described are found in the "free" fraction of the urine samples examined. No measureable amount of all these compounds could be detected in the conjugated part after enzymatic hydrolysis with β-glucoronidase [27]. A reason for this unusual behaviour may be the sterically hindered hydroxy group at C-17.

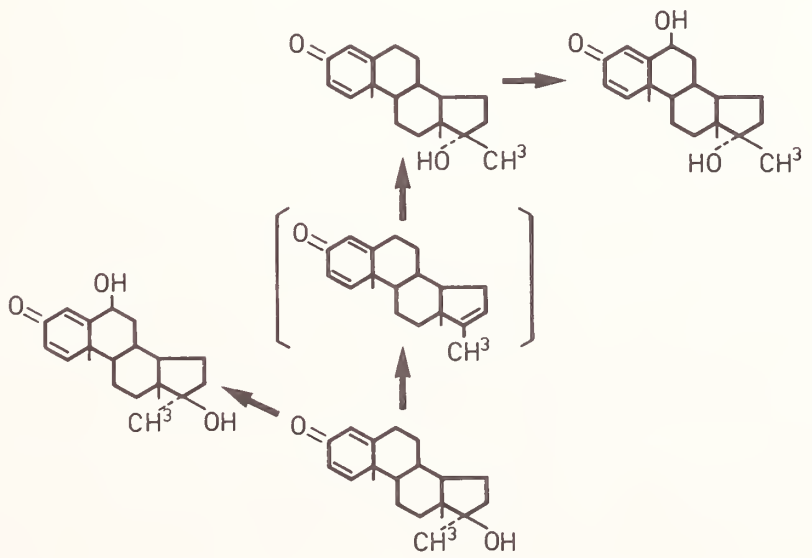

Figure 7. Metabolism of methandienone (major metabolites only).

V. General Conclusions

The results presented show that with gas chromatographic methods, either packed columns or glass capillaries, the administration of methandienone may be detected with excellent sensitivity, accuracy and precision. Thus, the methods applied offer a fast and reliable screening procedure which may be supported by hplc as an independent and convenient analytical alternative to the problem.

Recent results from extensive pharmacokinetic and metabolic studies of this and other compounds may be summarized in the following generalizable statements:

(1) Steroids with a tertiary hydroxy group at C-17 and no other OH-group present in the molecule are normally excreted in their unconjugated form. Thus, hydrolysis with β-glucoronidase [35] may be omitted, yielding a distinct improvement of accuracy and precision of the analytical result.

(2) For orally administered steroids the rate of metabolism seems to be more rapid than their excretion. Due to this fact their administration may be only detected via their metabolites. Therefore, without knowing the specific metabolism of each anabolic steroid administered, a reliable and comprehensive drug analysis seems to be impossible. Moreover, for the same reason, the date of sampling is of great importance as the drugs or their metabolites are totally excreted within a few days after the last administration.

(3) For methandienone the total amount of recovered material in the form of the two major metabolites is only about 5%. The fate of the remaining 95% is still unknown and requires further investigation.

VI. Acknowledgment

The authors are indebted to Mrs. H. E. Frischkorn (clean-up and hplc), W. Leymann (basic studies in capillary chromatography) and Mrs. I. Ohst (clean-up and enzymatic hydrolysis) for their skillful and engaged assistance.

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DEVELOPMENT OF A STANDARD REFERENCE MATERIAL FOR ANTIEPILEPSY DRUGS IN SERUM

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This paper describes one of the procedures developed in the process of producing and certifying an anticonvulsant drugs-in-serum Standard Reference Material (SRM). Four drugs, diphenylhydantoin, ethosuximide, phenobarbital, and primidone are used in the serum standards.

A human serum base is prepared from pooled human plasma. Methods for removing and testing for interferences are described. Uniformity of dispersed sample size is also tested.

Drugs added to the serum matrix are quantitated by gas chromatography using internal standards and bracketing techniques, and by liquid chromatography using calibration standards. The SRM will be supplied as a set of three samples, each having different concentration levels of the four drugs. A serum bank is also included.

Key words: Antiepilepsy drugs in serum; serum matrix; Standard Reference Material (SRM).

I. Introduction

In this paper we describe some of the procedures developed in the process of producing and certifying an anticonvulsant drugs-in-serum Standard Reference Material (SRM). In order to monitor epilepsy therapy, physicians want to know the concentrations of these drugs in patients' serum. The need for an SRM for these determinations is documented by reports of disturbingly large inter- and intra-laboratory measurement variabilities [1]. With the support of the National Institute for Neurological, Communicative Diseases and Stroke, work at NBS was begun with studies of various samples of four crystalline drugs (diphenylhydantoin, ethosuximide, phenobarbital, and primidone) to determine chemical and physical characteristics as analytical criteria for selecting supplies of each of the drugs used as crystalline standards and for use in the serum standards.

In the early stages of this work, bovine serum was considered as the serum matrix. However, human serum was ultimately selected because a bovine-based standard would not be compatible with a currently-used, clinical enzyme-based, immunoassay method which has cross-reacting antibodies to bovine components. Use of a human serum base required a pool of interference-free and drug-free serum. Further, it increased the problems associated with obtaining a uniform product as a vialed, freeze-dried SRM. Methods with sufficiently high analytical accuracy and precision to certify the drugs-in-serum SRM were also needed.

II. Preparation of a Human Serum Matrix for Use in Drug Standards

Because of the dietary and social habits of man, pooled human serum is never found free from a variety of drugs that could interfere with clinical laboratory measurements. In a reference material which may be used for instrument and method calibration, it is preferable to remove interferences that may affect accuracy. However, the treated matrix should have handling properties that closely approximate those of clinical samples.

A. PROCEDURE

Certified hepatitis-free plasma from several donors is combined in a single container. Calcium chloride is added to a final concentration of about 1% (w/v), or until clotting is observed. The addition of glass microscope slides or glass beads often enhances the clotting. After the clot is retracted, the supernatant liquid is centrifuged at 4000×g to remove debris and provide a clarified pool of serum.

Aerosil 380, a SiO_2 product, is added (Degussa, Inc., Teterboro, NJ, 07608) to a concentration of about 10 g/L and the mixture heated to 56° C for 30 minutes. This step removes substantial portions of lipoproteins and deactivates some enzymes.

The mixture is centrifuged at 4000×g for 30 min to pellet the Aerosil. The supernatant liquid is transferred into previously boiled and washed dialysis bags for dialysis vs. deionized or distilled water at 4 °C. This procedure is continued with periodic changes of water until the conductivity of the dialysate is constant for 2 hours or more and near to the conductivity of the water used for the dialysis.

The serum, while still in the dialysis bags, is equilibrated, with 0.15 mol/L NaCl at pH 6.5 to 7.0 until the conductivity of the dialysate is constant (±1%) for 2 hours or more. The sera are combined and passed through charcoal filters to reduce the serum content of non-protein substances. For this procedure, coconut charcoal (6–14 mesh) may be packed in a long glass column and the serum pumped through the charcoal packing. Commercial columns such as the Hemodetoxifier unit (B-D Life Support Systems, Sharon, MA 02067) were found useful. Alternatively, finely-divided charcoal such as Norit may be added (1% w/v) and then removed by centrifuging at high speed (15000×g) for 1 hour or more.

Finally, the material is filtered through 0.22-μm pore-size membrane filters into sterile tared, glass containers.

B. TESTING FOR INTERFERENCES

One of the methods by which serum base is tested for possible interferences before the drugs are added, is by reversed-phase liquid chromatography (LC), using the same conditions described in Section 3A. Test limits: With a detector at 254 nm set at 0.05 Absorbance Units, Full Scale (AUFS), peaks in the chromatographic interval where the drugs elute should not be more than 2% of full scale above the blank baseline. Additionally, the serum base should be examined for interferences by gas chromatographic methods as described in Section 3B. If the material passes these specifications, it may then be used as the serum base for preparation of the SRM. Typical LC chromatograms of unprocessed and processed human serum are shown in Figure 1.

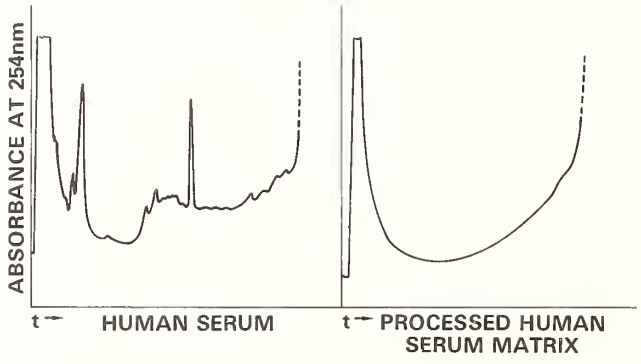

Figure 1. Reversed-phase liquid chromatograms of unprocessed, pooled human serum (left panel) and processed human serum matrix (right panel). Conditions described in Section 3A.

C. Addition of Drugs to the Processed Serum Base

Examples of values (in μg/mL) we have used as target levels are shown in Table 1. The appropriate amount of each drug required to give the desired concentration is added to a 100 mL volumetric flask. About 60 mL of chromatographically-pure ethyl alcohol is added and the drugs are dissolved with the aid of sonic oscillation to complete the dissolution of the crystals. The flask is then filled to the 100-mL mark with the alcohol, capped, and mixed thoroughly.

The solution of drugs is added to the tared serum matrix dropwise over a period of 10–15 min, while stirring the serum constantly, until 1% of the serum volume is added. For example, if 6000 mL of serum is available, 60 mL of the alcohol-drug solution is added.

TABLE 1. *Target values for drugs in serum (in μg/mL)*

	Subtherapeutic	Therapeutic	Toxic
Diphenylhydantoin	4	17	62
Ethosuximide	11	78	177
Phenobarbital	5	22	104
Primidone	3	8	19

D. Dispensing and Freeze-Drying

The drug-spiked serum is dispensed uniformly into vials by use of an automatic pipettor. Pre-calibration of this instrument is essential in assuring accuracy and precision of delivery. All vialed specimens are freeze-dried in a single operation to assure uniformity of the residual moisture. After completion of the freeze-dry cycle, the chamber of the freeze-dryer is repressurized with dry, filtered nitrogen to a slightly reduced pressure (approximately 9/10 atmosphere) and stopper the vials are stoppered.

E. Testing for Uniformity of Dispensed Sample Volumes

In order to estimate vial-to-vial variations in the volume of serum dispensed, a random sampling of about 25 vials from each lot is obtained. These are weighed before and after reconstitution to correct for variation in the addition of reconstituting water. The refractive index of the serum is about 0.01 greater than that of water, so to detect variations of 0.1%, refractive indices must be measured to better than 0.00001. This is readily and rapidly accomplished with a differential refractometer, by comparing the serum samples to a reference solution containing about 100 g/L of KCl in water. The sensitivity of the differential refractometer is about 0.000001, so filling errors are easily detected.

III. Analytical Techniques

One of the analytical goals of the certification procedure is to have two independent methods yield sufficiently accurate and precise data so that assignment of values will be unambiguous. Ideally, the methods used should give significantly better precision than the fill volume variation between vials. Accordingly, we use methods for achieving such accuracy and precision that might not ordinarily be utilized in clinical laboratory situations.

A. High Performance Liquid Chromatography

Quantitative analysis of drugs in serum is performed using reversed-phase LC. A Waters μBondapak C_{18} column (Waters Associates, Milford, MA 01757) is used. The flow rate is 2.0

mL/min; the amount injected is 50–200 μL, depending on the drug level; the gradient is from 0.01M phosphate buffer (pH=6.5) to 100% methanol in 45 minutes; detection is at 254 nm, 0.05 AUFS. Peak areas are determined by use of an electronic integrator. Peak heights are measured in the primidone analysis with a variable wavelength UV detector set at 210 nm in series with the 254 nm fixed-wavelength detector. Standards made up in 20% methanol—80% water and containing each of the four drugs are run alternately with serum samples. Calibration curves are thus obtained concurrently with the analyses and are applied to give the quantitative results. Figure 2 illustrates a typical chromatogram, recorded at 254 nm. The order of elution is ethosuximide, primidone, phenobarbital and diphenylhydantoin.

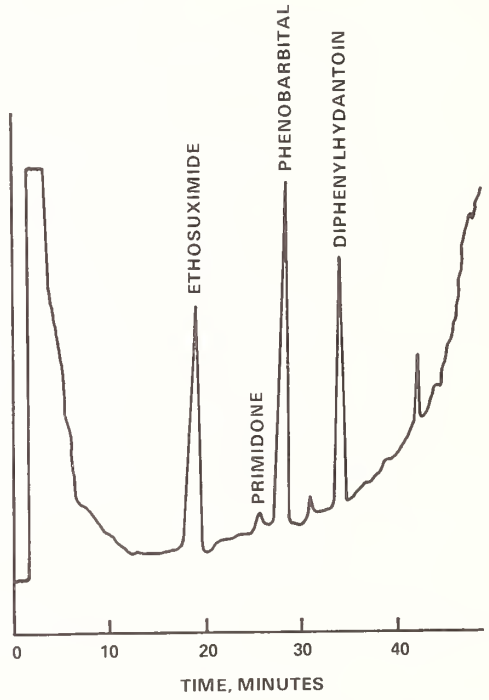

Figure 2. Reversed-phase liquid chromatogram of antiepilepsy drugs in processed human matrix (detection at 254 nm).

B. Gas Chromatography

1. Conditions

Our best analytical results were obtained using a special column packing, GP 2% SP-2510 DA on 100/120 Supelcoport, (Supelco, Inc., Bellefonte, PA 16823) developed to separate anticonvulsant drugs and their metabolites in an underivatized form.

For chromatography, a gas chromatograph equipped with flame ionization detectors was used with the following conditions: initial temperature—150 °C; temperature program rate—16 °C/min; final temperature—265 °C; final temperature—hold—4 minutes; injection temperature—300 °C; FID temperature—265 °C; helium carrier gas at 50 mL/min. The output is interfaced with a computing integrator.

2. Bracketing Technique

Analytical accuracy is improved by using a bracketing method performed as follows: Amounts of internal standards for each drug, calculated to be equivalent to the weighed-in target

values are placed into a 100 mL volumetric flask. The internal standards used for each of the four drugs in the SRM are listed in Table 2. The flask is filled to the mark with ethyl alcohol and the contents mixed thoroughly. An automatic pipettor is used to dispense 100 μL of the mixture into washed, clean centrifuge tubes (29 mm o.d. × 140 mm).

Similar solutions of drugs are prepared, one at a level about 2% lower than the weighed-in target value, and another about 2% higher than the target. The automatic pipettor is used to dispense 100 μL volumes of the drug mixtures into the tubes containing internal standards according to the protocol outlined in Figure 3.

The alcohol is allowed to evaporate to dryness, and the tubes are then capped. Thus, internal standards and drugs are in a dried state and may be kept for several weeks until time for use.

For use, 1.0 mL of the SRM is added to the tube containing only the internal standard. At the same time, 1.0 mL of a serum blank is pipetted into the tubes containing both the internal standards and the drugs at high and low bracketing concentrations. Usually, four SRM's and two each of the high and low bracketing tubes are processed as a group.

The sera are acidified with 3 drops 3 mol/L HCl to pH 1–2 and then extracted with 10 mL of high-purity chloroform. After shaking and centrifugation of the tubes, the aqueous upper layer is discarded and the chloroform decanted into a 15 mL conical centrifuge tube. The chloroform is evaporated at 20 °C under vacuum in a Rotary Evapo-Mix (Buchler Inst., New York, NY 10013). The extract is reconstituted with 50 μL of chloroform and vortex-mixed. One μL of this residue is then injected into the gas chromatograph.

TABLE 2. *Drugs and internal standards used in GC analysis*

Drug	Internal standard
Diphenylhydantion	5-(p-methylphenyl)-5-phenylhydantoin
Ethosuximide	α-Methyl, α-propyl-succinimide
Phenobarbital	5-Ethyl-5-p-tolyl-barbituric acid
Primidone	4-Methylprimidone

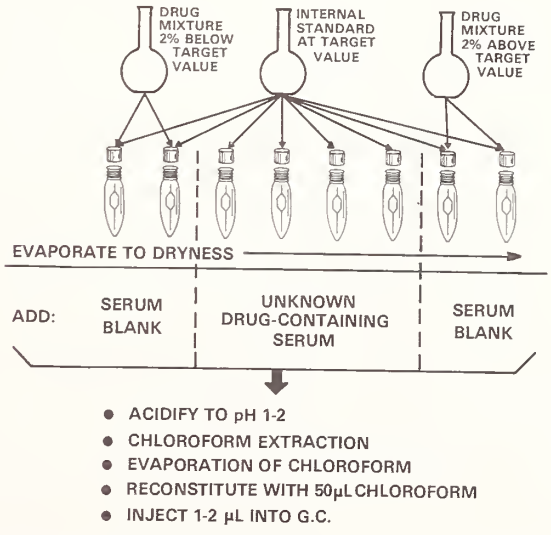

Figure 3. Procedure for preparation of tubes containing drugs for quantitative gas chromatography bracketing.

IV. Results and Calculations

A typical separation of drugs and their internal standards is illustrated in Figure 4. Peak areas are obtained and ratios of drugs to internal standard are calculated. Concentration of drugs in the serum is then calculated using the formula:

$$\frac{R_x - R_{low}}{R_{high} - R_{low}} \times (C_{high} - C_{low}) + C_{low} = C_x$$

where

$$R_x = \frac{\text{Area of Drug Peak}}{\text{Area of Internal Standard Peak}}$$

C_x = Concentration of Drug in μg/mL

Alternatively, peak height ratios can be used instead of peak area ratios. Preliminary, but not certified values obtained by GC and LC for the Therapeutic level of the proposed SRM are shown in Table 3.

TABLE 3. *Therapeutic level in SRM antiepilepsy drugs in serum*

	Drug			
	DPH	E-SUX	ϕ-B	PRIM.
Target weighed-in value (g/mL)	16.4	74.4	21.2	7.9
LC mean of 5 determinations	16.56	73.08	21.49	7.99
Rel. Std. Dev.	2.71%	2.19%	1.10%	4.09%
GC bracketing 5 samples	16.69	78.31	21.88	8.08
Rel. Std. Dev.	0.58%	0.41	0.95%	1.59%

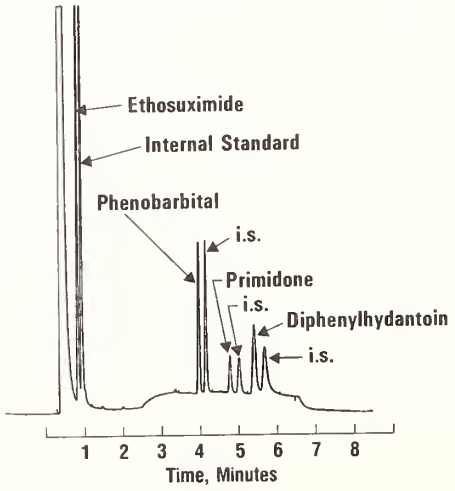

Figure 4. Gas chromatogram of antiepilepsy drugs. Conditions described in Section 3B.

V. Summary

By utilizing precise LC and GC methods, we are certifying the concentration of antiepilepsy drugs in serum as a Standard Reference Material. Procedures for eliminating interfering peaks from the pooled human serum for both LC and GC are developed and described in detail in this paper. Release of the material for public use is anticipated in 1978.

The SRM will be supplied as a set of three samples, each having different concentration levels of four drugs. A serum blank is also included.

VI. Acknowledgment

The authors acknowledge partial financial support from the Institute for Neurological, Communicative Diseases and Stroke.

VII. Reference

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POTENTIOMETRIC TITRATION OF MICROGRAM AMOUNTS OF PENICILLAMINE USING ION SELECTIVE ELECTRODES

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Over the last 20 years penicillamine (PA) has seen application in the treatment of Wilson's Disease, Cystinuria, and heavy metal poisoning. Recently it has shown promise as an effective therapeutic agent in the treatment of rheumatoid arthritis (RA). However, its mode of action in RA therapy is not known. To aid in more effectively monitoring patients treated with this investigational drug and to provide a tool for studying the fundamental aspects of PA therapy, we have sought to develop a sensitive analytical procedure applicable to physiological fluids.

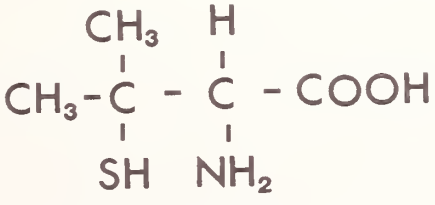
That PA is a strong metal chelating agent is the basis of its use in the treatment of heavy metal poisoning and Wilson's Disease. Very strong complexes are formed with Hg^{2+}, Cu^{2+}, and Pb^{2+}. This same characteristic was exploited in developing our analytical procedure.

The method utilizes an automatic titration system equipped with a first derivative mode for the recorder. The sample is maintained at a constant pH (usually 9) using a Tris buffer. Ionic strength is adjusted by addition of sodium perchlorate solution. Typically, the sample is titrated with a standard solution of lead perchlorate (analyzed with EDTA). Initially PA will complex Pb^{2+} as it is added to the system. When all available PA is complexed, excess titrant will add free Pb^{2+} to the solution. Pb^{2+} is detected by a lead specific electrode referenced to a double junction reference electrode containing a solution of sodium nitrate in the outer chamber. A first derivative endpoint is recorded. Less than 0.2 μg PA/mL can be determined.

Key words: Clinical chemistry; ion selective electrodes; penicillamine; potentiometric titration.

I. Introduction

Penicillamine (PA) is a thiol containing α-amino acid which occurs naturally as a hydrolysate of penicillin type antibiotics. It was first isolated and characterized by Abraham, et al. in 1943 [1]. PA is a white crystalline powder, stable in dry form and soluble in water and alcohol. Solutions are stable against oxidation at pH 2–4. The pK values for the three ionizable centers are 1.8, 7.9 and 10.6 corresponding to the carboxyl, amino and thiol groups respectively.



Penicillamine structure

PA was first used therapeutically by Walshe in 1956 [2] in the treatment of Wilson's Disease which is characterized by an excess of copper in organ tissues. Walshe had identified PA in the urine of patients being treated with penicillin for advanced hepatic disease [3]. He noted PA's stability in reduced form, ready excretion by the kidneys and chemical structure, and deduced that it should readily chelate Cu^{++} thus promoting its excretion. The capacity of PA to form strong chelates with heavy metals prompted Boulding and Baker [4] in 1957 to suggest it for

the treatment of lead poisoning. A third therapeutic application of PA is cystinuria which is characterized by the excessive urinary excretion of cystine (i.e., cysteine disulfide). Cystine has poor solubility in acid urine resulting in recurrent stone formation in the renal tract. PA was used by Crawhall et al. in 1963 [5] on the basis of its ability to undergo thiol exchange with cystine. This results in the formation of the more soluble mixed disulfide (PA-cysteine) which is then readily excreted. Cystinuria, Wilson's Disease, and certain types of heavy metal poisoning are the therapeutic applications of PA which have been granted approval by the Food and Drug Administration.

PA has recently gained attention from evidence that showed it to be effective in the treatment of rheumatoid arthritis (RA). The rationale for PA's initial application [6,7] to RA was its observed ability to dissociate macroglobulins in vitro. This was at a time when the possible role of the rheumatoid factor (RF) in the pathogenesis of RA was subject of much speculation. Upon treatment with PA, RF titres did decrease, and clinical improvement was noted. However, Jaffe subsequently determined that these two observations were not directly related [8]. Clinical trials continued to support the clinical effectiveness of PA in RA [9].

As of yet the mode of action of PA in RA is unknown. To effectively study the fundamental aspects of PA therapy a means is required to monitor its absorption, distribution, metabolism, and excretion by the body. In addition, a number of side effects have been noted during PA therapy including allergic reactions, loss of taste and, more seriously, proteinuria and bone marrow depression. Effective monitoring of patient levels of PA might help to decrease the incidence of such side effects. General therapy optimization would be served by bioavailability and pharmacological studies. All of the above require an analytical procedure applicable to physiological fluids which provides sensitivity, specificity, precision and accuracy. It is also important that cost and analysis time not be restrictive.

Previous analytical approaches have included ion exchange, automatic amino acid analysis, gas chromatography, immunological, radioimmunological and, most recently, high performance liquid chromatography techniques. Borner [10] carried out a performic acid oxidation then separation on an anion exchanger followed by measurement using ninhydrin. This method did not differentiate between PA and PA disulfide. Cation exchange with gradient elution was applied in automatic amino acid analysis by Crawhall, et al. [11] but was limited by the duration of analysis, up to 21 hours. Gas-liquid chromatographic analysis after derivatization with pivaldehyde was recommended by Jellum et al. [12], but attempts at duplicating the results in this laboratory was unsuccessful. Assem developed both immunological [13] and radioimmunological [14] procedures for PA in physiological fluids. The former involves a haemagglutination-inhibition technique while the latter use ^{14}C-labeled D-PA. A major limitation of both procedures is the lack of the availability of required materials, such as the PA antisera and the labeled compound, resulting in significant expenditure for implementation. Recently, a high performance liquid chromatographic procedure was published by Saetre et al. [15] which utilized a mercury-based electrochemical detector. Both the reduced and total PA can be determined by the latter procedure.

The colorimetric procedure of Pal [16] has been mentioned in the literature as a standard means for determining PA in biological fluids. In view of this, the procedure was more closely examined by us for potential improvements and comparison to any newly developed procedures of the research group. In the method of Pal PA is reacted with potassium cyanide and ferric chloride and read spectrophotometrically at 645 nm. Method familiarization revealed that the sequence of reagent addition was critical (i.e., potassium cyanide first), and reagent optimization studies resulted in doubling the potassium cyanide concentration in the reacting solution. In addition, technique modifications were applied including the use of volumetric glassware, strictly reproducible mixing procedures (use of vortex mixer) and a double centrifugation step prior to spectrophotometric readout. Using the modified procedure the following statistics were obtained. Precision: $N=10$, standard deviation$=0.003$ (absorbance), relative standard deviation$=1.3\%$. Linearity: $y=2.35x-0.001$ (x=millimoles PA, y=absorbance), correlation coefficient$=0.998$,

variation = 4.9%. The modified method demonstrated sensitivity to about 1 ppm (this was with an absorbance reading of 0.010).

The structure of PA is such that it is capable of forming strong complexes via sulfur and a second group with many transition metals. Particularly strong complexes are formed with Cu^{++} (log $K_1 = 17.5$) and Pb^{++} (log $K_1 = 13.5$) [17]. PA's complexation capabilities have been the basis of titrimetric procedures applied to its analysis. Doornbos [18] performed a complexometric titration with phenylmercuric acetate to an indicator (diphenylcarbazone) endpoint. More recently Forsman [19] utilized coulometrically generated mercury (II) for the titration of PA. Of more general application and of special interest, has been the potentiometric titration of amino acids using specific ion electrodes as endpoint indicators. Gruen and Harrap [20] reported the titration of cysteine with silver nitrate utilizing a Ag/S specific ion electrode, and Liteanu et al. [21] reported the titration of cysteine, arginine, leucine, and histidine with Hg^{++} solution using a custom prepared Hg^{++} sensitive membrane electrode.

The procedure presented in this paper utilizes a lead selective ion electrode. As previously stated, PA exhibits a strong tendency to complex with Pb^{++}. The stability constant of the 1:1 lead-PA complex is in the vicinity of 10^{14}. During the course of the titration, PA initially complexes with Pb^{++} as titrant is added to the system. The electrode system does not respond to the complexed lead. When all available PA has been complexed, excess titrant adds free Pb^{++} to the solution which is detected by the lead selective electrode.

II. Experimental

In the aqueous studies a 0.001 mol/L solution of PA was prepared using freshly distilled water bubbled with nitrogen for at least 30 minutes prior to use. An aliquot was transferred to a 100-mL titration vessel. The pH was adjusted to 9 with Tris buffer, the ionic strength adjusted to 0.2 with sodium perchlorate solution and the volume brought to approximately 50 mL with distilled water. A nitrogen purge was maintained throughout the determination. The sample was titrated with 0.001 mol/L lead perchlorate utilizing an automatic titration system consisting of: Radiometer Copenhagen Titrator TTT2 equipped with Autoburette ABU 12, Servograph REC 51, Servograph Pen Drive REA 310, High Sensitivity Unit REA 112, REA 201 Derivative Unit, and a 2.5 mL buret calibrated to dispense 0.001 mL increments. The titration was monitored with a lead selective ion electrode (Orion, Model 91-82) referenced against a double junction reference electrode (Orion, Model 90-01) containing a solution of 1 mol/L sodium nitrate in the outer chamber. A first derivative endpoint was recorded.

III. Results

A linearity study was completed in which aliquots of 0.20, 0.50, 1.00, 1.50 and 2.00 mL of a 0.001 mol/L stock solution of PA were taken through the experimental procedure as described above. Each concentration level was analyzed in triplicate, and linearity tabulations were made using the average titration values for each level. The following results were obtained: $y = 0.822x + 0.025$ (x = mL aliquot, y = mL titrant), correlation coefficient = 0.999, standard error of the estimate = 0.018, variation = 2.1%.

A sensitivity study was undertaken to define the procedure's limit. Aliquots ranging from 2.00 mL to 0.05 mL of a 0.001 mol/L stock solution of PA were titrated as described. 7.4 μg/PA (total) was determined to be the minimum amount titratable. This is equivalent to 0.15 μg/mL (0.15 ppm) in the system described. A linear regression performed on the values obtained gave the following results: $y = 1.03x + 0.025$ (x = mL aliquot, y = mL titrant), correlation coefficient = 0.999, standard error of estimate = 0.015, variation = 2.3%.

To determine the precision of the procedure 10 replicate analyses were done on aliquots of the same stock solution. The following results were obtained: N=10, standard deviation=0.018 mL, relative standard deviation=2.0% at the 5×10^{-6} mol/L concentration level.

In further work K^+, Mg^{++}, and Ca^{++} were investigated for potential system interference which might occur in actual samples (i.e., in physiological fluids). In separate trials 10 μmol of K^+, Mg^{++}, and Ca^{++} were added to aqueous solution aliquots containing 10 μmol PA and the titration performed. Results are presented in Table 1.

Preliminary urine studies were undertaken to evaluate the method's potential for application to physiological fluids. Increasing amounts of urine were substituted for water in titration samples of 50 mL total volume which contained approximately 10 μmol PA. All recoveries obtained were greater than 99% for 5, 10, and 15 mL urine substitutions. Relative standard deviation values ranged from 0.5–1.0% for replicate analyses of the 5 and 10 mL urine substitutions. The difficulties seen with direct application to urine were a depressed endpoint signal which could have consequences of affecting method sensitivity and a tendency for the signal depression to increase with successive sample runs. In view of this, steps were taken to develop a rapid clean-up procedure prior to application of the titrimetric method. A 2×2 cm column of a strong acid cation exchanger (Dowex 50–X8, 200–400 mesh, hydrogen form) was used. The column was washed with 0.01 mol/L hydrochloric acid to an acidic pH. The sample (25 mL urine containing approximately 10 μmol PA) was adjusted to pH 2 with 1 mol/L hydrochloric acid and passed through the column followed by a 100 mL distilled water wash. Two elution approaches were tested, elevation of the pH (Tris buffer) and the use of different concentrations of sodium perchlorate. Poor recoveries were obtained using the former. This was possibly the result of PA oxidation. Elution with 100 mL of 0.2 mol/L, 0.5 mol/L, and 0.8 mol/L sodium perchlorate provided 75%, 89% and 97% recoveries respectively. Samples were adjusted to pH 8–9 (Tris buffer) immediately prior to titration. The clean-up step was rapid, requiring about 1/2 hour, resulting in a total analysis time of less than 1 hour.

Current work is aimed at the further optimization of the procedure for urine, application to other physiological fluids, and the utilization of other titrant ions and ion selective electrode systems, viz. Cu(II) and Hg(II) which may further increase the sensitivity of the titration method.

TABLE 1. *Results of interference study*

Titration solution	mL titrant to endpoint
10 μmol PA	1.052
10 μmol PA+10 μmol K^+	1.028
10 μmol PA+10 μmol Mg^{++}	1.037
10 μmol PA+10 μmol Ca^{++}	1.056

IV. Acknowledgments

The suggestions and advice offered by Dr. Vincent Maddi were very helpful. We are indebted to Dr. Israeli Jaffe for informative discussions, comments, and important literature references. A grant from the NIH-BRSG fund of SUNY at Binghamton has enabled G.E.J. to continue this project. The donation of pure D-penicillamine for this work by Merck, Sharp and Dohme is gratefully acknowledged.

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DETECTION AND QUANTITATIVE DETERMINATION OF TRICYCLIC ANTIDEPRESSANTS AND BENZODIAZEPINES IN SERUM AND PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Development and some applications of a liquid chromatographic method for the determination of most of the tri- and tetracyclic antidepressives in use is presented. Detection limits range from 5 to 50 ng per sample. Routine application to the determination of Maprotiline in serum shows that patients can profit from this kind of therapy control.

Key words: Benzodiazepines; clinical use; derivatization; quaternary two-phase systems; serum; uv-detection; tricyclic antidepressives.

I. Introduction

The correlation between serum level and clinical response in the therapy of endogeneous depression is at the moment a point of argument: Asberg and Sjöqvist claim a curvilinear correlation for Nortryptiline [1,2], while Burrows et al. deny the existence of such a correlation for Nortryptiline for a group of patients but claim that each patient has his own optimal serum level in the treatment of depression [3]. Studies in our group show that in the case of Maprotiline, a drug of the second generation, a rather close interindividual correlation between serum level and clinical outcome can be shown [4]. No point of argument is the fact that the metabolism of the drugs is genetically determined and that it shows such big rate differences that nearly no prediction can be made about the attainable serum level at a given oral dose of an antidepressive drug.

Since each antidepressive drug on the market has its own therapeutic profile and each doctor has his own personal experiences with particular drugs we have tried to develop a method for the simultaneous determination of all or nearly all antidepressives currently in use. During method development it turned out that the chromatographic behaviour of the most common minor tranquilizers, the benzodiazepines, is very similiar to the antidepressives. This led to the possibility to test the serum of patients for unauthorized use of benzodiazepines which is a valuable help in the treatment of drug addiction.

Since antidepressives and benzodiazepines are rather big molecules we looked at the potential of High Performance Liquid Chromatography (HPLC), especially as there existed a rather broad experience with the determination of corticosteroids in serum of rat, guinea pig and man [5]. The separation in a two phase system of three or four components had proved its wide applicability [5–7]. So we decided to work in this direction.

During the development of the separation it turned out that the antidepressive drugs consist of two groups of different polarity. The difficulty to elute either the nonpolar and the polar substances in reasonable time and with sufficient sensitivity could be overcome by the column switching technique of Huber et al. [8].

A last difficulty was the poor absorption of Maprotiline at a reasonable wavelength. This difficulty could be overcome by a simple and efficient derivatization procedure [9].

II. Material and Methods

A. Chemicals

All antidepressives were in the form of hydrochlorides, the benzodiazepines in free form, both were supplied to us as the gift of pharmaceutic firms.

p-Nitroazobenzenecarbonylchloride was obtained as reagent grade chemical from Merck (Darmstadt, GFR), Ethanol, n-propanol, iso-propanol, tert. Butanol and benzene were obtained as reagent grade chemicals from Merck (Darmstadt, GFR) and used without further purification.

Morpholine (reagent grade) was obtained from Baker Chemicals (Gross-Gerau, GFR) and used without further purification.

Pyridine (reagent grade) was obtained from Merck (Darmstadt, GFR) and dried over solid reagent grade NaOH before use.

Hexane and dichloromethane were obtained in technical quality from Krämer and Martin (Siegburg, GFR) and distilled before use.

Chromatographic phases were recycled in the following way: A first quick distillation (40–50 °C) separated the lot of dichloromethane from the hexane. An intermediate fraction was collected from 50 to 60 °C, the third one consisted of the rest in the still and contained mostly the hexane. The first fraction was redistilled and the dichloromethane collected between 40 and 45 °C. This dichloromethane was sufficiently pure even for chromatographic purposes. Several batches of the second fraction were taken together and fractionated for the dichloromethane and the hexane fraction. Difficulties in reusing the third fraction even after repeated distillation showed that another purification of the recycled hexane was necessary. A simple and efficient means to do so was to extract the third fraction before the second distillation 3 times with water which removed the alcohols and the morpholine.

For the extraction of serum samples we used the Extrelut-Columns® (Merck, Darmstadt, FRG) which proved very reliable.

B. Apparatus

The measurements were carried out in a liquid chromatograph S 100 (Siemens, Karlsruhe, FRG) with a photometric detector (PM2D, Carl Zeiss, Oberkochen, FRG) at wavelengths of 246 nm for the free drugs and 337 nm for the derivatives. The chromatograph contained a column of 10 cm length and 3 mm i.d. connected via a two-way high pressure valve (Siemens, Karlsruhe, FRG) either with the detector directly or with a column of 30 cm length and 3 mm i.d. and the detector. The columns were filled with silica (Lichrosorb SI 100 (5 μ), Merck (Darmstadt, FRG)). The valve could be actuated by a manual contact or by the integrator (Varian CDS 111 (C), Varian, Darmstadt, FRG). The setup of the chromatograph is shown in Figure 1.

C. Preparation of the Mobile Phases

The necessary amounts of hexane, the alcohol, dichloromethane and morpholine are measured in a measuring flask and mixed. Then water is added while the mixture is stirred on a magnetic stirrer until all of the water is dissolved in the rest of the aqueous solution. It is necessary to wait until the mixture has regained room temperature or to heat it under warm tap water. The mixture is filtered through a folded filter and is then ready for use. During a week or more of shelf life no degradation of chromatographic properties of the phases could be observed when they were stored in brown bottles at room temperature. The best composition of the mobile phases depends somewhat on the individual columns and their "chemical" history but the valves found by us may comprise a good starting point.

Best composition found for free antidepressives and benzodiazepines: 1.1% n-propanol, 23% dichloromethane, 0.05% morpholine, 0.1% water and hexane ad 100%.

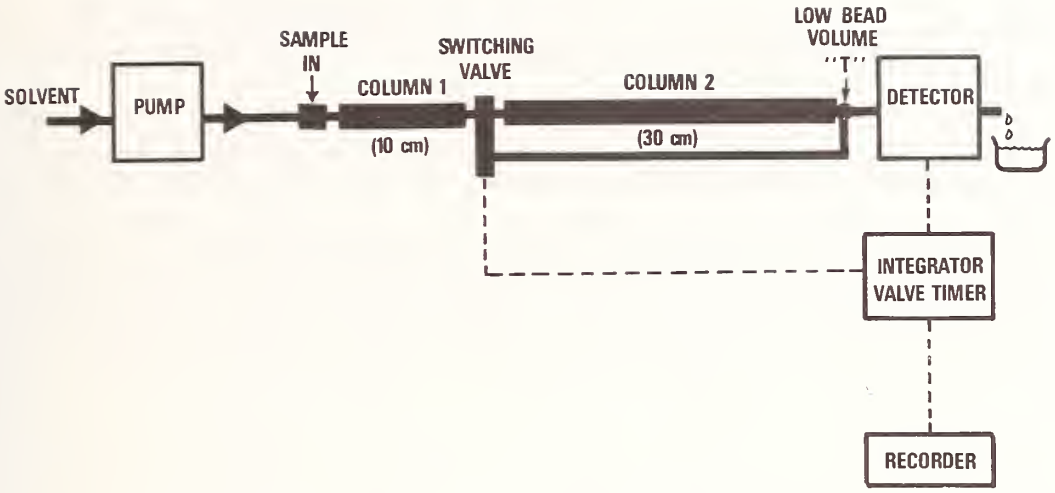

Figure 1. Setup of the liquid chromatograph with column switching.

Best composition found for the derivatives: 1.1% tert. butanol, 13% dichloromethane, 0.05% morpholine, 0.05% water and hexane ad 100%.

D. Sample Preparation for the Analysis

1. Without Derivatization

An amount between 1 mL and 5 mL serum is measured, 1 mL of a solution of the internal standard, 0.2 mL of 10% NaOH and water to make 19 mL are added, the mixture is saturated with solid NaCl and poured on an Extrelut-Column. After 15 min. equilibration time the lipophilic compounds are extracted by the addition of 40 mL of dichloromethane containing 10% iso-propanol (v/v). The filtrate is evaporated in vacuo to dryness, transferred to stoppered reagent glasses, again evaporated to dryness, dissolved in 0.1 mL of the mobile phase and analyzed in the liquid chromatograph.

2. Sample Preparation with Derivatization

Work as described in section 1. until after the evaporation in the stoppered reagent glasses. To the residue add 0.02 mL of 10% Na_2CO_3 in water and 0.2 mL of a solution of 40 mg of p-Nitroazobenzenecarbonylchloride in benzene, rinse the reagent glass inside with this mixture, put it on a vortex-mixer and hold at 37 °C (water bath, stoppered glasses). During the reaction time of about 1 hour the glasses should be agitated 5–6 times. After about 1 hour evaporate to dryness (rotary evaporator, bath temperature about 50 °C), fill with 0.1 mL mobile phase and analyze. It is important to evaporate in the final step to dryness since moisture will interefere with the analysis.

III. Results

Ternary two phase systems [6] soon proved to be not flexible enough. In order to work with the relatively acidic silica as support for the liquid stationary phase and with dichloromethane which is known to lose HCl with time, it was necessary to compensate in the mobile phase for these acid equivalents. Taking 25% aqueous ammonia instead of water proved a good way. However, the brass fittings of the apparatus became blue and green with this kind of mobile phase and the shelf life of such phases was only half a day to a day. Better results were obtained by the

addition of 0.05% of morpholine to the mixture of mobile phase. In Figure 2 is shown a chromatogram of the mixture of the "polar" group of antidepressives in a mobile phase containing ammonia.

Further relatively systematic studies showed that it was impossible to have a separation of the non-polar group of antidepressives (Minaserine, Amitryptiline, Dibenzepine, Imipramine) and of the polar group of antidepressives (Nortryptiline, Desipramine, Carbamazepine and Maprotiline) at the same time, in the same solvent and on the same column length without a gradient. Either the non-polar group was well resolved and the polar group had elution times of hours or the polar group was well resolved and the non-polar antidepressives appeared with the solvent. Here the combination of a short column with a long one proved to be the solution of this very special elution problem. In Figure 3 a separation of some antidepressives from both groups is shown.

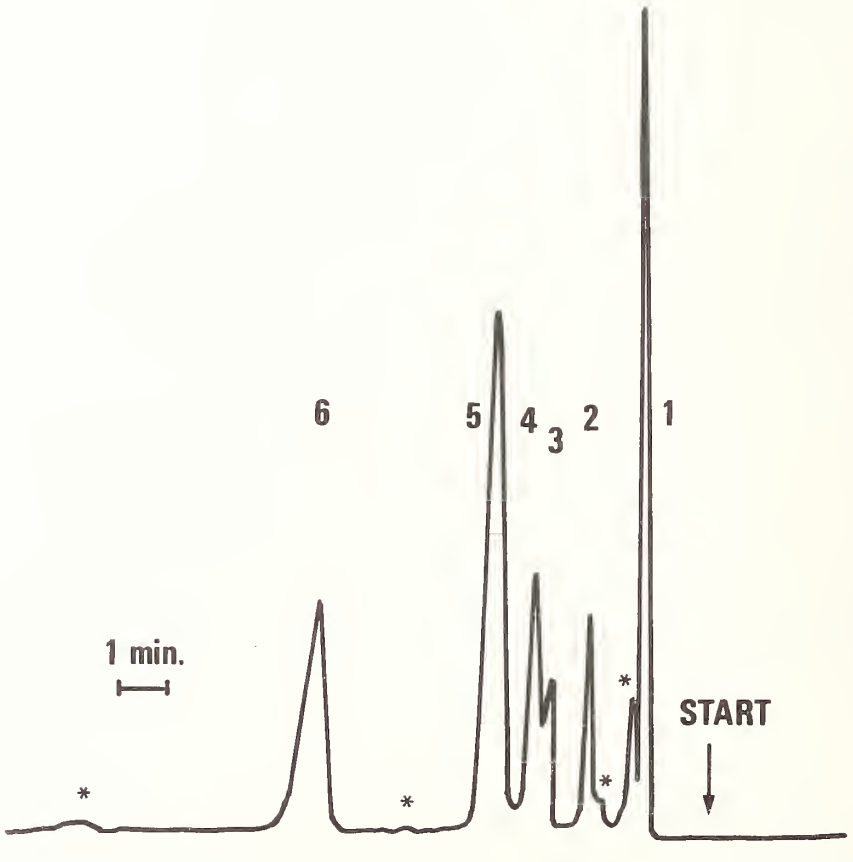

Figure 2. HPLC separation of antidepressives. Chromatographic conditions: Column: 30 cm x 3 mm stainless steel filled with Lichrosorb Sl 100, 5 μ, detection at 215 nm, mobile phase: 92.7% hexane, 2% dichloromethane, 5% tert. butanol, 0.3% 25% aqueous ammonia. 1=solvent, 2=Nortryptiline, 3=Dibenzepine, 4=Desipramine, 5=Maprotiline, 6=Carbamazepine.

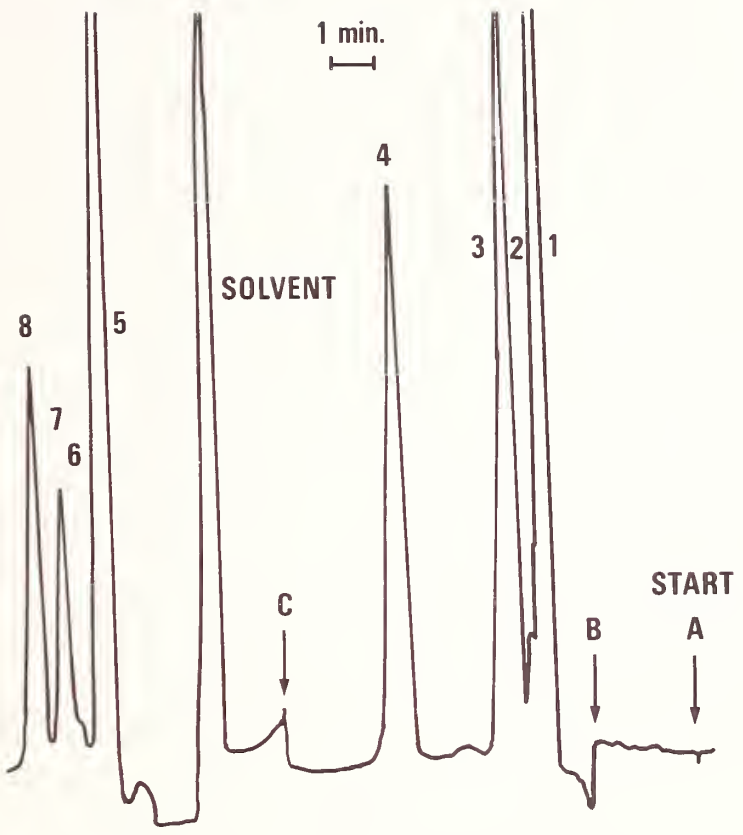

Figure 3. Separation of tricyclics with column switching. Chromatographic conditions: 10 cm+30 cm columns, connected by two-way switching valve, columns filled with Lichrosorb SI 100, 5 μ, detection at 246 nm; mobile phase: tert. butanol 1.5%, dichloromethane 25%, morpholine 0.05%, water 0.1%, hexane 73.35%. Column switching; A: start and long way, B: short way, C: long way. 1=Nortryptiline, 2=Dibenzepine, 3=Desipramine, 5=Amitryptiline, 6=cis-Doxepine, 7=trans-Doxepine, 8=Imipramine.

Figure 4 shows the chromatogram of a serum extract of a patient suspected of taking Diazepam (Valium). As can be seen there is a rather big peak of Diazepam but also of N-demethyldiazepam, the first (and also active) metabolite of Diazepam in the body. If the patient had taken potassiumdichloroazepate (Tranxene) there would be only the peak of N-demethyldiazepam.

Maprotiline (Ludiomil) is a new and very promising antidepressive drug widely used in our clinic. Because of its poor uv absorption at about 240 nm the quantitation of the drug was for the first time possible only at 215 nm which was beyond the energy reserves of the PM 2 D. With another photometer (PM 4 CHR, Carl Zeiss, Oberkochen, FRG) it was possible to overcome this problem, but the recorder trace was very noisy, so the detection limit was not the best one. Here we used the derivatization reaction with p-nitroazobenzenecarbonylchloride. This reagent attaches a group to the free methylamino group of Maprotiline which absorbs at about 337 nm very intensively. After rather unsuccessful trials with pyridine and reagent in the dichloromethane we found the reaction with the reagent in benzene according to Troschütz [9] to be the best way. Figure 5 shows the chromatogram of a serum extract.

Figure 6 is the representation of the calibration curve.

About 200 serum samples have been analyzed successfully in this way up to now. Since February 1978 the method has become a routine method in our laboratory.

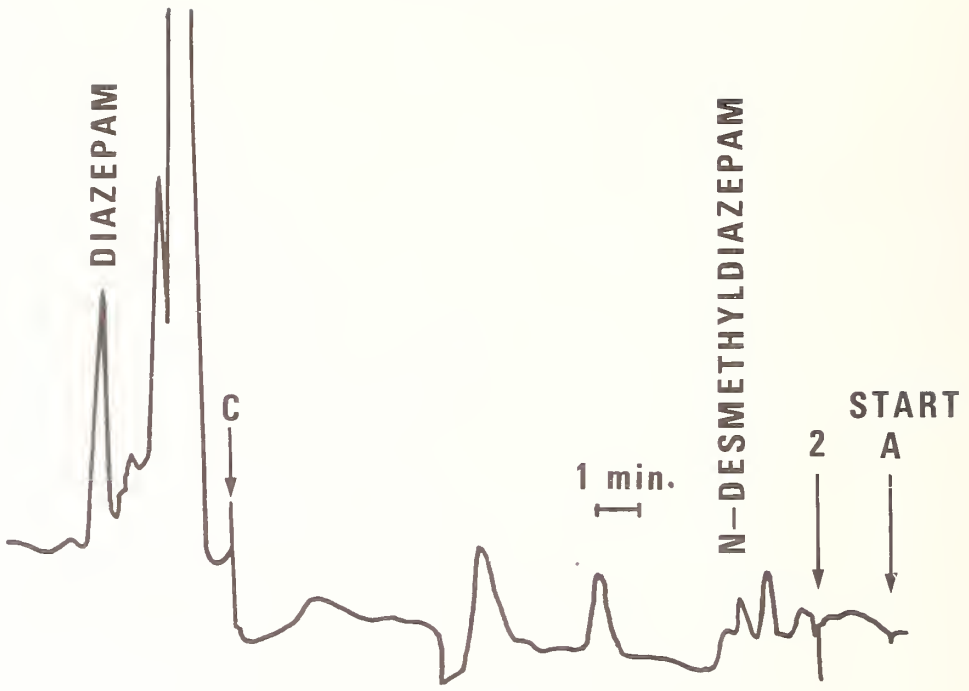

Figure 4. Separation of a serum extract of a patient who has been suspected of drug addiction. A, B, C, vide Figure 3.

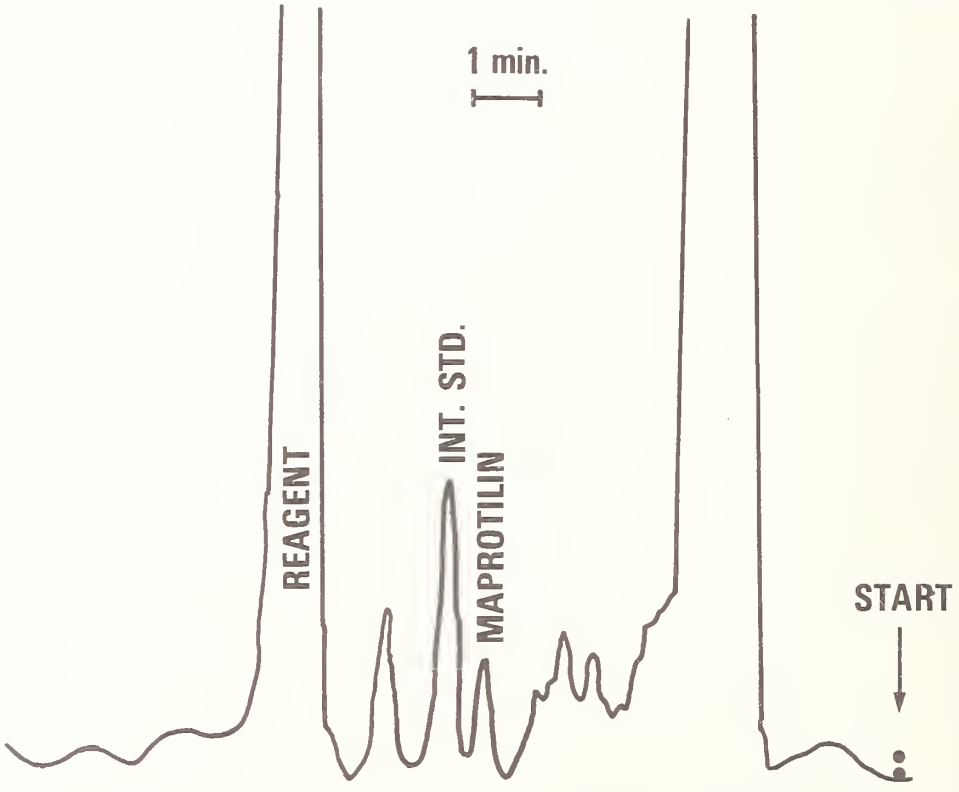

Figure 5. Chromatogram of a serum extract of Maprotiline with derivatization. Chromatographic conditions: 40 cm column, (10+30 cm) 3 mm i.d., detection at 337 nm, mobile phase: 1.1% tert. butanol, 23% dichloromethane, 0.05% morpholine, 0.05% water, hexane ad 100%.

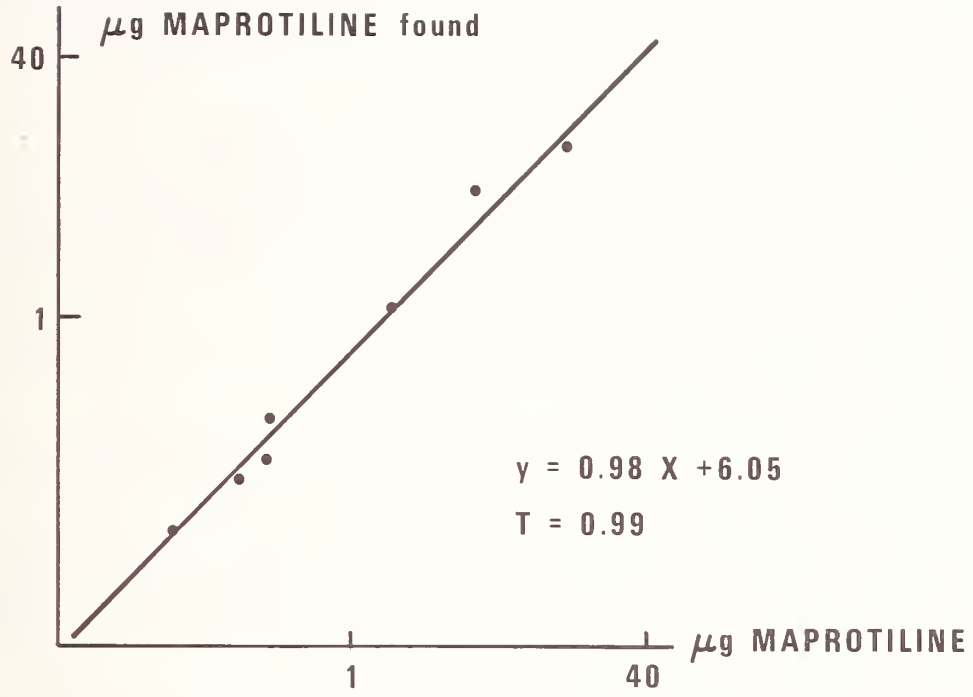

Figure 6. Calibration curve for the determination of Maprotiline in serum.

IV. Discussion

Quaternary two phase systems in fact seem to be a very flexible medium for the separation of many classes of compounds. A problem in the application of this method may be the extreme flexibility of these systems which offers the beginner too many choices. Close inspection of the possibilities has however shown a way in this fascinating area of separation concerning the kind of alcohol and a way for the systematic optimization of a separation system for a given sort of sample and a given column [10]. Up to now the silica in the short and the long columns was of the same kind. A greater span of polarities can however be obtained when the packing of the first short column consists of a less retaining silica than that of the second one. Studies here are in progress and rather promising. In our experience the reproducibility of the retention times with such phase systems is excellent, even with column switching about $\pm 1\%$ or better. The detection limits are 5 ng for Amitryptilin and 50 ng for underivatized Desipramine.

Clinical experience with Maprotiline in our patients shows that patients at a serum level of 100 ng/mL or less show no clinical response, at 200 ng/mL about 50% show a favorable response, whereas between 500 and 600 ng/mL nearly 90% of our endogeneous depressive patients showed a favorable result. Studies with patients who seemed to be resistant to antidepressive therapy with Maprotiline showed that they were underdosed in most cases and that sometimes an addition of about 10% of the oral dose raised the serum level about 100%. At a dose of 75 mg and 150 mg Maprotiline daily we observed about a 20 fold variation in serum level of the drug in the steady state measured immediately before the ingestion of the new dose. These findings are in agreement with the results found by the scandinavian workers for Nortryptiline [1,2].

In the case of Maprotiline, Maprotiline is the only active substance. In the case of Amitryptiline and of Imipramine, the first metabolites, Nortryptiline and Desipramine, are themselves antidepressives. Surely it is not adequate to take the sum of both drugs and correlate it

to clinical outcome. In our group a novel concept of evaluation is in development and will be applied to the therapy of depressive patients at the moment when the determination of all antidepressives has become as routine as that of Maprotiline.

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THE IDENTIFICATION OF ODORIFEROUS COMPOUNDS FORMED BY COITAL EJACULATE IN COLLAGEN SPONGE CONTRACEPTIVES

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The study analyzed the molecular mechanism of the formation of malodor related to the retention and decomposition of ejaculate in collagen sponge (CS) used as an intravaginal contraceptive barrier method. Parallel studies were done in vitro with sponges soaked with semen and incubated at 37 °C in a moist atmosphere. Gas samples from the vials containing CS used intravaginally or CS soaked with semen were analyzed by gas chromatographic and mass spectrometric methods. These analyses indicated that propylamine and butylamine were present in the gas samples analyzed. When ejaculate was incubated in vitro in sealed vials, not only were butylamine and propylamine detected, but also propanol. The incubation of radioactive polyamines with ejaculate and the isolation of radioactive products by derivatization and recrystallization to constant specific activity indicates that propylamine is derived from the spermine and spermidine in ejaculate, and that butylamine is derived from the putrescine contained in ejaculate.

It is concluded that the polyamines are converted to simple aliphatic amines by ejaculate enzymes and that the aliphatic amines are responsible, in part, for the malodor associated with collagen sponge worn postcoitally for several days by sexually active women.

Key words: Butylamine; collagen sponge; contraceptive device; gas chromatography; mass spectrometry; propylamine.

I. Introduction

We observed that 35–40% of the sexually active women who tested the intravaginal collagen sponge contraceptive (CSC) developed malodor [1] several days following coitus, whereas offensive odor was almost absent in sexually inactive women. Parallel with the incidence of malodor, the polyamines in ejaculate trapped in the sponge were significantly reduced.

Because ejaculate contains both polyamines and diamine oxidases which convert the polyamines to lower molecular weight compounds, some of which have offensive odor, we believed that under the given conditions, ejaculate was a primary cause of the malodor detected in the sponge [1–3].

This study describes a molecular mechanism for vaginal malodor formation, specifically that related to the incubation of ejaculate within the matrix of collagen sponge. We will show for the first time new metabolites typical for the malodorous environment and we will discuss the possible mechanism for their formation.

A. Collagen Sponges

The CSC used were made according to a previously described procedure [4,5].

B. Human Volunteers

A group of women, 18 to 45 years of age, with ligated fallopian tubes or using steroidal contraceptives, tested the collagen sponge (cylindrical shape 2.5 cm wide, 6 cm in diameter) during their sexual life. The sponge with entrapped ejaculate was retained in the vagina for various recorded time periods.

The sponge was removed either by the gynecologist or by the volunteer. It was placed at once into an airtight plastic bag or container and analyzed immediately for odor, or frozen (–18 °C) for subsequent evaluations.

In some cases a CSC that was used intravaginally was homogenized in 0.5 mol/L HCl using a Polytron homogenizer. The supernatant was obtained by centrifugation and stored at –18 °C until analyzed by gas chromatographic or gas chromatographic—mass spectrometric methods (gc or gc-ms). To 0.1–0.3 mL of the supernatant was added 0.5 mL of 4 mol/L NaOH, the 5 mL reaction vial was sealed with a Teflon lined rubber septum, and the mixture shaken and allowed to react for several minutes. The gas phase in the vial was sampled and analyzed on the gc-ms. Occasionally, the vial was heated to 67–70 °C before the sample was taken.

C. Sponge Handling During Chemical Evaluation

Small pieces of each sponge obtained from the various women participating in the study were placed in two reaction vials. Each vial was sealed with a screw cap having a Teflon lined rubber septum. Through this septum, using air sampling syringes, samples were obtained for gas chromatographic analysis.

This type of vial also allowed the addition of 0.5 mL of a sodium hydroxide (2.0 mol/L) solution to any one of the vials containing the moist sponge specimen. The addition of alkali to the sponge would cause an increased release of volatile amines into the gas and would block the release of volatile acids within the vial.

D. Sponge Incubation Studies

The incubation of ejaculate on CS has been described in our previous publication [2,3] and consists of adding 0.5 mL of pipes pH 7.4 buffer (0.1 mol/L) plus 0.5 mL ejaculate and 0.1 mL of tritiated spermine or spermidine or ^{14}C-putrescine substrate (10 μCi/μmol per mL) to 100–109 mg of sponge. After flushing the tubes with oxygen this mixture was allowed to incubate at 37 °C for 6–7 days in the sealed vial.

These incubations were carried out without the addition of radioactive substrates when ms analyses and gc analyses were done.

Gas chromatographic patterns of a sample of the gas phase in the vial containing the ejaculate before and after incubation were obtained from the vials containing no added substrates.

E. Gas Chromatographic Methods

A Hewlett-Packard gas chromatograph (gc) Model 402 with a 7127-A strip chart recorder and a 3373B integrator was used for gas liquid chromatographic separations of the various amines. A flame ionization detector was used throughout the study. A 6-foot glass column having 4 mm inside diameter containing Carbopack B 4% Carbowax 20 M and 0.8% KOH was used. The gas chromatography was run isothermally. The oven temperature, injection port temperature and flame ionization detector were maintained at 145°, 225°, and 245 °C, respectively. The He carrier gas was set at 22. A 3-foot column having 4 mm i.d. containing the same support was used for the gc-ms studies. The temperature was programmed from 70° to 220 °C at 8 °C per minute. A second 6-foot column having 4 mm i.d. containing chromosorb W AW, 10% Carbowax 20 M and 2% KOH was used for some of the gc studies. The gc was run isothermally at 79 °C. The injection port and flame ionization detection temperatures were maintained at 165° and 190 °C respectively. The helium carrier gas was set at 18. A third 6-foot Porapak Q column containing 0.8% KOH was used to obtain the gc-ms pattern of propylamine. Because the columns contained KOH, only neutral and basic volatile compounds would probably be eluted from these supports.

F. Mass Spectrometric Analysis

Mass spectrometric analyses of the various peaks arising from gc were done on a Finnigan Model 330–6100 ms interfaced with a 9500 gc using a glass jet separator.

The mass spectrometer was operated in the electron impact mode with 70 eV. The mass spectra were obtained by subtracting the "background spectra" obtained near the gc amine peak from the mass spectra obtained for each peak.

G. Derivation of Propylamine and Butylamine

For the radiometric determination of propylamine and butylamine formation, 2 mL of propylamine or butylamine was added as carrier to the vial after incubation of ejaculate with various radioactive polyamine substrates. The assay mixture and the added carrier were thoroughly mixed and the fluid poured into a reaction vial. The sponge in the vial was washed 2 times with 1.5 mL of water which was added to the reaction vial.

H. N-Propylbenzoylamide and N-butyl-p-nitrobenzoylamide Synthesis

To the washings from any one vial 7 mL of 6 mol/L potassium hydroxide was added. The mixture was cooled in a Dry Ice-acetone bath for 0.5 minutes and 3 mL of benzoyl chloride was added to the vial contents. After the addition of the acid chloride, the solution warmed due to the exothermic nature of reaction; the reaction mixture was stirred thoroughly and was allowed to stand at room temperature for 30 minutes. The amide crystals which usually formed during this time were extracted 2 times into 5 mL of hot toluene. The toluene extract was dried with anhydrous sodium sulfate. Then 2 mL hexane was added to the toluene extract and the mixture was cooled in a Dry Ice bath. Crystallization could be encouraged by scratching the side of the tube with a glass rod. The crystals were collected on sintered glass filters and washed several times with hexane. Recrystallizations were done by redissolving the benzoylamides in hot toluene followed by allowing the mixture to cool and collecting the crystals that formed by filtration then washing them with hexane. The uncorrected melting point of N-propylbenzoylamide obtained in this way ranged between 82.5–83.5 °C after 4 recrystallizations for N-propylbenzoylamide. The melting point for N-butyl-p-nitrobenzoylamide is reported to be 103–104 °C [6]. After three recrystallizations, we found the melting point to be 102–102.5 °C. This is an uncorrected value.

The crystals were dried *in vacuo* overnight. The specific activity of the crystals was determined by weighing some of each crystal into individual counting vials; adding scintillation fluid (3 g of POP and 150 mg of POPOP per liter of toluene) and determining the disintegrations per minute of each sample by using an internal tritiated or ^{14}C-toluene standard.

II. Results

A typical gc analysis of volatile malodorous compounds related to either *in vitro* or *in vivo* ejaculate decomposition is shown in Figure 1. Figure 1 also shows a standard gas chromatographic pattern of propylamine, butylamine and pentylamine.

The gas chromatographic patterns indicate 5 peaks emanating from the collagen sponge worn intravaginally and 5 peaks emanating from ejaculate incubated for 7 days at 37 °C. Chromatographic peaks number 1, 3 and 4 obtained from ejaculate have the same retention time as peaks 1, 2 and 3 from the CS worn intravaginally (see Fig. 1). Two of the peaks (ejaculate peaks 3 and 4 and intravaginal CSC 2 and 3) have the same retention time as propylamine and butylamine respectively. Peak #1 in the intravaginal CSC was a mixture of 2 or more compounds. None of these compounds were detected in fresh ejaculate.

A 6-foot Carbopack B column containing 4% Carboxwax 20 M and 0.8% KOH was used to try and resolve peak #1 above. Using this column we were able to resolve peak #1 (Fig. 1) into 2

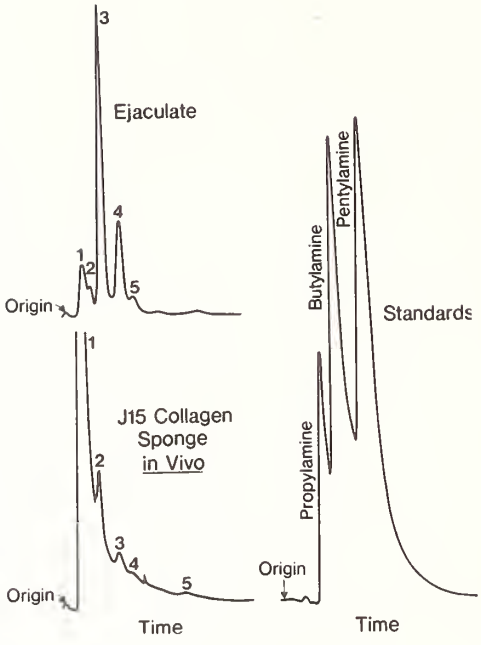

Figure 1. The 3 gc patterns were obtained on a Chromosorb W AW column as described under methods. All patterns were obtained under identical conditions. The top left gc pattern is of a gas sample from a sealed vial containing ejaculate which had been incubated for 7 days at 37 °C. The bottom left gc pattern is of a gas sample from a sealed vial containing a piece of collagen sponge which was worn intravaginally. The gc pattern to the right is from an amine standard containing propylamine, butylamine and pentylamine.

peaks as shown in Figure 2. Thus, a total of 6 peaks was obtained. Peak #1 has the same retention time as the first peak obtained when a 90% solution of aminobutyraldehyde diethylacetal was injected onto the Carbopack B column. This solution has 3 peaks on another column but only 1 elutes on this column. This peak has not been identified. Peaks 3 and 6 have the same retention time as propylamine and butylamine respectively (see Fig. 2).

When a gas chromatographic mass spectrometric analysis was determined on gas samples from the vials containing collagen sponge used by the women and on gas samples from vials in which ejaculate was incubated a propylamine peak was identified mass spectrometrically (see Fig. 3). Major ions at m/e 30 and 59 with a minor cluster of ions around m/e 42 were observed; m/e 59 is the parent ion. The spectrum obtained from the samples was compared to the spectrum of propylamine standard (see Fig. 3).

A gas sample from a vial containing ejaculate was injected onto gc-ms. The total ion current is shown in the center of the figure. Nine compounds are eluted from the Carbopack B column. Peak 4 (spectrum #105) is propylamine and peak 8 (spectrum #210) is propyl alcohol (see mass spectra, Fig. 4).

Major ions of m/e 31, 29, 42 and a parent ion at m/e 60 were observed: CH_2^+ –OH (m/e 31), CH_3–CH_2^+ (m/e 29) and CH_3–CH=CH_2^+ (m/e 42). Finally, the spectrum obtained from the sample was compared to the spectrum of a propyl alcohol standard (see Fig. 4).

Peak #9 (spectrum #315) gives a mass spectrum very similar to butylamine; however, this peak appears to contain two compounds. We suspect the other component of this peak to be Δ-l-pyrroline. Δ-l-pyrroline retention time would suggest that it is the leading edge of peak #9. Further work with other gc columns will have to be done before a pure mass spectrum from the components of this peak can be resolved. Peak numbers: 3 (spectrum #55); 5 (spectrum #125, the largest peak) gives major mass ions at m/e 31 suggesting that these compounds are alcoholic in nature. Their identification is still under investigation.

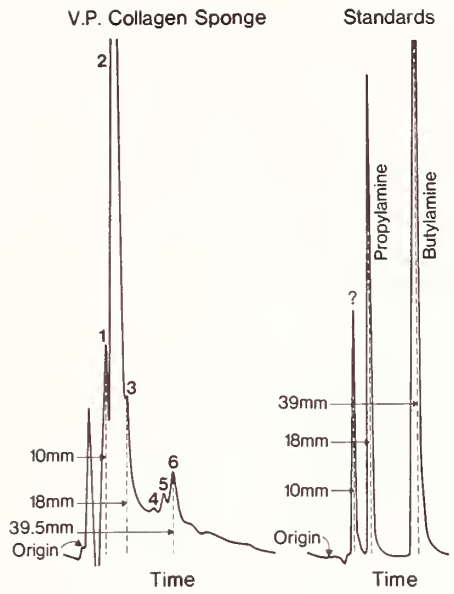

Figure 2. The 2 gc patterns were obtained using a Carbopack B column as described under methods. Each pattern was obtained under identical conditions. The gc pattern on the left is of a gas sample from a sealed vial containing a piece of collagen sponge which was worn intravaginally. The gc to the right is a mixture of a sample containing a 90% solution of aminobutyraldehyde diethylacetal, propylamine and butylamine.

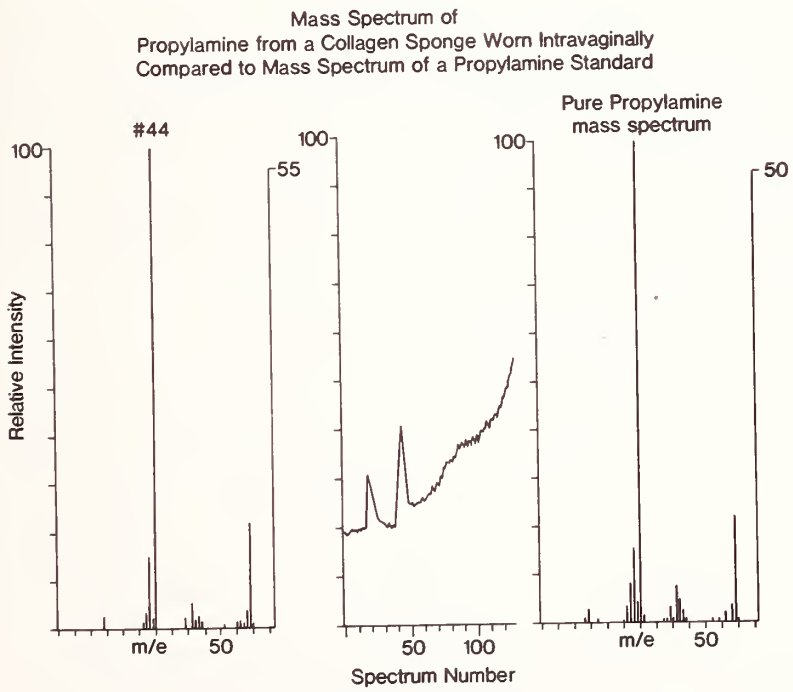

Figure 3. The gc-ms patterns were obtained by injecting a gas sample from a vial containing a piece of collagen sponge worn intravaginally by a volunteer who had coitus several hours before the sponge was removed. The gc was done isothermally on a Porapak Q column containing 0.8% KOH. A standard propylamine sample was used to obtain the ms-propylamine standard ms pattern on the right. The number 44 above the mass spectrum is the gas chromatographic total ion spectrum number. This number indicates that the mass spectrum is of that material which was eluted from the column at that point of the gas chromatogram.

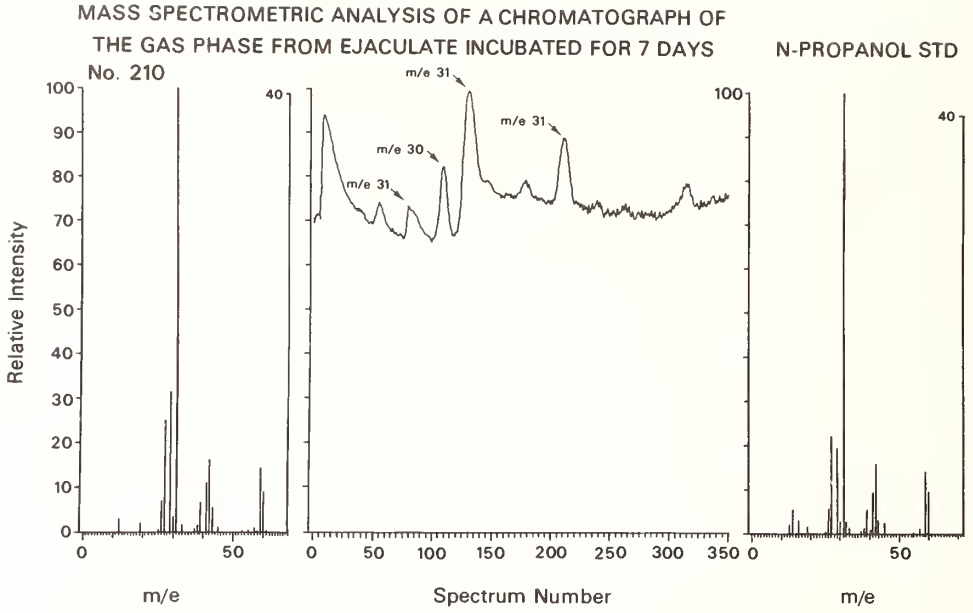

Figure 4. The gc-ms patterns were obtained as described in Figure 3 except a 3 foot Carbopack B column was used. The temperature was programmed (8 °C/min.) from 70° to 200 °C. A standard propanol solution was used to obtain the standard propanol pattern on the right.

To determine which compounds in ejaculate were precursors for butylamine and propylamine, analyses of the polyamines were done before and after incubating ejaculate for 7 days. We observed, as others have [7], that the polyamines decreased after incubating ejaculate for various periods of time. Next, an ejaculate soaked sponge was incubated at 37 °C with radioactive spermine or spermidine, or putrescine. Following the incubation, amide derivatives were made of propylamine and butylamine. The amides were synthesized by adding a large excess of propyl or butylamine to the mixture following the incubation and reacting them with benzoylchlorides. The amides were repeatedly recrystallized to constant specific activity. These studies indicate that propylamine was a metabolic product of spermine and spermidine, while butylamine was a metabolic product of putrescine (see Figs. 5 and 6).

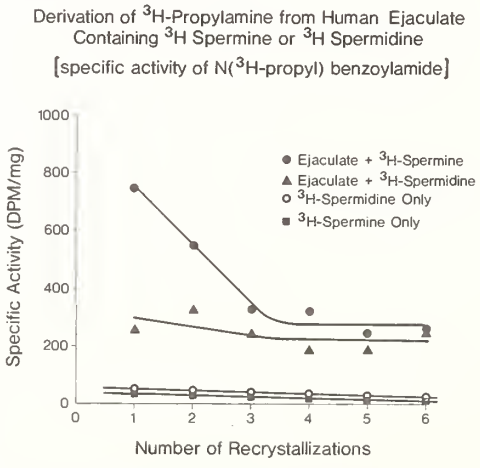

Figure 5. The crystals of N-(^{3}H-propyl) benzoylamide were obtained by adding carrier propylamine to an ejaculate sample which was incubated for 7 days with tritiated spermine and spermidine. The amide of propylamine was obtained by using benzoyl chloride. The resulting crystalline product was recrystallized and the specific activity of the crystals determined following each crystallization.

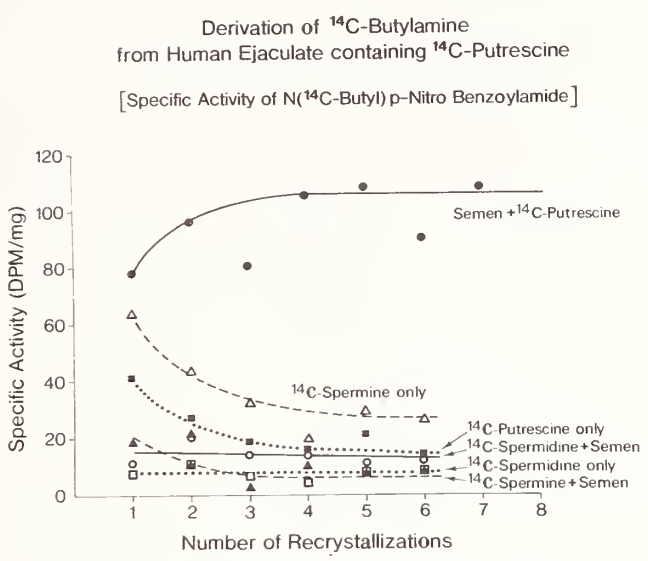

Figure 6. The crystals of N-(^{14}C-butyl) p-Nitrobenzoylamide were obtained by a method similar to that described for Figure 5 except that p-nitrobenzoyl chloride was used to form the amide from butylamine.

III. Discussion

We have identified butylamine, propylamine, and propyl alcohol as three of the compounds which contribute to malodor in collagen sponge contraceptives worn intravaginally as a sperm barrier.

The formation of propylamine and butylamine from the polyamines has not been described. These products are not the typical products of diamine or monoamine oxidase action [8–10]. The radiometric-recrystallization studies indicate that spermine and spermidine are precursors for propylamine formation and that putrescine is the primary precursor for butylamine formation.

We postulate a new enzymic action is probably responsible for the formation of propylamine and butylamine from polyamines normally found in human ejaculate. The enzymic action could possibly be a new action mechanism for one of the amine oxidases. The net result of the enzymic action appears to cleave a bond between a carbon and a secondary or primary amine. As yet no enzyme having this action has been described. Because of the complex nature of the reaction we suspect a multiple enzyme system is responsible for propylamine and butylamine production from the polyamines.

In addition, Δ-l-pyrroline formation is known [8] to involve the action of diamine oxidase on putrescine, a substrate for this enzyme. Putrescine is first converted by diamine oxidase into γ-aminobutyraldehyde which cyclizes spontaneously by means of a Schiff base to form Δ-l-pyrroline. We have not yet identified Δ-l-pyrroline in these studies but suspect it too contributes to the malodor associated with the CS contraceptive. Recent studies of Dupre et al. [9] using an enzyme system containing purified diamine oxidase, putrescine, NADH and liver alcohol dehydrogenase resulted in reduced formation of Δ-l-pyrroline. He suggested that the intermediate γ-aminobutyraldehyde formed by diamine oxidase was reduced to the γ-aminobutanol by alcohol dehydrogenase rather than the aldehyde cyclizing to form Δ-l-pyrroline. This study suggests this possibility for the biochemical formation of propanol (see Fig. 7), or possibly butanol.

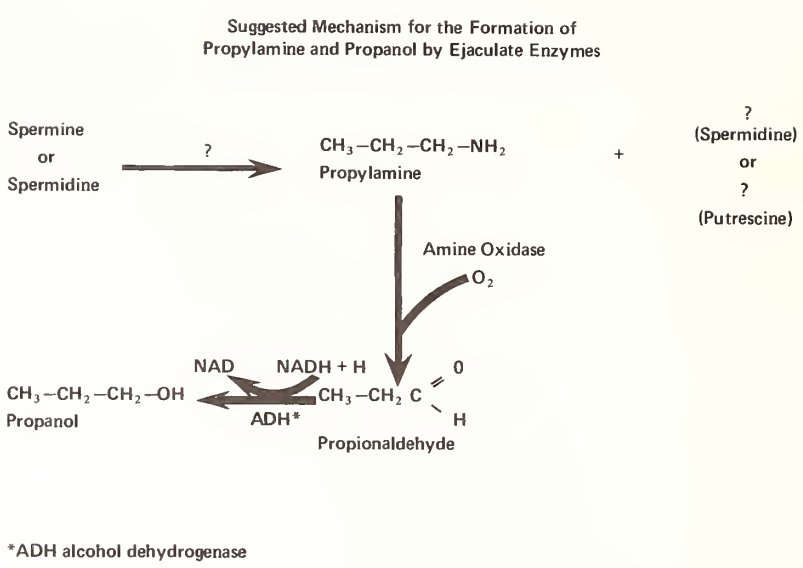

Figure 7. This is a proposal of the ejaculate biochemical reactions leading to the formation of propylamine, butylamine, propanol, and possibly butanol.

The formation of propanol and possibly butanol in ejaculate from propylamine and butylamine is enzymically conceivable because amine oxidases occur in semen. Amine oxidase activity on propylamine and butylamine would result in aldehyde formation, i.e., propionaldehyde and butyraldehyde. The reduction of the aldehydes to alcohols by alcohol dehydrogenase and NADH, then, could result in the corresponding alcohols (see Fig. 7).

IV. Acknowledgments

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THE ASSAY OF TRIAMTERENE IN HUMAN BLOOD BY ION-PAIR EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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The potent diuretic, triamterene (T), is often used in cardiac patients in conjunction with quinidine. It is metabolized in the body and appears in the urine mainly in the form of the sulfate ester of the hydroxylated drug. Most methods of assay of T in blood take advantage of its strong fluorescence, but difficulties may arise from interference by its metabolites or quinidine. This paper describes an assay which is highly specific and very rapid. Plasma or whole blood (0.5 mL) is acidified with perchloric acid, extracted with methylisobutyl-ketone and an aliquot of the organic phase passed on HPLC using silica gel with dichloromethane-hexane-methanol-perchloric acid (57:35:8:0.1) as mobile phase. The fluorescence detector is set at 350 nm excitation (monochromator) and above 470 nm emission (sharp cut filter). Recovery after a single extraction was 88.6 percent, and the calibration curve was linear from 1–1000 ng/mL (slope: 0.59, intercept: 0.06) with a C.V. of 3–9 percent over the entire range. The method was used for one non-fasted human volunteer given 50 mg T. The profile showed a peak at 3 h both for plasma (16.2 ng/mL) and whole blood (20.3 ng/mL), declining to 6.1 and 10.8 ng/mL at 7.6 h for plasma and blood, respectively.

Key words: Clinical analysis; fluorescence detection; liquid chromatography; triamterene assay.

The diuretic, triamterene, has been used clinically since the early 1960's [1]. It is generally used in conjunction with other diuretics since it reduces potassium depletion [2], and is of great value in certain types of heart conditions [3]. Early assay methods for biological fluids were based on total fluorescence of the material assayed, since the drug is an intense fluorophor. However, because of interference from some metabolites, newer, more specific methods were required to permit the study of its pharmacokinetics and pharmacodynamics [4].

In a recent study on the assay of quinidine in plasma [5] it was noted that high performance liquid chromatography (HPLC) with fluorescence detection would be suitable for the quantitative estimation of triamterene. The present paper describes a rapid and very sensitive assay for triamterene in plasma using ion-pair extraction and HPLC.

The method used was essentially the same as reported previously [5] except for the mobile phase, which consisted of dichloromethane-hexane-methanol-70% perchloric acid (57:35:8:0.1). The increase in the methanol concentration was found to shorten analysis time. The fluorometer monochromator (excitation) was set at 335 nm using a 7-54 filter (broad band pass) to remove second order radiation. Emission was monitored from 470 nm up using a sharp cut filter. While the maximum emission of triamterene is probably closer to 430 nm, better sensitivities were obtained at 470 nm due to a quieter baseline.

The retention characteristics of triamterene, as well as quinidine and some of its metabolites, are shown in Table 1. Of all the substances tested, only one, a metabolite of quinidine, eluted somewhat close to triamterene; however, no significant interference would be expected at the wavelengths used.

The extraction of the plasma was accomplished in the presence of perchloric acid at a final concentration of 1 mol/L, and a single extraction with 1.33 volumes of methyl isobutyl ketone. This single ion-pair extraction, accomplished in a little over 10 minutes, extracted 88.6% of the triamterene into a small enough volume of methyl isobutyl ketone to permit direct chromatography of the extract without the necessity of reconcentrating.

Chromatograms using the method described (Fig. 1) indicate that blank plasma (trace A) does not contain any naturally occurring fluorescent interference. Chromatogram B shows blank

TABLE 1. *Capacity factors for triamterene, quinidine and some of their metabolites*

Substance	Capacity factor, k′ 8% methanol	5% methanol
dihydroquinidine	2.4	4.0
2′-quinidinone	2.4	5.0
quinidine	2.7	5.0
3-hydroxyquinidine	4.2	12.1
triamterene	4.6	10.3

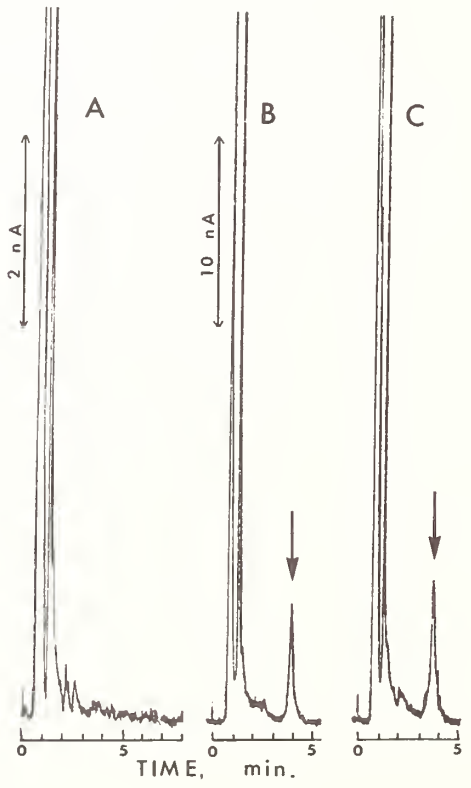

Figure 1. Chromatograms of plasma extracts. A, blank plasma; B, blank plasma spiked with triamterene, 10 ng/mL; C, plasma from a human volunteer taken 5 h after administration of 50 mg drug (estimated concentration: 13.9 ng/mL). Extraction and chromatography as described in the text.

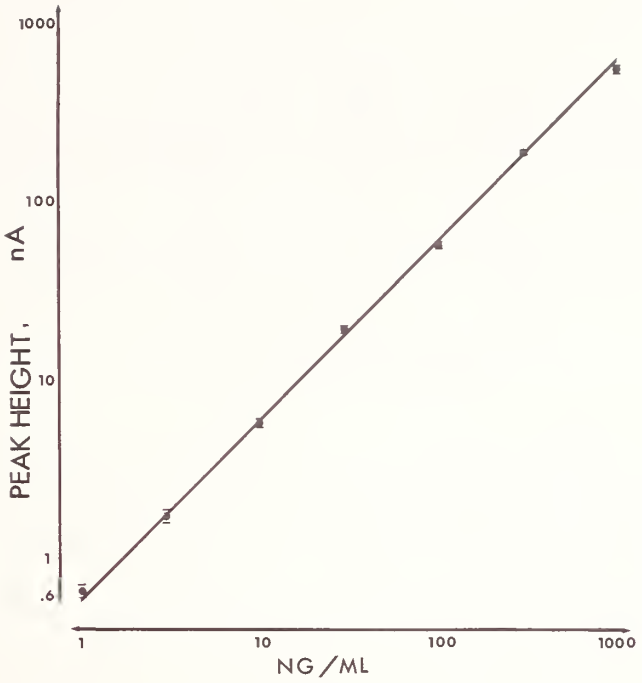

Figure 2. Calibration curve for triamterene in plasma. Blank plasma was spiked with triamterene to give the following concentrations: 1, 3, 10, 30, 100, 300 and 1000 ng/mL. Each point represents the mean and standard deviation of four determinations.

TABLE 2. *Statistical analysis of the triamterene calibration curve*

Regression	Coefficients	95% conf. interval
exponential ($Y=BX^{B'}$)	$B=0.662$	--
	$B'=0.993$[a]	--
weighted linear[b] ($Y=A+BX$)	$A=0.0625$[c]	$A\pm0.266$
	$B=0.590$	$B\pm0.040$

[a] Linearity, i.e., $B'=1.0$, $p>0.05$
[b] Weight, $W=1/S^2$
[c] Equivalent to approx. 0.1 ng/mL

plasma spiked with triamterene (10 ng/mL) and chromatogram C is from a plasma sample from a volunteer taken 5 h after ingestion of one 50 mg triamterene tablet. This plasma was estimated to contain 13.9 ng drug per mL.

A calibration curve for plasma spiked with 1–1000 ng/mL triamterene is shown in Figure 2, and the corresponding statistical analyses are summarized in Table 2. The least square regression curve fitted with an exponential equation showed the line to be straight and the intercept, calculated by a weighted linear regression, was not found to be significantly different from 0. The coefficients of variation associated with the points in Figure 2 ($N=4$ each) ranged from 8.9% at 3 ng/mL to 1.6% at 1000 ng/mL, with an overall mean of 4.3%.

The blood profile for triamterene in a human volunteer is summarized in Table 3. The concentration of unmetabolized drug in plasma after administering 50 mg of the drug to a non-fasted subject (light breakfast 1 h prior to the dose) reached a peak value of 16.2 ng/mL at 3 h after the dose. Somewhat higher concentrations were found in whole blood. Blood samples were

TABLE 3. *Blood profile for triamterene*[a]

Time, h	Drug conc., ng/mL[b] Plasma	Whole blood
0.5	1.33	3.88
1	1.98	3.41
2	6.46	8.65
3	16.23	20.29
5	13.9	16.63
7.7	6.1	10.75

[a] Human volunteer, normal adult; dose: 50 mg.
[b] Means of duplicate determinations.

taken until 7.7 h after the dose, which appeared to be too short an interval to draw meaningful conclusions as to the drug kinetics in this volunteer. More recently, another volunteer was given 200 mg drug after fasting overnight. Preliminary results indicate peak concentrations of almost 1000 ng/mL at 1 h, which is in accordance with results obtained by other workers [6].

The absence of interfering metabolites under the triamterene peak was ascertained by collecting the effluent corresponding to the peak and rechromatographing by thin layer chromatography according to the method of Grebian et al. [7]. Only one fluorescent spot could be detected with an R_f identical to that of authentic triamterene standard. Similar results were obtained using the 0–7 h combined urine of the second volunteer (200 mg dose), which was shown to contain large amounts of metabolites prior to extraction and chromatography.

Thus, the method of ion-pair extraction used in conjunction with HPLC for the determination of triamterene is fast, specific and sensitive, and should be useful in clinical and toxicological, as well as in pharmacokinetic studies.

Acknowledgment

The authors wish to thank Dr. W. Tostowaryk for the statistical analysis of the data.

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

THE GAS CHROMATOGRAPHIC MEASUREMENT OF DEXTROMETHORPHAN LEVELS IN HUMAN PLASMA

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A highly sensitive method for the determination of plasma levels of the antitussive, dextromethorphan (DM), is described. Following the administration of a therapeutic dose (30 mg) of dextromethorphan hydrobromide (DM·HBr), plasma levels of intact drug can be expected to be low due to rapid and extensive demethylation. To reliably and accurately measure such levels, an ultra-sensitive means of determining the concentration was developed. A preliminary extraction with hexane, a back-wash into acid, a final basic extraction into hexane with subsequent measurement by a gas chromatograph equipped with a nitrogen sensitive detector provided the required selectivity, sensitivity and speed of analysis. Less than 5 minutes of instrument time was required to reliably measure 1 ng DM/mL of plasma. Plasma levels of DM were determined from human subjects receiving 30 mg orally every 6 hours for a 1-week period.

Key words: Antitussive; dextromethorphan; gas-liquid chromatography; human plasma; nitrogen-sensitive detector.

I. Introduction

Dextromethorphan hydrobromide (DM·HBr), a non-narcotic antitussive, has been accepted as safe and effective by the O.T.C. Cough-Cold Panel. Even though it is contained in many cough-cold preparations, the levels of intact DM in plasma from human subjects receiving orally administered doses in the therapeutic range have not been reported. The drug is metabolized by O-demethylation rapidly and extensively by humans, therefore, as might be expected, the plasma levels of intact drug are extremely low.

A fluorometric method for measuring levels of this O-demethylated metabolite in plasma has been described by Ramachander et al. [1]. It was felt, however, that, to follow drug disposition, it would be necessary to develop a method capable of measuring low levels of intact DM. This report describes the results.

A convenient, ultra-sensitive, selective and reliable procedure to measure low concentrations of intact DM in human plasma was developed. The extraction of the drug is a relatively simple three-step procedure that results in the drug contained in a 2 mL plasma volume being extracted into a 0.5 mL volume of hexane. The separation and measurement was done on a gas chromatograph equipped with an ultra-sensitive and selective nitrogen thermionic detector. As the name implies, the detector enhances selectivity of the separation by being sensitive only to those compounds eluting off the column which contain nitrogen and/or phosphorous atoms. This selectivity prevents certain non-nitrogen containing compounds, which are endogenous to plasma, from acting as an interference in the DM measurement. Chromatographic resolution and efficiency is remarkably improved with the conspicuous absence of a non-nitrogen solvent front and the normally occurring signals resulting from lipid-like substances in plasma extracts.

The contribution of all of these factors provided sensitivity that was at least 20 times greater than the conventional flame ionization detector for a nitrogen-containing substance such as DM.

The data was processed immediately after each analysis by employing an electronic-computing integrator. Use of this combined system has provided the selectivity, sensitivity and speed of analysis needed to analyze plasma levels of DM in human subjects. Less than 5 minutes of instrument time was required to reliably measure 1 ng DM/mL of plasma. On this basis, one person could conveniently prepare and assay a dozen samples in less than 3 hours.

II. Experimental

A. Instrumentation

The gas chromatograph employed was a Hewlett-Packard 5711A equipped with a nitrogen-phosphorous/FID detector and a glass column (1.2 m×6 mm o.d.×2 mm i.d.) packed with 2% OV-101 on 100–220 mesh Chromosorb W-HP.

The operating temperatures were: 215 °C for the column; 300 °C for the detector and 200 °C for the injection port. The carrier gas, helium, flow rate was 30 mL/min with an inlet pressure of 55 psig. The flow rate of the detector gas (8% hydrogen in helium) was 30 mL/min with an air flow of 100 mL/min.

The output signal was processed by Autolab Systems I computing integrator (Spectra-Physics) for quantitation.

B. Extraction of Dextromethorphan from Plasma

The extraction of the DM from the plasma is a relatively simple procedure and may be outlined as follows:

1. 2 mL heparinized plasma/15 mL centrifuge tube.
2. Add internal standard—20 μL of a 0.01 mg/mL water solution of chlorcyclizine·HCl.
3. Add 0.2 mL 1 mol/L NAOH/5.0 mL hexane/extract on rotary-mixer for 30 minutes/centrifuge.
4. Pipet off 4.5 mL hexane into a 13 mL centrifuge tube/discard plasma.
5. Add 1.0 mL 0.1 mol/L HCl to hexane/extract on rotary-mixer for 15 minutes/draw off hexane and discard.
6. Add 0.2 mL 1 mol/L NAOH/0.50 mL hexane/extract on rotary-mixer for 15 minutes.
7. Aliquot 3 μL and inject into the chromatograph.

C. Standard Curve

A conventional standard curve was constructed using hexane solutions containing known amounts of DM and internal standard to determine the chromatographic linearity of response to DM and the internal standard. Also using the described procedure, the feasibility of measurement and efficiency of the extraction were determined by adding known quantities of DM (5–40 ng/mL plasma) and the internal standard to plasma. The described procedure was then followed and the parameter of interest obtained.

D. In Vivo Studies

A study was initiated in order to determine the steady state levels of DM in the plasma of subjects after 7 continuous days of receiving DM according to an accepted dosage regimen.

Beginning at 8 a.m., and every 6 hours for 7 days, 5 normal, healthy volunteers received 30 mg of DM·HBr as a solution. On days 1 and 7, blood samples were drawn at 0, 0.5, 1, 2, 4, 6, 6.5, 7, 8, 10 and 12 hours after the 8 a.m. dose. On days 2 through 6, a 24-hour blood sample was drawn immediately prior to the 8 a.m. dose. The plasma fraction was separated from the cells and frozen until assayed for DM according to the methodology.

III. Results and Discussion

Typical chromatograms of human plasma extracts from the study are shown in Figure 1. The sample on the left (A) is a control plasma extract from a subject prior to dosing on the first day. The sample on the right (B) is from the same subject 4 hours after being dosed with 30 mg of DM·HBr. The indicated peak corresponds to a residual DM plasma level of 13.5 ng/mL. The chromatograms are markedly clean due to the specificity and selectivity of the nitrogen detector. Instrument time expenditure is less than 5 minutes per sample.

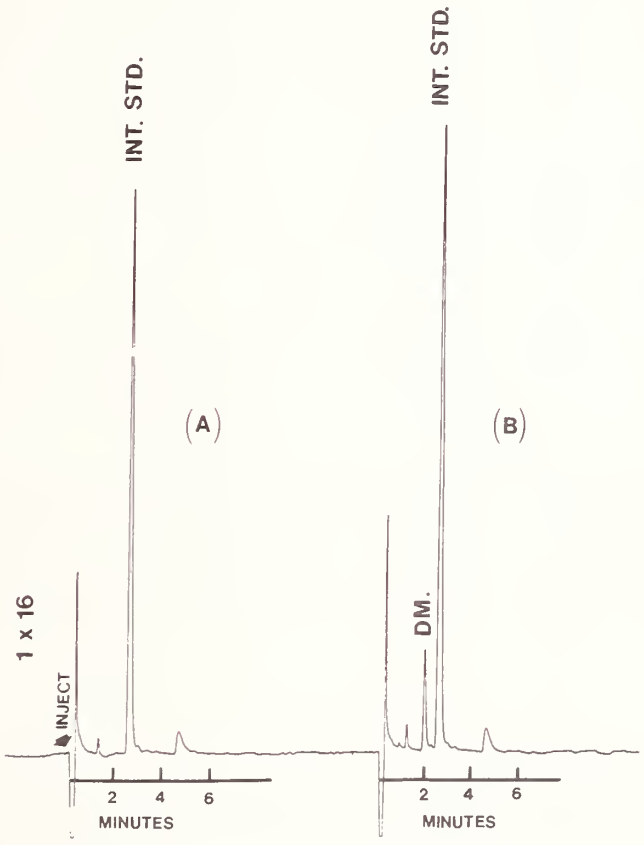

Figure 1. Typical chromatograms of human plasma extracts.

In order to demonstrate the reliability of measuring drug levels, known quantities of DM·HBr were added to human plasma and assayed according to the procedure. The results are shown in Table 1. As can be seen from the data, the technique provides a precise and reliable measurement of the drug in plasma.

After 5 subjects received 30 mg of DM·HBr at 6-hour intervals for 7 continuous days, their DM plasma levels were measured and compared. A graphical representation of the data was made by plotting and drawing composite curves.

Figure 2 shows a comparison of the plasma levels of DM, over a 12-hour period, on the first day of the study with those on the seventh day. The individual data points display the mean and standard error of the DM levels of the five subjects at each sampling time. As expected, the Day 7 values are significantly higher than those of the pre-steady state conditions of Day 1.

TABLE 1. *Recovery of dextromethorphan from human plasma*

Concentration (ng/mL)		
Added	Recovered	% Recovery
5.0	4.6	92
"	4.4	88
"	5.1	102
"	4.1	82
"	4.8	96
10.0	8.0	80
"	9.6	96
"	8.0	80
"	8.8	88
"	9.6	96
20.0	16.0	80
"	16.0	80
"	15.5	78
"	15.0	75
"	17.5	88
40.0	37.0	92
"	33.0	82
"	36.5	91
"	34.5	86
"	32.5	81
		Mean = 87
		S.D. = 7

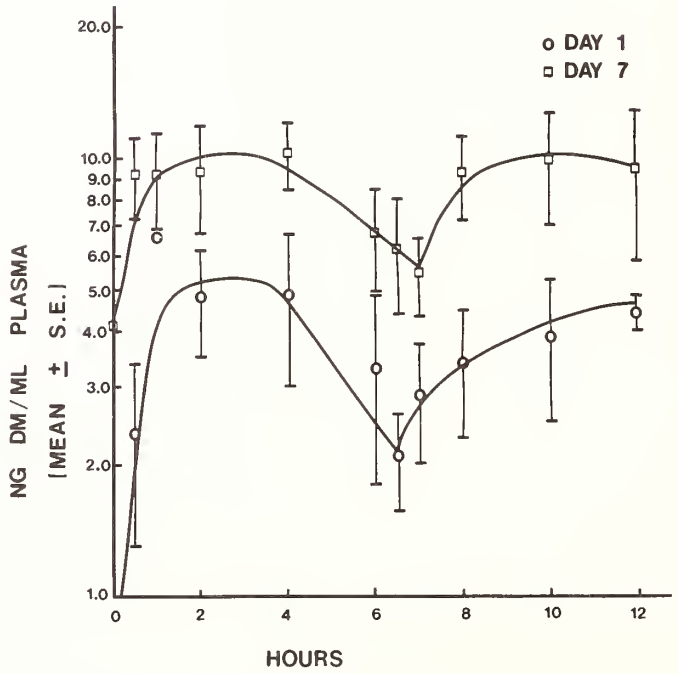

Figure 2. Comparison of Day 1 and Day 7 plasma levels of DM—Mean±S.E. of 5 subjects.

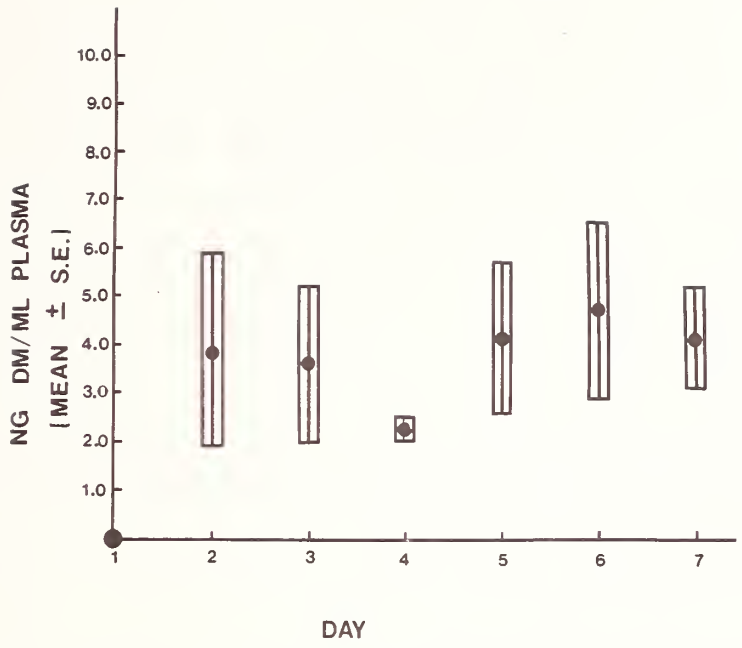

Figure 3. Comparison of DM plasma levels at 8 a.m. on Days 1 through 7—Mean±S.E. of 5 subjects—showing attainment and maintenance of steady state.

In order to establish at what point a steady state condition of DM plasma levels had been attained, a blood sample was drawn from each subject immediately prior to the 8 a.m. dose on days 1 through 7. The data, as shown in Figure 3, indicate that a steady DM level was achieved by the second day of the study with no significant change from that point through 7 days. This suggests that drug disposition, elimination and metabolism is not altered with chronic administration of DM.

IV. Conclusion

Using a sensitive and selective thermionic N-P detector, a sensitive and reliable G.C. procedure was developed permitting the measurement of plasma levels of DM after administration of therapeutic doses of this antitussive. The described methodology was employed to analyze plasma samples of subjects who had undergone a dosage regimen of 30 mg of DM·HBr at 6-hour intervals for 7 continuous days. The data generated support the fact that drug disposition is not altered by chronic administration of DM according to an acceptable dosage regimen.

V. Acknowledgments

The authors wish to acknowledge Dr. Lawrence Lillienfield of Georgetown University Medical School and Dr. Lee M. Sharp of Clinical Sciences Inc., who conducted the clinical investigation. In addition, we wish to thank members of the Pharmaceutical Development Department for preparation of the test formulations, as well as members of the Medical Department for their assistance in preparing the protocols.

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Section IV. ANALYTICAL SYSTEMS FOR TRACE ORGANIC ANALYSIS

NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

CHROMATOGRAPHIC METHODS IN ORGANIC TRACE ANALYSIS: CURRENT SITUATION AND PERSPECTIVES OF FUTURE PROGRESS

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Gas chromatography and high-performance liquid chromatography now belong among the most important methods of trace organic analysis. The analytical aspects of the column efficiency, detection sensitivity and ancillary techniques are reviewed for both methods. Future methodological directions are indicated.

Key words: Ancillary methods; bonded phases; chromatographic preconcentration; detector sensitivity; gas chromatography; glass capillary columns; high-performance liquid chromatography; LC microcapillary columns; selective detectors.

I. Introduction

Throughout the entire period of the development of chromatographic separation methods, any possibilities to work with ever decreasing amounts of samples have always been a welcome event to the chemist. As A. J. P. Martin recollects [1], approximately half a kilogram of protein was necessary for a reliable amino acid analysis prior to the inception of partition chromatography. To say that the reliable analyses can be currently performed by both gas and modern liquid chromatography at the nanogram level is no overstatement; less than 4 decades of progress reduced the sample size by an approximate factor of 10^{12}. With the invention [2] and further development of the electron capture detector, terms such as "femtogram" (10^{-15} g) or "attamole" (10^{-18} mol) are becoming common in the chemist's vocabulary. Some current technological innovations strongly suggest that further increases of the measurement sensitivity may be available in selected instances.

Chromatographic methods have rapidly become the most important means of organic trace analysis. The reasons for this situation are not difficult to understand. For sure, sensitivity alone is not entirely unique to chromatographic techniques, even though some of the most sensitive measurement systems were developed primarily for this method. Its ultimate strength resides with the unique capability to separate and simultaneously determine the individual components of even the most complex samples. The rare exceptions of extreme selectivity notwithstanding, the sensitivity limits of other instrumental techniques are frequently imposed by the presence of interferences from other molecular species. A separation step prior to the compound final determination thus further improves the analytical reliability even when a conventional (off-line) measurement method is ultimately used (e.g., in the radioimmunoassay). Thus, chromatography in principle extends the utilization of many other analytical methods.

While a combination of the chromatographic column and the detecting (measuring) device is the most crucial one to the analyst, the modern chromatography is perceived as including the specialized sampling systems, ancillary techniques and computational accessories for both the acquisition and evaluation of chromatographic data. Thus, the present-day chromatographs will typically include all these functions centered around the separation columns. To this extent, an important prediction of A. J. P. Martin [3] from 1957 is fulfilled in that "...here you have the uniting instrument of the gas chromatograph in the center with its slaves clustered around. The

calculating machine in the background will have the records of all previous substances separated...."

Efforts for better compound resolution and sensitivity in chromatography have been particularly intensified during the last decade. This is primarily due to an increasing emphasis on the trace analysis of organic substances in complex sample matrices and the need for their characterization. It appears that important advances are crucially dependent on the quality of chromatographic determinations in the fields as diverse as human physiology, pharmacology, food and environmental analysis, fuel technology, etc.

While some directions of the chromatographic trace analysis have matured to secure their significant utilization, other directions are at present in their developmental stages. The present article summarizes the most important advances in chromatographic trace determinations, while pointing at some potential developments in this field.

II. Advances in Gas Chromatography

While the earlier scientific efforts in the field of gas chromatography were primarily focused at the fractionation of major low-boiling compounds and a better understanding of the physical phenomena involved in the separation process, the beginnings of gas chromatography as a powerful trace analytical method fall into the middle 1960's. Undoubtedly, the three major areas of development responsible for this progress were: (a) introduction of highly sensitive ionization detectors; (b) dramatic improvements in GC column technology; and (c) chemical derivatization methods and improvements in sample preparation techniques. In addition, the significant contribution of the instrument industry to gradually improving the measurement reliability of analytical gas chromatographs, preparation of new types of separation columns, devising novel sample micro-manipulation techniques, etc., is widely recognized.

In terms of efficiency and sensitivity, gas chromatography has been unique among all analytical methods. The capillary column that is currently considered one of the most important analytical tools was invented more than 20 years ago, but developed to its full potential only recently. Instead, some of the most typical trace organic determinations of the past years are largely due to the availability of well-deactivated chromatographic packings coated with thin films of thermostable polymers. Thin-film packed columns have found particular appreciation in biochemical analysis [4] and environmental applications. Such columns are still widely used.

Because of their low resolving power, conventional packed columns will find only limited application in future multicomponent determinations and will gradually be replaced by capillary columns. The relatively insignificant number of capillary applications during the 1960's was a result of both insufficient column technology and a lack of suitable sampling techniques. The first difficulty has now been largely overcome by the development of stable glass capillary columns, while the satisfactory methods for direct sample introduction onto small diameter (0.2–0.5 mm, i.d.) columns have also been devised. Thus, the situation has now been reversed: while the injection splinters (used exclusively in the earlier capillary work) resulted in an undesirable sample waste and impaired quantitation, the direct sampling methods now permit a full utilization of the inherent advantages of capillary columns in trace analysis. Namely, the high inertness of glass capillary columns and the improved sensitivity and reproducibility due to the narrower peak profiles and less column bleed. The latter aspect is also important for a reliable and sensitive operation of the combined gas chromatograph/mass spectrometer.

An effective identification and a reliable quantitation of a trace component within a very complex mixture is largely derived from our capability of separation. Various surface treatment methods and an improved understanding of the behavior of thin stationary-phase films on glass surfaces have now been reflected in highly efficient and inert capillary columns.

The overall theoretical plate numbers as high as several hundred thousand are becoming common to many laboratories together with complex chromatograms containing hundreds of

resolved peaks. Alternatively, short glass capillary columns can serve extremely well in applications where lesser numbers of plates are required, while retaining some distinct advantages of their own such as high inertness, speed of analysis, minimum column bleed and the capability to analyze relative high-boiling mixture components. Several aspects of capillary gas chromatography in general [5,6] and in relation to environmental analysis [7] have been recently reviewed by this author.

Importance of preconcentration and sampling techniques in conjunction with capillary gas chromatography (and, indeed, analytical chromatography in general) cannot be overemphasized. While the amounts of the trace components of interest are typically within the operating concentration range of capillary chromatography, they are present in great dilution in most media that we analyze. The frontal chromatographic concentration technique of Novak et al. [8] has been most widely used in air and water pollution analytical problems, aroma research, and some biomedical applications. Different chromatographic materials have been used as the preconcentrating media by different investigators together with a great degree of variation concerning the most effective sample transfer into a chromatographic column.

Thermostable porous polymers have been particularly popular in connection with gas chromatography. While their principal advantages for the preconcentration of trace organics from both gaseous and aqueous media were demonstrated several years ago [9–11], much additional research has to be carried out concerning the quantitative aspects of such methods. An example of analysis from an aqueous medium is shown in Figure 1. The chromatograms [12] are due to urinary volatile constitutents, analyzed on the glass capillary columns with two different stationary phases; prior to chromatography, the samples were preconcentrated on the porous polymer, Tenax GC, and thermally desorbed into a column in the injection port of a gas chromatograph.

In addition to their concentration effect, chromatographic precolumns serve frequently the purpose of sample "clean-up." For example, the headspace concentration on a porous polymer precolumn avoids the problems with the formation of sample artifacts due to the thermal decomposition of extractable non-volatiles in the injector. Similarly, precolumns with a chromatographic packing material can be useful in removing an excessive amount of solvent as well as preventing the mechanical migration of non-volatile interfering material into the analytical column [13]. The requirements for sample clean-up are considerably greater in capillary gas chromatography than they have been in the packed-column work. This is an aspect of analytical work where compromises will be hard to make. On the other hand, effective sample purifications need not be necessarily difficult.

Sufficient sample volatility is a prerequisite of a successful gas chromatographic analysis. Although this is frequently considered to be a serious limitation of this method, gas chromatography spans an impressive volatility range. The introduction of thin-film column packings earlier by VandenHeuvel et al. [14] and a gradual development of procedures for reliable chemical derivatization of polar and labile molecules have been particularly important. Today's biochemical analysis through gas chromatography is a typical example of such advances.

It should be noted that the elution of heavier molecules can be more easily accomplished with capillary columns due to their different phase ratios. Figure 2 exemplifies this situation, showing the separation of polynuclear aromatic hydrocarbons on a short glass capillary column at relatively low column temperatures. Elution of coronene would typically require a column temperature over 300 °C if the packed column were used.

Improvements in sample derivatization are being still made (for a review see ref. [15]) with the objectives of improving reaction yields and reproducibility, thermal stability of the chromatographed solutes and chromatographic resolution. Characteristic structural features can be introduced into the studied molecules through derivatization for the analysis by the electron capture detector, other selective GC detectors, or mass spectroscopy.

In the area of GC detection, the flame ionization detector has survived for years practically unchallenged as the most universal (yet, very sensitive) and useful chromatographic detector.

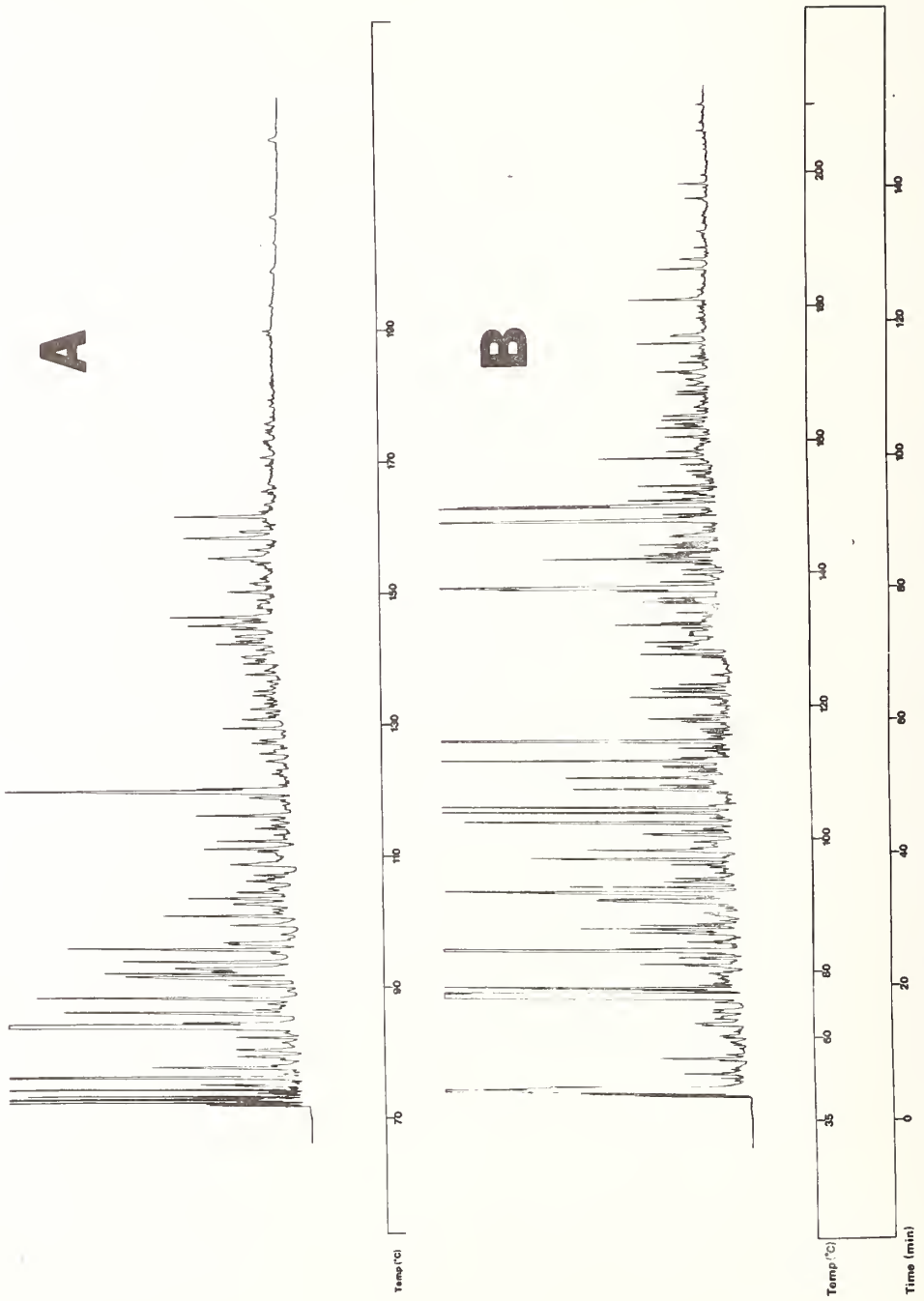

Figure 1. Chromatograms of preconcentrated urinary volatiles from a normal male as recorded by two glass capillary columns of different polarity. Reproduced from M. Novotny et al., *Clin. Chem.* **20**, 1105 (1974).

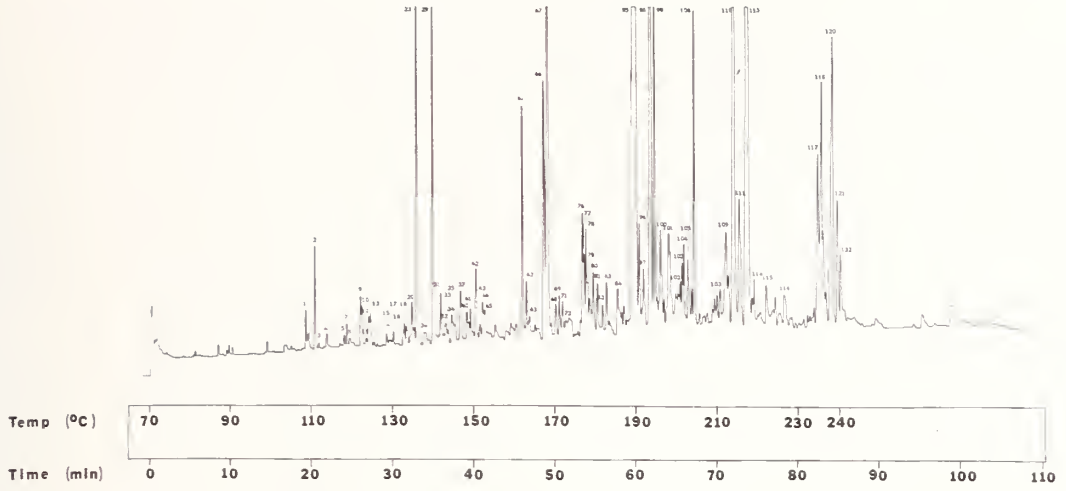

Figure 2. Capillary-column gas chromatogram of the total polynuclear aromatic fraction of airborne particulates. Reproduced from M. L. Lee et al., *Anal. Chem.* **48**, 1566 (1976).

However, due to their virtually nondestructive nature, some earlier types of ionization detectors with more or less general response (e.g., argon or photoionization detectors) could find some utilization in preparative capillary GC.

While selective GC detectors were frequently developed to alleviate the problems resulting from the excessive sample complexity and a lack of column resolving power, the current availability of efficient capillary columns does not diminish their importance. Firstly, the inherent sensitivity of many of these devices can only be strengthened due to the already mentioned desirable features of inert capillary columns for trace analysis. Secondly, in some instances the performance of a selective detector in measuring the solute concentration is affected by the presence of interfering compounds even when they are not detectable alone. Once again, the column resolving power is desirable.

In the analysis of complex mixtures, a simultaneous use of a universal detector with those giving a specific response can provide much complementary information. Figure 3 [16] demonstrates this situation rather dramatically. The non-selective, high-resolution GC profile shown on the upper chart is consistent with the level of complexity expected from this type of sample (sewage effluent); the electron capture recording is capable of tracing important environmental pollutants which would not be otherwise seen. The very large peak in the middle part of the lower chromatogram demonstrates a remarkable difference in response of the two detectors. A parallel use of up to four different detectors connected to a conventional wall-coated capillary column is now feasible through a proper design of the effluent splitter [17].

Numerous other advantages can be derived from the use of selective GC detectors. The high sensitivity aspect is perhaps the most important one for trace organic analysis. For example, a better understanding of the detection mechanisms with the electron capture detector [18] has led to significant sensitivity improvements; the detector is currently the most sensitive from all devices used for detection or organic molecules. Unfortunately, at this stage no ancillary tools of comparable sensitivity are available to reveal the structures of such low-level mixture components, although improvements can be expected from the current studies on ion-molecule reactions.

Mass-spectroscopic detection techniques, although generally expensive to many investigators, appear to offer the utmost in combination of sensitivity and selectivity at present. Since its development in the late 1960's, mass fragmentography has become one of the most important high-sensitivity detection techniques and shaped significantly many directions of

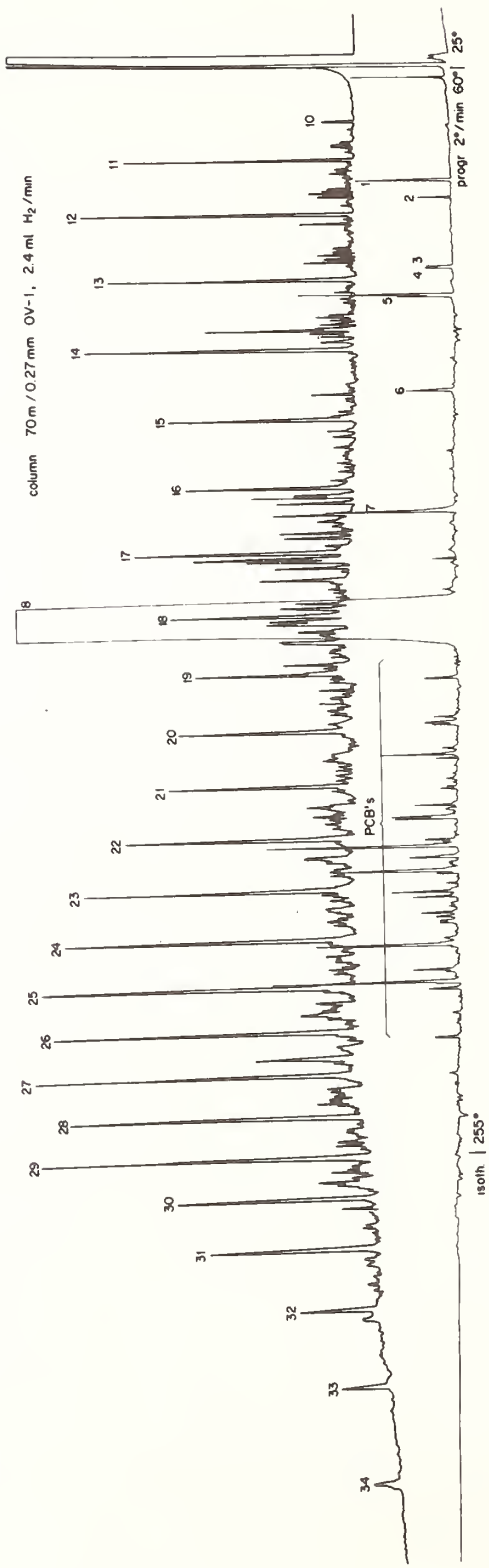

Figure 3. Chromatographic analysis of the nonpolar fraction of sewage extract. The organic components are simultaneously detected with the flame ionization detector (upper chart) and the electron capture detector (lower chart). Reproduced from K. Grob, *Chromatographia* **8**, 423 (1975).

pharmacological, toxicological and environmental research. The gradual development of various ionization techniques has further expanded its capabilities.

A number of useful selective GC detectors are available to the analytical chemist. Besides the already mentioned electron capture detector, the most commonly used detection principles are those of flame photometry, thermionic effects, microwave emission, electrochemistry, and the gas-phase optical spectroscopy. The most widely needed are selective detection methods for the compounds containing halogens, nitrogen, phosphorus, and sulfur. However, developments have also been evident with other organic substances of environmental interest such as those containing tin, lead, mercury, selenium, etc. It is expected that the future investigations into improved selective GC detection will be both popular and necessary.

While certain identification power of GC retention data (e.g., in isomer identifications) is recognized, sensitive ancillary techniques are vital to structural elucidations of trace organic mixture components. Evidently, some structural information can occasionally be extracted from an enhanced response with a selective GC detector (e.g., a presence of sulfur, nitrogen, phosphorus, etc., in a molecule). Sample derivatization methods can further enhance chances of identification with both sample selective detectors and ancillary tools.

The combined GC/MS is currently considered to be the most powerful method of organic analysis. Very significant technological advances have lately been made in this field, including a reliable coupling of capillary columns, different ionization methods, and the extensive use of computers in data handling and presentation. While the combination of capillary column GC with a medium-resolution MS has now become common, significant sensitivity improvements have also been achieved in capillary GC/high-resolution MS. However, it is also recognized that some structural assignments cannot be made as based on mass-spectral data alone; other ancillary methods are needed. Since an IR spectrum gives frequently information complementary to a mass spectrum, the recent sensitivity improvements in the Fourier-transform IR spectroscopy are seen as a significant breakthrough.

III. Advances in Liquid Chromatography

While there is every indication that the more fundamental developments in gas chromatography are rapidly approaching a plateau, numerous innovations have been characteristic for the liquid chromatography of recent years. The LC "renaissance" of the late 1960's and the emergence of high-performance liquid chromatography (HPLC) have had their eventful continuation until the present time. The utilization of higher inlet pressures and smaller particle sizes have been reflected in a great number of new efficient separations. The advent of chemically bonded stationary phases has further expanded the range of applications.

The recent progress in HPLC has been significantly aided by studies of both theoretical and empirical nature. For example, while the optimum particle size was earlier predicted by Knox and Saleem [19], the development of efficient columns packed with 5 to 10 μm particles is a result of many tedious experimental efforts over several years.

Using small particles of porous nature, LC separations have been considerably improved and the plate-height values as low as 20 μm have been reported. The selectivity has also been available with changing from the conventional absorbents to various chemically bonded stationary phases. Chromatographic properties of various bonded phases were extensively studied, ranging from hydrophobic to very polar packings and ion-exchangers. Although kinetic properties and the retention mechanism under different chromatographic conditions are not always adequately understood, the analytical utility of such column substrates is now beyond dispute. Hydrophobic packings in the reversed-phase chromatography have been by far most popular in analytical work.

It became soon evident in HPLC that the mobile-phase interactions are a very powerful means to affect the solute retention. Therefore, much current research has been concentrated into this area. The current emphasis on the ion-pairing chromatography and similar techniques is evidence of an increasing importance of this direction.

An adequate technology of small-particle columns, availability of chemically bonded phases usable under the conditions of gradient elution, and an improved understanding of mobile-phase phenomena have all had a very significant impact on the quality of current LC analyses. A chromatogram shown in Figure 4 of a mixture of selected amino acids and peptides [20] attests to the remarkable improvements becoming recently available.

It is widely recognized that very substantial advances in bio-chemistry and polymer chemistry are due to the development of gel chromatography. While technology of hydrophilic gels has been steadily improving over the years to the great benefit of researchers in the field of protein chemistry and nucleic acids, a more recent availability of lipophilic gels [21] is a welcome advance in the fractionations of medium-sized molecules. Although chromatography on lipophilic gels is seldom used as an analytical method by itself, many effective fractionation and sample purification schemes are now crucially dependent on it. Even when the final determinations are made by either GC or HPLC, the precision of such high-sensitivity measurements is strongly dependent on the high inertness of lipophilic gels, as demonstrated by determinations of steroids and other substances in small plasma samples [21].

Since the conventional gel chromatography is not compatible with the high-pressure operation, other column materials have been investigated for high-speed analytical work. These primarily include the porous glass, macroporous silica, and some highly cross-linked organic resins. Since chemical derivatization of the siliceous materials is often desirable, the column technology somewhat overlaps with the preparation of conventional bonded-phase materials for HPLC. Although much work remains to be done in this area, some promising high-speed separations were already obtained, including synthetic polymers, proteins, nucleic acids, and polysaccharides [22,23].

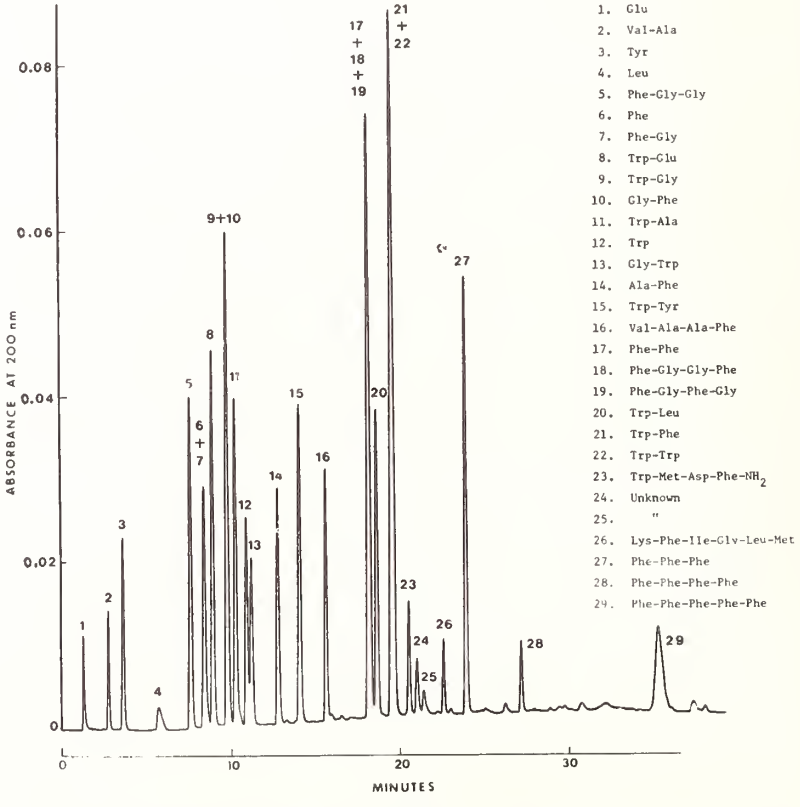

Figure 4. Liquid chromatogram of nonpolar amino acids and small peptides obtained on a reversed-phase bonded packing with gradient elution. Reproduced from I. Molnar and C. Horvath, *J. Chromatogr.* **142**, 623 (1977).

Advances in LC detection appear to fall far behind the column developments. As we enter the second decade of HPLC, still no sensitive, universal detector is available. Obviously, the sensitivity and detection conveniences in GC (due to gas-phase ionization phenomena) are not easily paralleled in LC.

Many chromatographers resort to a wider use of selective detectors. This approach is particularly fruitful while detecting suspected solutes, but it is less valuable in the analyses of the samples with unknown composition. While a number of LC detectors were suggested during the past decade, various spectrosopic LC monitors enjoy most application. Availability of flow-cell spectrophotometers and spectrofluorometers with adjustable wavelengths has significantly aided many LC analytical applications, although some problems of a limited compatibility of chromatographic and detection conditions occasionally exist. An extension of the UV spectral range down to 190 to 200 nm results in numerous detection possibilities with a proper selection of a non-absoring mobile phase. Similarly, responses of other spectroscopic detectors can be maximized through a proper selection of the involved parameters.

Enhancement of detection sensitivity is frequently achieved through a chemical alteration of solutes. Thus, the molecules of interest can be "tagged" with a suitable chromophore. A recently advanced fluorometric detection of amino acids and peptides [24,25], serves as an outstanding example of such efforts. Pre- and post-column derivatizations are becoming increasingly common, but some limitations exist with both approaches. Specifically, while the advantages of selective chromatographic interactions are often lost with the precolumn derivatization, the post-column approach can encounter difficulties with the incompatibility of column and detection conditions.

Whereas detection sensitivity figures known to gas chromatographers are generally less common in LC applications, some impressive detection limits have been obtained. In the measurement sensitivity aspect, the spectrofluorometric and electrochemical detectors [26] are clearly leading the way.

Just as in GC identification efforts, on-line ancillary techniques are sought in HPLC for the purposes of structural elucidation. Thus far, possibilities to combine HPLC with mass spectrometry or Fourier-transform IR spectroscopy have provoked most interest in the recent years. Although some positive indications are emerging from the current research efforts, the problems are technologically involved. Less difficulties are encountered with an on-line acquisition of UV-visible or fluorescence spectra. However, the diagnostic power of these data is generally less than with other spectral methods.

While assessing the current potential and limitations of LC analytical methods, recent technological advances in the field of thin-layer chromatography (TLC) should not be excluded from consideration. Recent improvements in the sample application techniques, quality of thin layers, developing conditions, and improved detection caused some investigators to use the term "high-performance TLC" [27]. Whether or not the separation characteristics of LC in columns and TLC can be judiciously compared is a matter of numerous current discussions. However, both methods have their inherent advantages. An attractive feature of TLC operation for trace analysis is the capability of zone concentration at the sharp boundary between two different stationary phases as recently shown by Halpaap and Krebs [28]. Figure 5, reproduced from their work, shows a difference between two TLC runs: one with, and the other without the zone concentration effect.

IV. Some Future Directions of the Chromatographic Analysis

Various analytical separation methods have now achieved a degree of maturity in both resolution and sensitivity. Even some techniques that were considered relatively sophisticated just several years ago (e.g., glass capillary techniques, combined GC/MS, or mass fragmentography) are rapidly becoming routine. In certain directions, chromatography instrumentation advanced

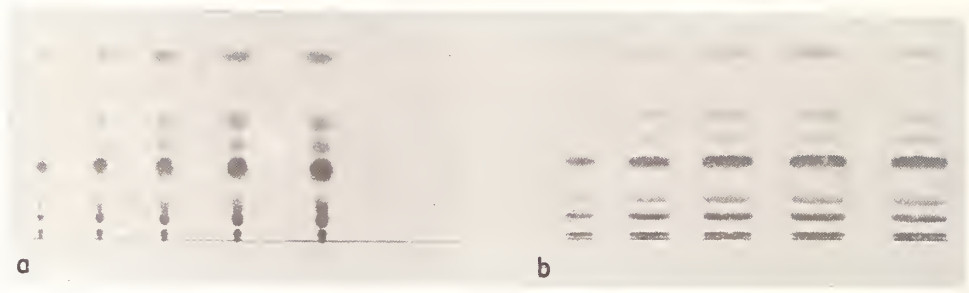

Figure 5. Chromatograms of the mixture of lipophilic pigments obtained on (a) conventional silica gel plate, and (b) silica gel plate with a concentrating zone. Reproduced from H. Halpaap and K.-F. Krebs, *J. Chromatogr.* **142**, 823 (1977).

dramatically. The question inevitably arises as to which directions should be pursued to extend the analytical scope of chromatographic methods.

The category of improvements that is clearly visible at present includes technological and design advances in the area of chromatographic instrumention. The ever-increasing number of routine analytical applications will be strongly dependent on this direction. Effective analyses of complex mixtures with sufficient accuracy and precision will remain among important subjects of investigation for some time. One fundamental key to a more reliable quantitation will be a better understanding of the solute interactions with sample matrices.

In efforts to improve measurement precision, it is essential to control various instrumental parameters quite rigorously on a day-to-day basis. This is now becoming increasingly more realistic due to the progress and economy of computer-controlled instrumentation.

There is, no doubt, sufficent room for improvements and new developments of ancillary methods. Various physical methods become gradually compatible with the amounts of chromatographed substances. It is generally felt that also the chemist's imagination will continue to have a major impact on structural identification through sample derivatization. Micromanipulation techniques will undoubtedly improve with time. It should be noted that while new structural techniques are becoming rapidly available, the physical dimensions of interfacing separation columns must also be optimized.

An increasing importance of data treatment methods in connection with multicomponent analyses will soon be widely recognized. While the separation, detection, or even identification of hundreds of compounds in a single run is an impressive achievement of today's chromatography, the amount of data generated may frequently be ahead of our present interpretation capability. In order to appreciate the meaning of such chromatograms to find the correlation of multicomponent data with the needed answers, a wider application of computational methods will be necessary. Presently, we are only at the beginning of such efforts. A demonstration of this direction can be seen in the application of pattern recognition to metabolic profiles generated by capillary GC [29] through a reliable automated equipment [30].

What are the future trends in chromatographic resolution? The recent advances in capillary GC have evidently pushed the status of this method quite close to its theoretical limit, at least in terms of column efficiency. Many selective interactions between solutes and mobile phases have also been explored, including separations on optically active substrates [31,32] or liquid crystalline phases [33]. Although further design of highly selective systems is desirable, this approach has only limited application in the analysis of complex mixtures.

The extreme complexity of some environmental and biological samples suggests that additional theoretical plates would be of advantage. An shown by Desty et al. [34] long ago, a decrease of the capillary column diameter improves its efficiency quite dramatically. Figure 6, demonstrating the resolution of heptane insomers in less than a minute, remains one of the most impressive separations ever achieved. The column diameter was reduced to 100 μm. Now, with

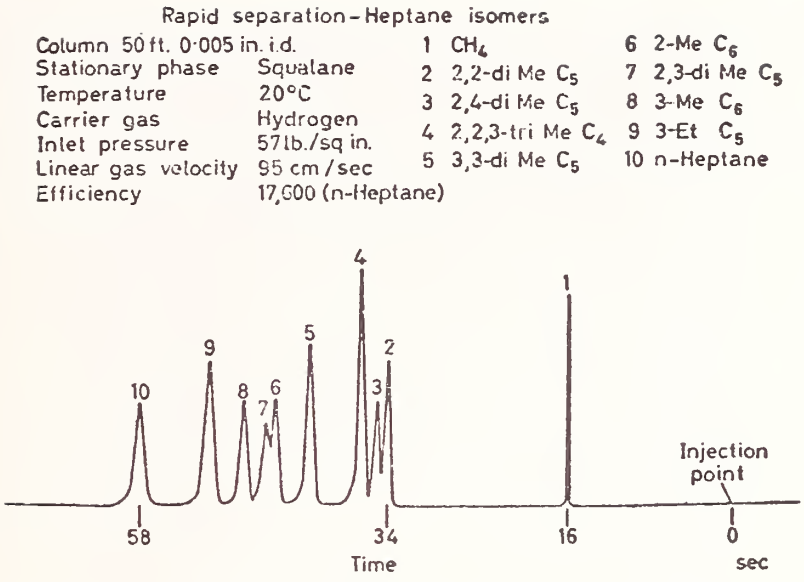

Figure 6. Fast analysis of heptane isomers with a small-bore capillary column. Reproduced from D. H. Desty and A. Goldup, in "Gas Chromatography 1960," R. P. W. Scott, Ed., Butterworths, Washington, D.C., 1960, p. 162.

less emphasis on the speed of separation in capillary GC, simple calculations show that the plate numbers well over one million should be feasible with moderately high inlet pressures.

While non-conventional sampling, detection and recording methods will be needed in work with GC capillary columns of 100 μm internal diameter or less, there are some very promising attributes of this direction. Will it become possible to resolve every single mixture component within given volatility range? If possible, would it become feasible to make structural assignments as based on high-precision chromatographic data alone?

Whereas LC is an obvious substitute of GC for samples with lower volatility, its separation task are even more difficult. With increasing molecular weight of solutes, the number of possible isomers increases dramatically. Yet, conventional high-performance LC falls short of capillary GC in terms of theoretical plates. For sure, some impressively low plateheight values have recently been reported. However, the preparation of conventional (long) LC columns with an overall high efficiency may encounter numerous problems. The ultimate limits of the small-particle technology were discusses by Halasz et al. [35]. Capillary LC in either (packed) semi-permeable [36] of micron-size open capillaries [37] may provide a solution to such problems.

In the theoretical analysis by Giddings [38] of the efficiency limits of GC and LC, the product of the mobile-phase viscosity and the solute diffusivity for both methods determines in the major part the number of theoretical plates. While LC should have an advantage of about three orders of magnitude over GC, the inlet pressure becomes the most important variable in controlling the column efficiency. Thus, future utilization of appreciably higher pressures in LC is a very distinct alternative.

Recently developed packed microcapillary columns [36] are consistent with the above considerations. Figure 7 shows a microphotograph of such a column, demonstrating its semi-permeable nature together with a relatively regular packing structure. High efficiencies can be obtained with such columns through a properly modified equipment. In addition, the low flow-rates typical for microlumns (of the order of several μL/min) present some new opportunities for the design of novel detection and ancillary techniques.

Whereas the presently available chromatographic methods have dealt adequately with relatively small molecules, the area of separation of large molecules, molecular aggregates, particles, and subcellular units has been relatively neglected. An importance of this area to various

Figure 7. Microphotograph of a section of alumina-packed microcapillary (75 μm, i.d.) for liquid chromatography. Reproduced from T. Tsuda and M. Novotny, *Anal. Chem.* **50,** 271 (1978).

aspects of scientific research has been increasingly obvious. It may well become the next frontier in the separation science. While outstanding separations by electrophoretic techniques (e.g., the high-resolution, two dimensional electrophoresis shown recently) are already at the scene, much improvement in the area of the chromatographic separations will be necessary.

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FLOW INJECTION ANALYSIS—A NEW APPROACH TO QUANTITATIVE MEASUREMENTS

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The reason for automation of chemical analysis should not only be a necessity to analyze a large number of samples, but also less apparent advantages of automated sample handling, i.e., better reproducibility, instant availability of analytical result, and new opportunities for innovation. The continuous flow analysis is in comparison to batch approach closest to this ideal as it is easily programmable, yet its conventional form based on air segmentation is aimed to meet the first two goals, but certainly not the rest of them. It is the nonhomogeneity of the carrier stream, segmented by air, which causes pulsation and irregular movements of the liquids in the system, which requires a "steady state" readout to be taken if sufficient reproducibility of the measurement is to be achieved.

The new method of continuous flow analysis is based on an entirely different approach and utilizes three principles: 1) sample injection, 2) controlled dispersion, and 3) reproducible timing. In the absence of air, the sample zone, injected into a carrier stream of reagent will disperse on its way towards the detector. This process of zone dispersion can be used (rather than unselectively retarded by air bubbles) for various analytical purposes. Following the principles of chemical reactor engineering and the theory of laminar flow in narrow tubes, limited, medium, or large dispersion can be achieved. This allows a new approach to design of analytical methods based on spectrophotometric, fluorescence, nephelometric, voltammetric, and ion-selective electrode measurement. As the flow system is miniaturized, the sample has a dwell time in it between 3 to 30 s which makes the readout instantly available.

Emphasis will be given on explaining the theory of the dispersion and its relation to the flow conditions in the system. The ways to utilize the dispersion patterns for analytical purposes will be illustrated by examples of methods from clinical, agricultural, and pharmaceutical analysis.

Key words: Automation; continuous analysis; controlled dispersion; flow injection analysis.

The concept of continuous flow analysis is to many people synonymous with the concept of Skeggs [1] who 20 years ago suggested the use of air segmentation to prevent carry over and to preserve the identity of individual samples. The effectiveness of this approach was so obvious and its realization in the AutoAnalyzer system was so successful that this type of continuous flow analysis became firmly established in countless clinical and industrial laboratories. The main conceptual features of the system, i.e., air segmentation, proportional sampling and steady state readout became the unquestionable parts of the philosophy of automation.

Yet, there are definite disadvantages inherent in Skeggs concept (Fig. 1). Air has to be added and, unless "Computerized out" [2], it has to be removed again prior to measurement in a flow through cell. What is worse, however, is the compressibility of air which necessitates the use of relatively long dwell times of the sample in the system (2–10 min) in order to average the small irregularities caused by stream compressibility and pulsation. To this long dwell time contributes also the proportional sampling, which is based on aspiration of the sample into the system by the peristaltic pump, which simultaneously introduces air and reagents through parallel channels. Neither the metered sample volume nor the volume of the sample in the transfer line to the detector is exactly defined, due to the already mentioned stream compressibility. Therefore, the concept of reaching a steady state signal becomes a necessity, as any readout attained at the

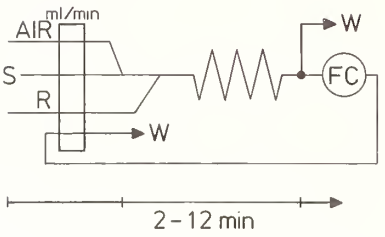

Figure 1. Schematic diagram of a segmented flow continuous analyzer.

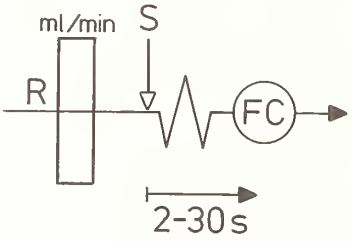

Figure 2. Schematic diagram of a non-segmented flow continuous analyzer.

steeply ascending part of the curve would be uncertain. Furthermore, the effectiveness of air segmentation in limiting the sample zone dispersion was so obvious that it was never tried, or considered, to use the dispersion patterns for various analytical purposes.

There is, however, a way [3,4] to perform continuous flow analysis of discrete samples using a carrier stream which is not segmented at all (Fig. 2). In such a system where the sample is injected into a carrier stream after a pump, the dwell time can be radically shortened, which means that the analytical readout is available nearly instantaneously after the sample has been injected. At the present stage of development the sample volumes range from 3 to 30 microlitres and the pumping rates vary from 0.5 to maximum 1.5 mL/min per channel, while typical sampling frequencies are 120 determinations per hour.

Although it might appear that the main feature of the Flow Injection method [3,4] is the absence of air segmentation, or perhaps the feat of injecting the sample into a system, it is important to realize that this technique is founded on a combination of the following three principles: 1) sample injection, 2) reproducible timing, 3) controlled dispersion.

The purpose of the *sample injection* is to place a well defined sample zone into a continuously moving stream in such a way that the movement of this stream is not disturbed. The amount of sample, although not necessarily needed to be accurately known, has to be injected with a high precision so that the volume and length of the sample zone at the point of injection is well reproducible. The injection technique has progressively evolved from the use of a syringe and a flap valve to the use of a rotary valve with a bore acting as sample container, and furnished with a bypass of a higher hydrodynamic resistance than the sampling volume (Fig. 3). In the latter device the carrier stream flows entirely undisturbed while filling of the volumetric bore takes place, because the pumped stream bypasses the valve through a shunt, and only after the core of the valve has been turned is the sample zone injected into the system by the stream which now follows the flow path of lower resistance. This way of injecting the sample into a continuously flowing stream is more reproducible than the aspiration technique used in the air segmented systems, and therefore the injection technique allows the conventional concept of the "steady state" to be abandoned and the sampling frequency to be increased while the sample consumption is reduced.

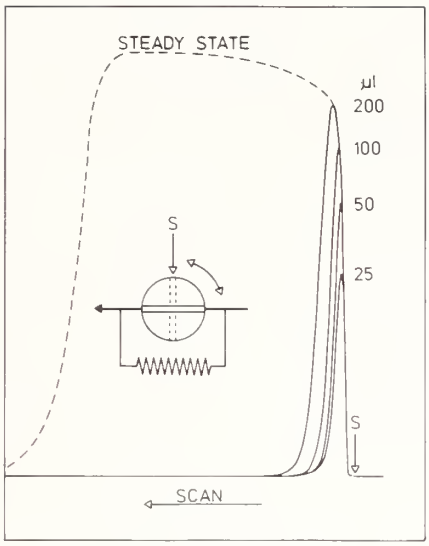

Figure 3. Diagram of rotary valve and response to various injection volumes.

If a solution of a dye of a concentration C_o^o were injected into a colorless carrier stream, the rising part of the curve could be described by the equation [6]:

$$C = C_o^o (1-e^{-kS}) \tag{1}$$

where $S_{1/2}=0.693^{k-1}$ is the volume of sample necessary to reach 50% of the "steady state" (Fig. 3). Thus for sample volumes smaller than $S_{1/2}$ the increase of S would nearly linearly increase the peak height (C^{max} or H^{max}).

As the signal is no longer to be read at the flat part of the response curve, but on its steep ascending part, a highly *reproducible timing* is essential as any imprecision in residence time of the sample on its way to the detector will be reflected in the readout. It is well recognized that precise timing is vital in continuous flow analysis, where the chemical reactions employed are very seldom allowed to reach equilibrium. Therefore, besides purely physical reasons, related to the length of the sample zone and its dispersion as it flows towards the detector, the chemistry involved also requires a reproducible travel pattern of the sample from the point of introduction through the system and the detector. In the absence of air segmentation the only source of pulsation in a nonsegmented stream can be attributed to the use of an imperfect pump, a problem which can easily be avoided. Besides, because the sample does not pass through the pump on its way to the detector (Fig. 2) its path through the system is well defined, and the dispersion of the sample zone and the residence time can be chosen at will to suit exactly the requirements of the chemistry involved. Not only long, but extremely short residence times can be reproducibly maintained.

The *controlled dispersion* of the sample zone which occurs during its passage through the system towards the detector results in a response curve having a peak shape type which is characteristic of the Flow Injection system. As expected, the sample zone broadens as it moves downstream and changes from the originally asymmetrical shape to a more symmetrical and eventually Gaussian form. By changing the flow parameters, the dispersion can be easily manipulated to suit the requirements of a particular analytical procedure so that optimum response is obtained at minimum time and reagent expense. Furthermore, the concentration gradients in the interfacial regions between the sample and the carrier solution can be employed to develop entirely new analytical techniques. Generally, dispersion can be classified as limited, medium and

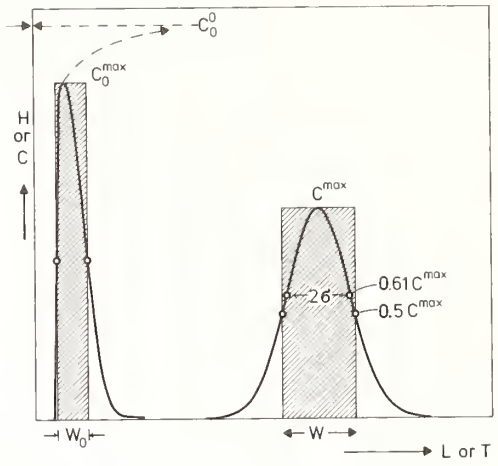

Figure 4. Dispersion curves.

large, and as the analytical readout in Flow Injection analysis is mostly based on peak height measurement, it is advantageous to define the dispersion as *the ratio of the concentrations before and after the dispersion process has taken place in those elements of fluid which correspond to the maximum of the dispersion curve* (Fig. 4). By denoting C_o^o as the original concentration of the injected sample solution and C^{max} as the concentration in the element of fluid corresponding to the peak maximum, the dispersion is related to the peak height H by:

$$D = \frac{C_o^o}{C^{max}} = \frac{\text{const. } H_o^o}{\text{const. } H} \tag{2}$$

where const. is a conversion factor between instrument readout and concentration as obtained by calibration (In colorimetry—providing that Lambert-Beer's law holds for the dye and concentration range chosen—H can be expressed as the recorded peak height, e.g., in mm, and H_o^o as the distance between the baseline and the signal recorded with the flow cell filled with the dye of the original concentration).

Using this approach the *total dispersion* D_t might be imagined to consist of:

$$D_t = D_i \cdot D_f \cdot D_d \tag{3}$$

where D_i is the dispersion due to the injection system, D_f is that of the flow arrangement, and D_d is the dispersion ascribed to the detector. Using the same flow cell throughout, the latter is constant, and in the present context, and based upon experimental evidence, D_d might be assigned the value of 1 as the detector volume is negligible. The D_i value is obtained from equation (1) by replacing C by C^{max}, while the D_f value was found to be, for an open narrow tube and $S \leqslant S_{1/2}$

$$D_f = \text{const } \sqrt{t_i}\,\sqrt{T}\,F \tag{4}$$

where const and $\sqrt{t_i}$ are constants for given flow geometry and intensity of mixing, F is the linear flow velocity and T is the mean residence time—coinciding with peak top appearance. By using tracer color injection, the D_i values in different flow geometries at different pumping rates were measured and the rules for construction of the Flow Injection system were suggested [4].

If the original composition of the sample solution is to be measured, e.g., pH, pCa, conductivity, a *limited dispersion* ($D_i = 1\text{-}3$) of the sample zone is required in order to ensure that

the readout as obtained at the center of the sample zone is not affected by any mixing with the surrounding carrier stream. Also when the Flow Injection system is to serve merely as a means of reproducible introduction of the sample into the detector, (e.g., in atomic absorption or flame photometry) the conditions of limited dispersion are most suitable.

If however, one or several chemical reactions resulting in color formation, change of pH, adjustment of ionic strength, complex formation, etc., are to take place, a *medium dispersion* (3–10) of the sample zone is called for. The reason is that the center of the sample zone must effectively be mixed with the carrier stream and often with several reagents in sequence. A suitable compromise between the requirements of mixing and reaction time on one hand and maximum acceptable broadening of the sample zone on the other hand must be found as the increase of the zone width decreases the sampling frequency. Medium dispersion encompasses a very broad area of application as on one end it can accommodate very fast chemical reactions like those employed in turbidimetry, where the sample is mixed with reagent and immediately measured before the crystals start to cluster from a colloidal precipitate, while on the other end it might be used to promote several reactions in sequence like in the enzymatic analysis of urea measured through the formation of indophenol blue.

Although this reaction proceeds in several stages:

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} CO_2 + 2NH_3 \; (pH = 7) \quad (1)$$

$$NH_3 + ClO^- \rightarrow NH_2Cl + OH^- \; (pH = 12) \quad (2)$$

$$2OCl^- + NH_2Cl + C_6H_5O^- \rightarrow OC_6H_4NCl + 2Cl^- + OH^- \quad (3)$$

$$OC_6H_4NCl + C_6H_5O^- \rightarrow \underset{\text{INDOPHENOL BLUE}}{OC_6H_4 = N\text{–}C_6H_4O^-} \quad (4)$$

at various pH, it was possible to design a flow system (Fig. 5) and the reagent compositions in such a way that the analytical readout was available 15 seconds after sample injection (Fig. 6) and the method was found suitable for urea determination in the range of 2 to 8 mmol/L, thus making an analysis of urea in undialyzed serum possible at a rate of 100 determinations per hour (see group of peaks at right in Fig. 6).

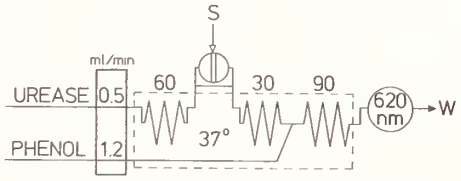

Figure 5. Flow injection analyzer for urea.

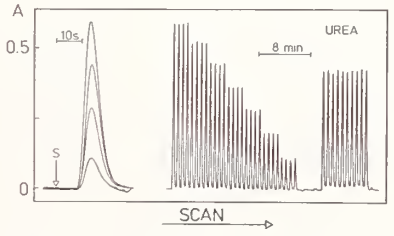

Figure 6. Analytical readout for urea analyzer.

Stop Flow Injection Analysis

It follows from the theory that the dispersion increases with the square root of the residence time T, but this is, of course, true only if F is constant and larger than zero. Should the carrier stream cease to move, the dispersion of the sample zone will stop (except for a negligible contribution caused by the radial molecular diffusion) and D_t will remain constant, i.e., independent of T. In other words, by stopping the flow one would gain the reaction time. Thus, providing that the movement of the carrier stream can be exactly controlled from the situation of complete standstill to the operational pumping rate used, one could let the sample zone travel through the line until the reagent has been mixed with the sample material to the desired degree (D_t), then stop the flow to allow the reaction to proceed, and then restart the flow to remove the measured sample and to introduce a new one. In absence of air, there is no movement of liquid in the conduits of the manifold after the pump has been stopped, and the formation of the reaction product can be followed.

The specific reaction of glucose dehydrogenase with β-D-glucose was used for the kinetic determination of glucose in order to verify the idea of the stop flow injection principle. A commercially available System Glucose enzyme set (Merck, Germany) was used in which the coenzyme nicotinamide adenine dinucleotide (NADH) serves as a chromogen which can be measured spectrophotometrically at 340 nm. Further details on this chemistry, the reagent compositions and the experience with one and two point kinetic measurements were published previously [5]. The manifold used in the present experiment is shown in Figure 7, and the response curves in Figure 8.

In these experiments the concentration of glucose in the injected sample (S=30 microlitres) was increased from 1 to 20 millimoles/litre and the sample zone was stopped in the flow cell as

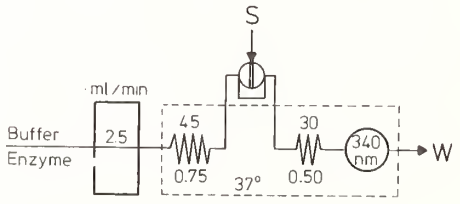

Figure 7. Flow injection analyzer for glucose.

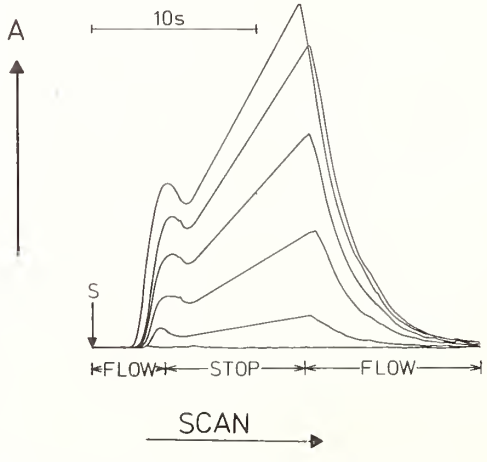

Figure 8. Glucose analyzer kinetic response curves.

soon as the recorded curve reached its maximum, that is, when $t=T$. As the pump was thus stopped for 9 seconds, the measuring cycle consisted of three parts: (1) the sample injection, dispersion and transport into the detector (4.5 s); (2) the measuring period with the stopped flow (9 s); and (3) the washing period (10 s) at the end of which the next sample was injected. Further details of this method, which allows a multipoint kinetic determination to be performed at a rate of 150 samples/h, will be published shortly.

The application of Flow Injection Analysis is not limited to measurement of homogenous solutions or one phase equilibria.

Thus *solvent extraction* was successfully developed for the determination of caffeine in acetylsalicylic acid preparations as well as of codeine and vitamin B_1 at a rate of 100 determinations per hour using sample volumes 12 to 25 microlitres [7].

Gas permeation through a porous Teflon membrane, followed by a spectrophotometric measurement of the color change of a bromocresol green pH indicator was used for ammonia determination, executed at a rate of 100 determinations per hour, using 30 microlitre samples and a pumping rate of 0.6 mL/min.

The Flow Injection Analysis is still in the initial stages of development, and new surprising applications of this method will undoubtedly be discovered in the near future. The use of the concentration gradients formed within the sample zone on its way towards the detector appears to allow yet another degree of freedom to be utilized for developing of new analytical methods. Further development of the theory of dispersion will be based upon the work of Sternberg [8] on dispersion in chromatographic columns and the work of Bullham [9] on flow systems.

Acknowledgment

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ION-PAIR HPLC OF DRUGS AND RELATED ORGANIC COMPOUNDS

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The principles for high performance liquid chromatography (HPLC) based on ion-pair distribution are indicated. Ion-pair HPLC can be performed in straight phase mode with an organic mobile phase and an aqueous stationary phase and in reversed phase mode with an aqueous mobile phase and a nonpolar surface or liquid as the stationary phase. The retention can be regulated by the kind and concentration of the counter ion added to the aqueous phase.

The design and preparation of stable systems for ion-pair HPLC is indicated and the influence of the composition of the phases on the separating efficiency and selectivity is demonstrated.

Straight phase systems for separation of amines, amino acids and dipeptides as well as anionic compounds (carboxylates, sulphates, sulphonates, sulphamates, conjugates of glycine and glucuronic acid) are demonstrated. By use of counter ions with high UV-absorbance, down to a few nanograms of non-absorbing compounds can be detected.

Reversed phase systems are given for separation of psychotropic amines (e.g., imipramine, amitriptyline, fluphenazine, zimelidine) and their metabolites, catecholamines and related phenylethylamine derivatives (e.g., adrenaline, dopamine), sulphonamides, barbituric acids and their metabolites, amino acids, dipeptides and metabolites of biogenic amines (phenylacetic, mandelic and indoleacetic acid derivatives).

Key words: Amino acids; barbiturates; carboxylic acids; catecholamines; conjugates; detection sensitivity; dipeptides; drugs; HPLC; ion-pair adsorption; ion-pair chromatography; ion-pair partition; metabolites; psychotropic amines; selectivity; sulphonamides.

The isolation of trace amounts of organic compounds from complex samples is of rapidly increasing importance in biochemical and pharmacological investigations. Liquid chromatography based on ion-pair distribution, usually called ion-pair chromatography, has during the last years found a wide application in such studies due to its high selectivity and versatility which makes it suitable for ionic samples of widely different kinds.

The use of the technique in drug research has been summarized in some recent reviews [1,2].

The principle of the technique can be demonstrated by an expression for the distribution of a sample ion, Q^+, between the two phases in a chromatographic system

$$Q^+_{aq} + X^-_{aq} = QX_{org}$$

where *aq* signifies a polar liquid phase and *org* a non-polar surface or liquid phase. The prerequisite for the transfer of Q^+ to the non-polar phase is that it is accompanied by an equivalent amount of a counter ion, X^-. The equilibrium constant for the distribution process, $K_{ex(QX)}$, usually called the extraction constant,

$$K_{ex(QX)} = \frac{[QX]_{org}}{[Q^+]_{aq} \cdot [X^-]_{aq}} \qquad (1)$$

can be influenced by the nature of the counter ion, X^-, and the properties of the two phases.

I. Control of the Retention

Ion-pair chromatography can be performed "straight phase" with a non-polar mobile phase and an aqueous solution of the counter ion as the stationary phase. The sample, Q^+, migrates as the ion-pair QX and its capacity ratio, k'_Q, given by:

$$k'_Q = \frac{V_s}{V_m} \cdot \frac{1}{K_{ex(QX)} \cdot [X^-]_{aq}} \tag{2}$$

"Reversed phase" mode can also be used. The mobile phase is then a solution of the counter ion in a polar liquid while the stationary phase is non-polar. The sample, Q^+, is retained as ion-pair and migrates as ion. When the stationary phase is a liquid (non-polar), the capacity ratio is given by

$$k'_Q = \frac{V_s}{V_m} \cdot K_{ex(QX)} \cdot [X^-] \tag{3}$$

Reversed phase ion-pair chromatography can also be performed with a non-polar adsorbent as the stationary phase. The absorption ability of the non-polar surface is limited and adsorption of other components of the mobile phase as uncharged molecules or ion-pairs reduces the retention of the sample [3]. This can be illustrated by the following expression for the capacity ratio of the sample

$$k'_Q = \frac{K_1 \cdot K_{ex(QX)} \cdot [X^-]}{1 + K_{ex(AX)} \cdot [A^+] \cdot [X^-]} \tag{4}$$

which shows the influence of the counter ion X^- and another mobile phase component, A^+ (e.g., a buffer component), adsorbed as ion-pair with X^-.

When the adsorption of the ion-pair AX dominates, due to high $K_{ex(AX)}$, $[A^+]$ or $[X^-]$, eq. (4) assumes the following form:

$$k'_Q = \frac{K_1 \cdot K_{ex(QX)}}{K_{ex(AX)} \cdot [A^+]} \tag{5}$$

The retention can no longer be regulated by $[X^-]$ but by the concentration of A^+, i.e., by an ion with the same charge as the sample. This chromatographic situation is similar in effect to ion-exchange chromatography, but it is obviously only a special case of ion-pair chromatography.

Mixed retention mechanisms including both liquid-liquid distribution and adsorption have been observed in many cases. The degree of ion-pair adsorption increases with decreasing volume of stationary liquid phase on the solid phase but it depends also on the structure of the ion-pair [4,5].

Mobile phases that are mixtures of water hydrophilic organic solvents, such as methanol or acetonitrile, are often used when the stationary phase is a non-polar adsorbent. The organic component will, to some extent, be adsorbed to the solid phase and it has been proposed that this will give rise to a liquid stationary phase that can retain the sample [6,7]. The retention mechanism in these systems is still rather unclear.

It must be emphasized that additional equilibria, not covered by the equations above, can have influence on the retention. One example is ion-pair formation in the aqueous phase. This is usually of minor importance, since the formation constants in most cases are rather low (~ 5) [8,9], but it will have influence on eqs. (2)–(5) at higher ion concentrations.

II. Choice of Chromatographic Conditions

In chromatographic systems containing two liquid phases, it is often possible to base the choice of counter ion on extraction constants determined by batch extraction. If, however, an estimation of the distribution properties of the sample can be made from published data,

chromatographic test runs will often be a more convenient way. This is also the normal procedure by use of a solid adsorbent, since no constants are known for ion-pair adsorption equilibria.

The test runs are easily done in reversed phase systems, where the counter ion is present in the mobile phase. The capacity ratio is regulated to a suitable level by changing the concentration and/or the nature of the counter ion. An illustration is given in Figure 1, which shows the change of k′ of some hydrophilic phenylethylamine derivatives with the concentration of octyl sulphate as counter ion in a liquid-liquid system where eq. (3) should be valid.

In systems where the stationary phase is a non-polar adsorbent, and eq. (4) can be valid, a plot of k′ versus the counter ion concentration will give a non-linear curve. To facilitate the choice of counter-ion concentration, it is then convenient to transform eq. (4) into a linear form:

$$\frac{1}{k'_Q} = \frac{K_{ex(AX)} \cdot [A^+]}{K_1 \cdot K_{ex(QX)}} + \frac{1}{K_1 \cdot K_{ex(QX)} \cdot [X^-]} \qquad (6)$$

A plot according to eq. (6) is given in Figure 2, which shows the regulation of the retention of some hydrophilic anions by the concentration of tetrabutylammonium as counter ion in an aqueous mobile phase when a non-polar adsorbent is used as stationary phase.

The same approach can be used in systems where the mobile phase contains a mixture of water and a water-soluble organic liquid such as methanol or acetonitrile. An example is given in a study by Tilly-Melin et al. [10] where the retention of acetylsalicylic acid and semi-carboxylic acids on a non-polar adsorbent (LiChrosorb RP-8) is regulated by the concentration of quaternary alkylammonium ions in an acetonitrile-containing mobile phase.

In the straight phase systems, where the counter ion is present in a stationary aqueous phase, a regulation of the retention by change of the nature or concentration of the counter ion is a bit more complicated and it is often more convenient to regulate the capacity ratio by the composition of the non-polar mobile phase. An example is given in Figure 3. The samples are an amino acid, a dipeptide, a secondary and a tertiary amine, which migrate as ion pairs with naphthalene-2-sulphonate in a mixture of chloroform and 1-pentanol. Log k′ changes linearly with the logarithm of the pentanol concentration, as is often the case when the mobile phase is a mixture of two components of different polarity. The slope of the lines changes with the degree of substitution of the amino group due to different solvation by the pentanol. The separation selectivity will obviously change considerably with the pentanol concentration.

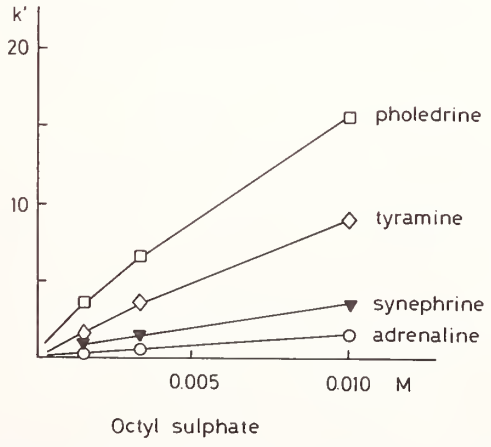

Figure 1. Retention control by counter ion: liquid-liquid system [5]. Mobile phase: octyl sulphate in phosphate buffer pH 3.0. Stationary phase: 1-pentanol on LiChrosorb RP-18 (10 μm).

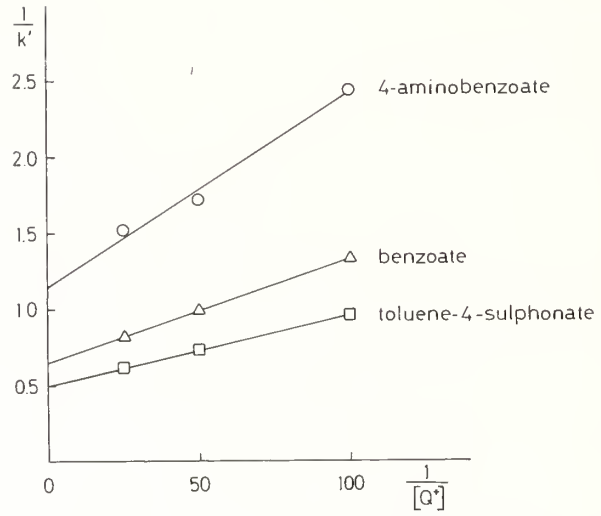

Figure 2. Retention control by counter ion: liquid-solid system. Mobile phase: tetrabutylammonium (Q^+) hydrogensulphate in phosphate buffer pH 7.4. Stationary phase: LiChrosorb RP-2 (10 μm).

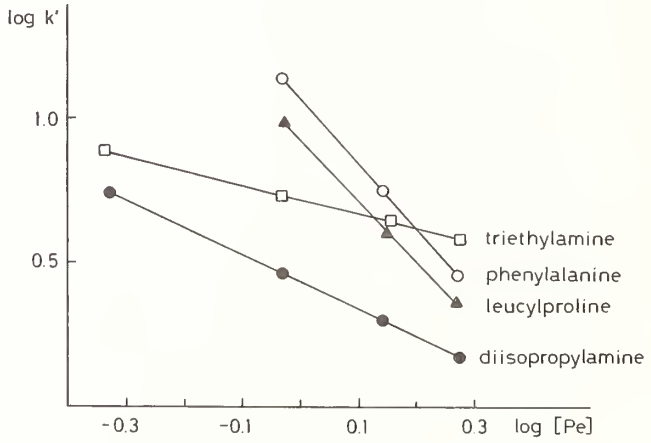

Figure 3. Retention control by pentanol in mobile phase: liquid-liquid system [11]. Mobile phase: chloroform + 1-pentanol (Pe). Stationary phase: naphthalene-2-sulphonate 0.1 mol/L, pH 2.1 on LiChrospher SI 100 (10 μm).

III. Preparation of Columns

The preparation of columns for ion-pair chromatography is outlined in Table 1. Reversed-phase columns are easily prepared by pumping the mobile phase saturated with the stationary non-polar liquid through a column packed with non-polar adsorbent [12,13]. Preparation of a straight phase column can be made either by pumping technique (1) or injection (2), which seem to give columns of equally good quality [8]. The injection technique is also suitable for restoring a column when liquid stationary phase has been stripped off.

In liquid-liquid systems, a careful equilibration of the liquid phases with each other and efficient thermostating of the whole system is of vital importance for the long term stability of the column. Under such conditions columns have been used for years without change of properties.

TABLE 1. *Column preparation*

System	Liquid-liquid[a]		Liquid-solid
Solid phase	Straight phase silica: particle diam. 5–10 μm pore diam. 100–200 Å	Reversed phase hydrophobized silica: particle diam. 5–10 μm pore diam. 60–100 Å	Reversed phase hydrophobized silica: particle diam. 5–10 μm pore diam. 60–100 Å
Liquid stationary phase	aqueous solution of the counter ion	organic solvent	--
Coating	1) 50 mL of stationary phase pumped through the column, followed by organic mobile phase 2) 30 μL portions of stationary phase injected during pumping of mobile phase	mobile phase, saturated with stationary phase, is pumped through the column	--
Stable k′ after a mobile phase volume of	500–1000 column volumes	30–200 column volumes	about 10 column volumes

[a] Mobile and stationary phase carefully equilibrated with each other. The whole chromatographic system carefully thermostatted.

IV. Reversed-Phase Separations

A. Anionic Samples

Acidic metabolic products of biogenic amines can be separated as ion pairs in systems with pentanol as the stationary phase and tetrabutylammonium as counter ion in the aqueous mobile phase. The retention of some derivatives of benzoic acid, phenylacetic acid, mandelic acid and indoleacetic acid are given in Table 2 (cf. [12,14]). The selectivity is good: 3- and 4-substituted benzoic acids are easily separated as well as the different dihydroxybenzoic acids. These systems with tetrapropylammonium as counter ion have been used for separation of steriod glucuronides [15].

Systems of this kind are also highly suitable for separation of sulphonamides. They are weak acids with $pK_a = 6–8$ and can migrate both as acids and ion pairs with tetrabutylammonium. Changes of pH in the mobile phase will under these circumstances give rise to rather drastic changes of k′ as demonstrated in Figure 4. The sulphonamides migrate almost entirely as acids at pH

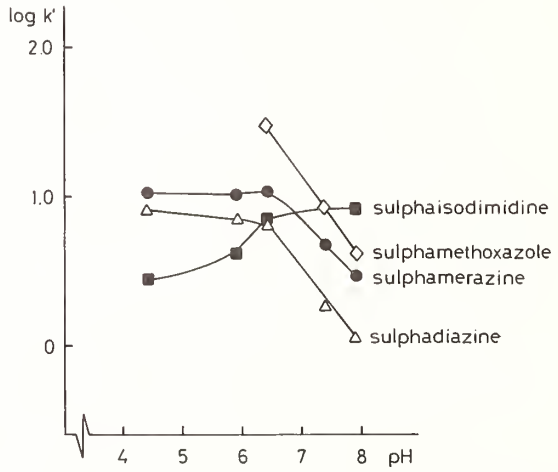

Figure 4. Change of selectivity with pH in ion-pair chromatography [16]. Mobile phase: tetrabutylammonium 0.03 mol/L in phosphate buffer. Stationary phase: butyronitrile on LiChrosorb RP-2 (10 μm).

TABLE 2. *Reversed-phase chromatography: carboxylic acids as ion pairs with tetrabutylammonium*

Mobile phase: tetrabutylammonium 0.03 mol/L, pH 7.4

Stationary phase: 1-pentanol on LiChrosorb RP-2

Sample	log k′	Sample	log k′
4-aminobenzoic acid	–0.24	3,4-dihydroxy-PAA	–0.02
3-aminobenzoic acid	–0.05	4-hydroxy-3-methoxy-PAA(HVA)	0.11
4-hydroxybenzoic acid	0.28	3,4-dimethoxy-PAA	0.43
3-hydroxybenzoic acid	0.49	3,4,5-trimethoxy-PAA	0.59
benzoic acid	0.82	4-methoxy-PAA	0.71
		phenylacetic acid (PAA)	0.68
3,5-dihydroxybenzoic acid	0.20		
3,4-dihydroxybenzoic acid	0.30	5-hydroxyindole-3-acetic acid	0.28
2,5-dihydroxybenzoic acid	1.05	indole-3-acetic acid (IAA)	0.86
4-hydroxy-3-methoxy-MA (VMA)	–0.24		
3-hydroxy-4-methoxy-MA (IVMA)	–0.20		
mandelic acid (MA)	0.28		

below 4, but the fraction that migrates as ion pair increases with increasing pH. The relation between k′ and pH will be highly specific for each sulphonamide since it depends on its acidic strength and distribution properties as acid and ion pair. Separation of sulphonamides and their N^4-acetyl metabolities as well as phenobarbital and its *m*- and *p*-hydroxy metabolites have also been performed in these chromatographic systems [14,16].

Reversed-phase ion-pair chromatographic separations with hydrophobized adsorbents are often made with a mobile phase containing the counter ion in a mixture of an aqueous buffer and a polar organic solvent. Westerlund et al. [7] have separated naphthylacetic acid derivatives with tetrapropylammonium as counter ion in a methanol-containing mobile phase. Knox et al. [17] have made separations of sulphonic acids with acetyltrimethylammonium as counter ion in systems with a high content of propanol. Ascorbic acid has been isolated from food and vitamin products in ion-pair chromatographic systems with tridecylammonium in a mobile phase containing 50% of methanol [18].

Reversed-phase ion-pair chromatography opens up good possibilities to gradient elution by decreasing the counter ion concentration [19]. The gradient is formed without changing the solvent composition which means that practical problems such as formation of gas bubbles in the mobile phase or stripping of liquid stationary phases can be avoided. The gradient technique can be used to concentrate large volumes of sample at the top of the column thereby making a quantitation of low-concentration samples possible. An example is given in Figure 5 which shows the result of injecting a 1 mL sample on a small analytical column using a stepwise gradient.

B. Cationic Samples

Hydrophilic cationic compounds can be separated in reversed-phase systems with organic sulphates, sulphonates and sulphamates as counter ions [5]. The retention of adrenaline and some related phenylethylamine derivatives as ion pairs with octyl sulphate and dicyclohexylsulphamate with pentanol as stationary phase are given in Table 3 (cf. [5,20]). The excellent selectivity of the octyl sulphate system is demonstrated in Figure 6 A-C which shows the separation of nine amino acids (Fig. 6A), three hydrophilic dipeptides (Fig. 6B) and dopamine, tyrosine and corresponding amines (Fig. 6C). The detection has been made fluorimetrically after post-column derivatization using a packed bed reactor [21] with ortho-phtalaldehyde and the minimum detectable amount is less than 1 nanogram.

Separation of cationic compounds with alkylsulphates as counter ion has also been performed by Knox et al. [22] and others [23,24].

Quaternary ammonium ions and also some cations of hydrophobic character show strongly tailing peaks and abnormally high retention in ion-pair systems constructed according to the principles given above. Studies of retention mechanisms [4] have shown that these disturbances are due to interactions with the solid support. Good peak symmetry and normal separating efficiency can be obtained if a long chain tertiary or quaternary alkylammonium ion (e.g., N,N-dimethyl-octylamine or N,N,N-trimethylnonylammonium) is added to the mobile phase. The regulation of the retention can then be made in accordance with normal principles for ion-pair chromatography, i.e., by changing the nature and the concentration of the counter ion. The retention of a series of amines in systems with dihydrogenphosphate and bromide as counter ions and pentanol as the stationary phase is demonstrated in Table 4. N,N-dimethyloctylamine was added to prevent peak deformation.

The abnormal behaviour of the hydrophobic amines is due to a mixed retention mechanism comprising both liquid-liquid distribution (eq. (3)) and adsorption (eq. (4)) where the adsorption effect causes the tailing. The added long-chain amine cancels out the disturbing effect of the adsorption [4].

In a system with a low content of stationary liquid phase, where an adsorption mechanism dominates, the retention can be regulated by the added long-chain amine (cf. [5]) in accordance

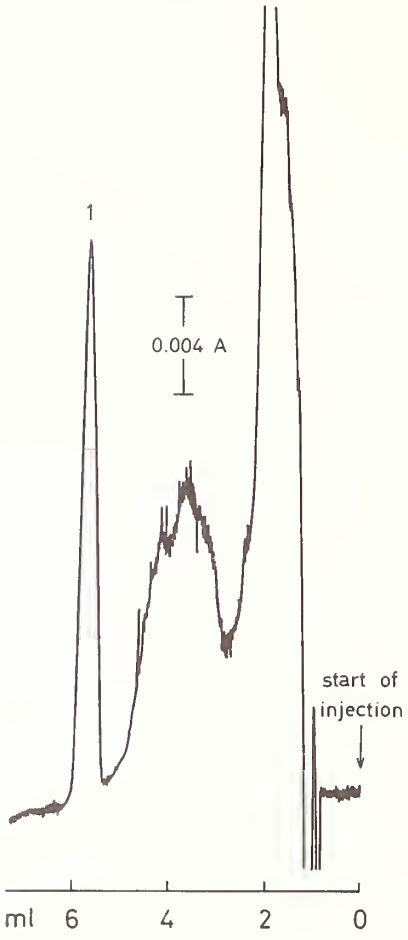

Figure 5. Stepwise gradient to increase sample volume [19]. Sample: toluene-4-sulphonate 5 μg in 1.1 mL of tetrabutylammonium 0.1 mol/L. Mobile phase: tetrabutylammonium hydrogensulphate 0.0075 mol/L in phosphate buffer pH 7.4 (void volume 1.0 mL). Stationary phase: 1-pentanol on LiChrosorb RP-2 (10 μm).

TABLE 3. *Reversed-phase chromatography: phenylethylamine derivatives as ion pairs with octyl sulphate and dicyclohexylsuphamate*

Mobile phase: counter ion in phosphate buffer

Stationary phase: 1-pentanol on LiChrosorb RP-18

Sample	Counter ion	
	Octyl sulphate 0.003 mol/L, pH 4.2	Dicyclohexylsulphamate 0.007 mol/L, pH 4.5
	k'	
Adrenaline	0.95	0.90
Synephrine	1.4	1.7
p-Hydroxyephedrine	2.2	3.0
Pholedrine	4.8	-
Ephedrine	5.9	7.2
Norephedrine	5.4	7.4
N-Ethylephedrine	7.3	7.9
Amphetamine	12.6	15.6

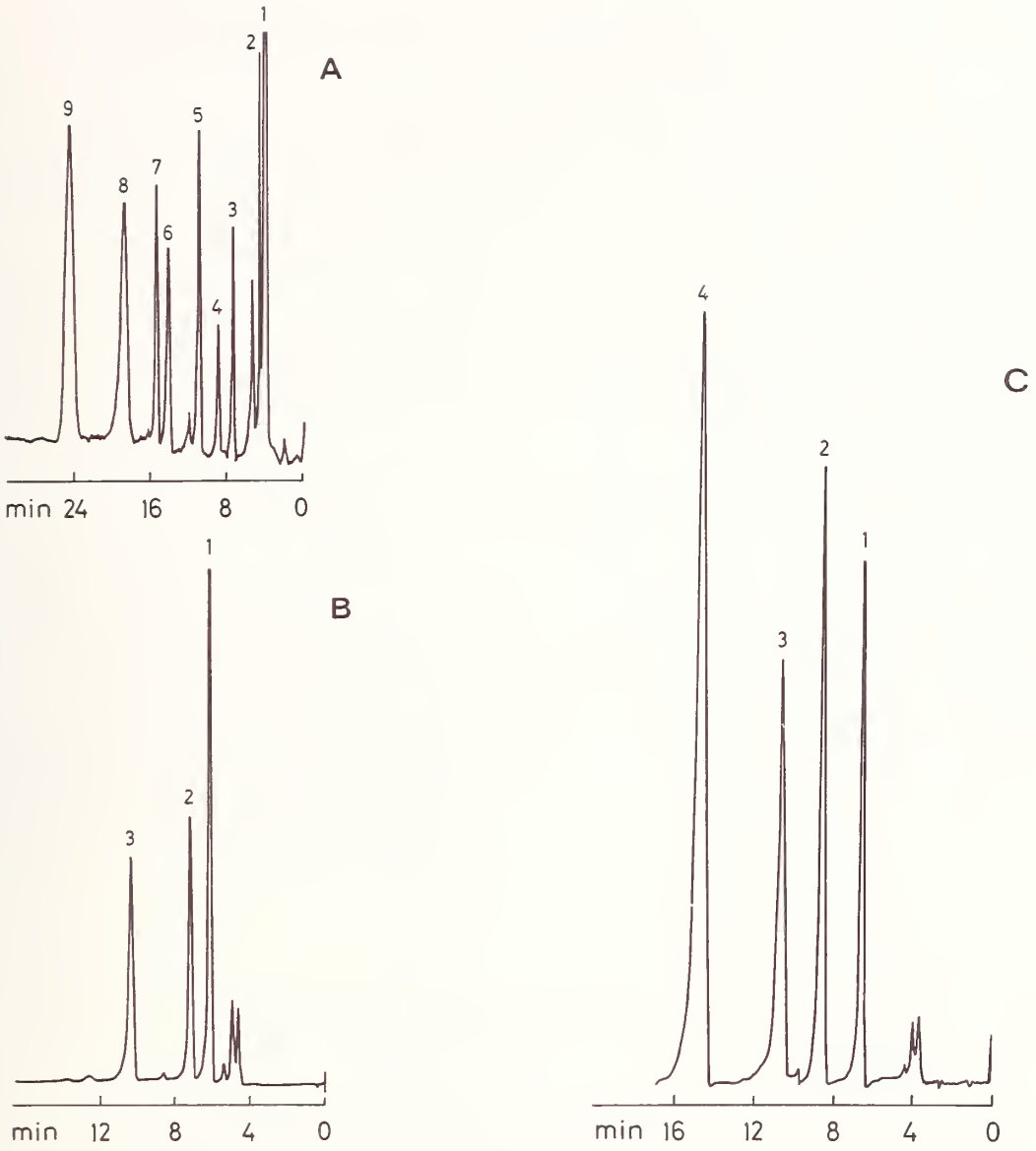

Figure 6. Reversed-phase separation of hydrophilic cations as octyl sulphate ion pairs [20]. Mobile phase: octyl sulphate 0.01 mol/L in phosphate buffer pH 2.5, saturated to 50% with 1-pentanol. Stationary phase: LiChrosorb RP-8. Detection: fluorimetrically after post-column derivatization with *o*-phtalaldehyde.

Samples:

A
1. glycine
2. alanine
3. valine
4. tyrosine
5. histidine
6. lysine
7. iso-leucine
8. leucine
9. arginine

B
1. alanylglycine
2. alanylalaine
3. valinylalanine

C
1. DOPA
2. tyrosine
3. dopamine
4. tyramine

TABLE 4. *Reversed-phase chromatography: hydrophobic ammonium compounds as bromide and phosphate ion pairs [4]*

Mobile phase: counter ion + dimethyloctylamine 0.028 mol/L
in phosphate buffer, pH 2.0

Stationary phase: 1-pentanol on LiChrosorb RP-8

Sample	Counter ion $H_2PO^-_4$ 0.10 mol/L	$H_2PO^-_4$ 0.10 mol/L Br^- 0.043 mol/L
	k'	
dixyrazine	0.19	1.05
perphenazine	0.47	2.52
fluphenazine	0.92	4.75
imipramine	1.72	9.12
N-methylamitriptyline	1.42	9.37
desipramine	2.38	12.6
amitriptyline	3.01	16.4
nortriptyline	4.09	22.1

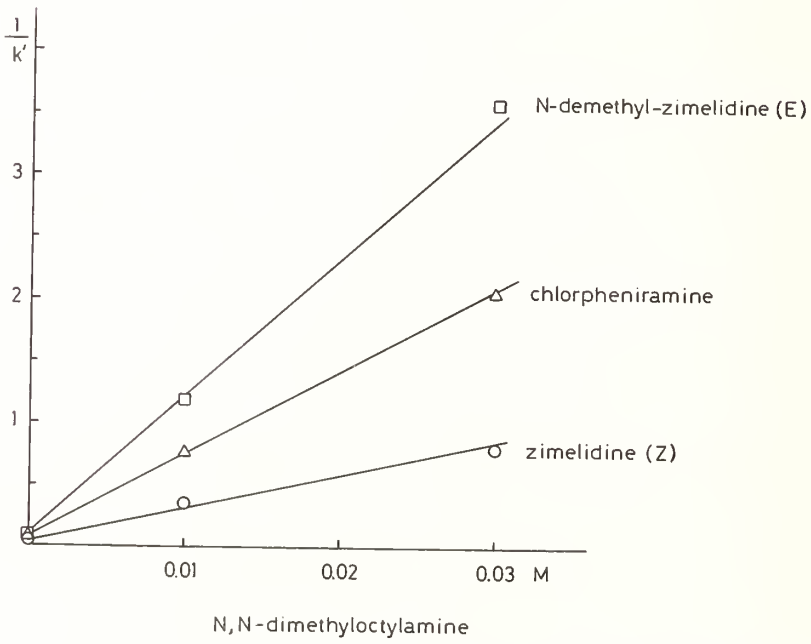

Figure 7. Regulation control in liquid-solid system by an ion of the same charge as the sample. Mobile phase: N,N-dimethyloctylamine in cyclohexyl-sulphamate 0.05 mol/L in phosphate buffer pH 2.2, saturated to 50% with 1-pentanol. Stationary phase: LiChrosorb RP-8.

with eq. (6). The relationship betweeen the capacity ratio of the sample (Q^+) and the concentration of the added amine (A^+) is demonstrated in Figure 7.

V. Straight Phase Separations

The straight phase systems have the advantage of giving a more free choice of organic phase than the reversed systems which improves the possibilities to a control and variation of the selectivity. Halogenated hydrocarbons or hydrocarbons mixed with higher alcohols have so far mainly been used as mobile phases. The selectivity of a system of this kind is demonstrated in

Figure 8, which shows the isolation of zimelidine and its demethylation product from a plasma sample with perchlorate as the counter ion. The amines, that have molar absorptivities of about 20,000 at the measuring wavelength of the detector, give peaks well resolved from other sample components, even when the amine concentration is as low as 100pg/mL of plasma.

A unique feature of the straight phase systems for ion-pair chromatography is that the counter ion can be used to improve the detection sensitivity. The sample is eluted from the column as ion pair and a sample ion without UV-absorbance can be measured with high sensitivity by a UV-detector if the ion pair through the counter ion has a high absorptivity.

For cationic compounds, an excellent response increase has been obtained by using naphthalene-2-sulphonate as counter ion. The sensivitity and the separating efficiency of the system is demonstrated in Figure 9A-C, which shows the separation of alkylamines of different degrees of substitution but the same number of carbons (Fig. 9A), amino acids (Fig. 9B) and dipeptides (Fig. 9C). The noise is very low in spite of the high detector sensitivity due to the careful thermostating of the system, and the minimum detectable amount of the samples is of the order of 1 ng.

The selectivity of the system for amino acids and dipeptides is demonstrated in Table 5. A change of sequence order between leucine and phenylalanine in a dipeptide gives a separation factor of about 1.3.

Corresponding systems for anionic samples, with a cation of high absorbance in the stationary phase, have also been developed. The selectivity of a system with 1-(1-napthylethyl)trimethylammonium as counter ion is demonstrated in Table 6. The non-absorbing butyl sulphate can be quantified with reasonable precision down to 20–30 ng by a UV-detector.

The straight phase systems can also be used for separation of anionic compounds formed by conjugation processes with glucuronic, sulphuric and phosphoric acid as well as glycine. Some examples are given in Tables 7 and 8 (cf. [14]). Tetraethylammonium is used as counter ion for the rather hydrophobic sulphates, while the more hydrophilic glucuronic acid conjugates require a larger counter ion, tetrapropylammonium.

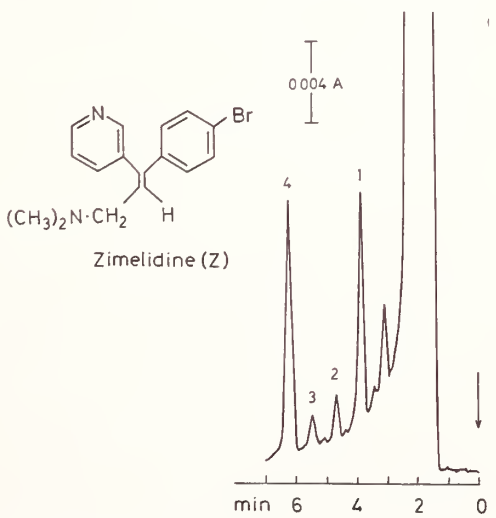

Figure 8. Isolation of amines from plasma as perchlorate ion pairs [25]. Mobile phase: methylene chloride + *n*-butanol (89 + 11). Stationary phase: $HClO_4$ 0.2 mol/L + $NaClO_4$ 0.8 M on Partisil 5. Sample: extract from 5 mL of plasma

1. internal standard
2. zimelidine (Z) 100 pg/mL
3. N-dimethylzimelidine (Z) 100 pg/mL
4. internal standard

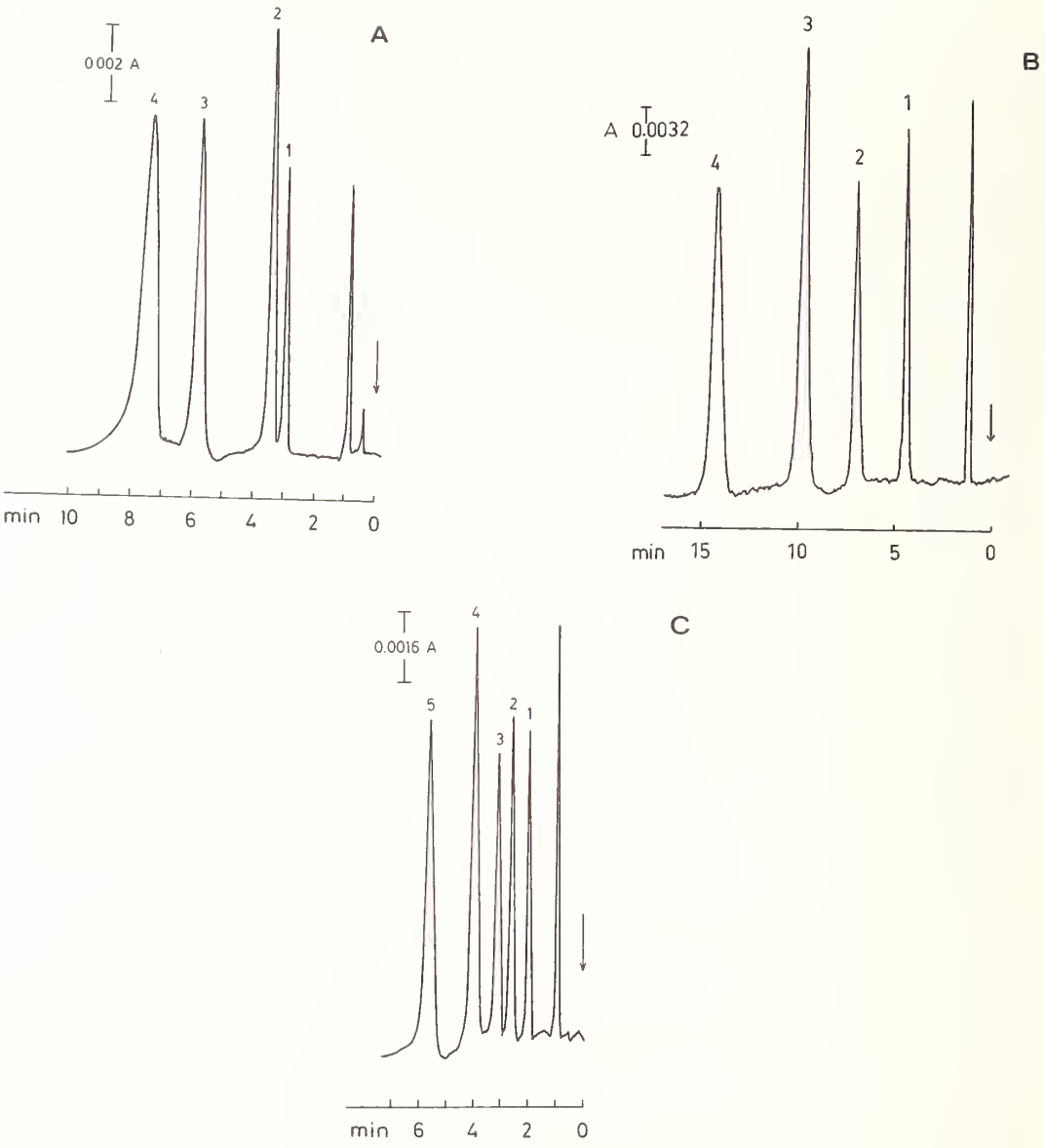

Figure 9. Separation of organic cations as ion pairs with naphthalene-2-sulphonate [8,11].
Mobile phase: chloroform + 1-pentanol (9 + 1).
Stationary phase: naphthalene-2-sulphonate 0.1 mol/L, pH 2.3 on LiChrospher SI 100 (10 μm).
Samples:

A
1. *n*-hexylamine (31 ng)
2. di-*n*-propylamine (63 ng)
3. diisopropylamine (80 ng)
4. triethylamine (107 ng)

B
1. 8-aminocaprylic acid
2. phenylalaine
3. leucine
4. tranexaminc acid

C
1. leucylleucine (74 ng)
2. phenylalanylalanine (104 ng)
3. valylphenylalanine (106 ng)
4. leucylvaline (170 ng)
5. methionylvaline (214 ng)

TABLE 5. *Straight phase chromatography: amino acids and dipeptides as ion pairs with naphthalene-2-sulphonate [8,11]*

Mobile phase: chloroform + 1-pentanol

Stationary phase: naphthalene-2-sulphonate 0.1 mol/L, pH 2.2 on LiChrospher SI 100 (10 μm)

Sample	Pentanol content of mobile phase (%)	
	10	15
	k'	
8-Aminocaprylic acid	8.1	3.3
6-Aminocaproic acid	-	32.7
Tranexamic acid	35.7	13.2
Phenylalanine	13.5	5.7
Leucine	-	8.6
Tryptophane	-	6.0
Isoleucine	-	8.5
Methionine	-	19.5
Valine	-	29.8
Phenylalanylphenylalanine	0.6	-
Phenylalanylleucine	0.8	-
Leucylphenylalanine	1.0	-
Leucylleucine	1.2	0.5
Methionylleucine	1.8	0.7
Phenylalanylvaline	1.9	0.8
Leucylmethionine	2.2	0.9
Valylphenylalanine	2.5	1.1
Leucylvaline	3.4	1.4
Valylleucine	3.9	1.5
Phenylalanylproline	4.6	1.9
Methionylvaline	5.3	2.2
Leucylproline	9.6	4.0
Phenylalanylalanine	-	4.4
Leucylalanine	-	8.9

TABLE 6. *Straight phase chromatography: organic anions as ion pairs with a UV-absorbing quaternary ammonium ion* [26]

Mobile phase: chloroform + pentanol (19 + 1)

Stationary phase: 1-(1-naphthylethyl)trimethylammonium 0.02 mol/L, pH 6.6 on LiChrospher SI 100 (10 μm)

Sample	log k'
Butanesulphonate	1.40
Pentanesulphonate	0.82
Butyl sulphate	0.61
Camphor-10-sulphonate	0.79
3-Bromocamphor-10-sulphonate	0.34
Cyclohexylsulphamate	1.14
Cyclohexanecarboxylate	0.34

TABLE 7. *Straight phase chromatography: organic sulphates as ion pairs with tetraethylammonium*

Mobile phase: CH_2Cl_2 + 1-pentanol (9+1)

Stationary phase: tetraethylammonium 0.10 mol/L, pH 7.4 on LiChrospher SI 100

Substance	log k′
6-bromo-2-naphtyl sulphate	–0.85
2-naphtyl sulphate	–0.32
4-methylumbelliferyl sulphate	–0.16
3-estradiol sulphate	–0.44
17-estradiol sulphate	0.00
17-estriol sulphate	0.15
3-estriol sulphate	0.75

TABLE 8. *Straight phase chromatography: glucuronic acid and glycine conjugates as ion pairs with tetrapropylammonium*

Mobile phase: methylene chloride + 1-pentanol (9+1)

Stationary phase: tetrapropylammonium 0.10 mol/L pH 7.4 on LiChrospher SI 100

Substance	log k′
salicyluric acid	0.48
hippuric acid	1.00
N-methylhippuric acid	1.08
nicotinuric acid	2.10
6-bromo-2-naphtyl glucuronic acid	0.46
2-naphtyl glucuronic acid	1.28
8-hydroxyquinoline glucuronic acid	1.70

VI. Comparison of Reversed and Straight Phase Systems

The regulation of the retention time by the type and concentration of the counter ion (cf. eqs. (2) and (3)) is easily done in reversed phase systems where the counter ion is dissolved in the mobile aqueous phase. It is more difficult in straight phase systems, however, since a new aqueous stationary phase then must be applied.

In straight phase systems, the regulation of the capacity ratio is easily done by changing the polarity of the mobile organic phase (Fig. 3). A change of the solvent composition of the mobile phase can also be utilized for this purpose in those reversed-phase systems where the mobile phase is a mixture of methanol or acetonitrile with water.

The separation selectivity is governed by the nature of the organic phase and therefore easily controlled by the composition of the organic mobile phase in straight phase systems (Fig. 3). In reversed-phase systems, these possibilities are limited since the choice of stationary phase is less free.

A major advantage of the reversed-phase systems is that hydrophilic samples and samples already present in aqueous media, e.g., biological material, often can be dissolved directly in the mobile phase. In the straight phase mode the samples must be transferred to an organic solvent (the mobile phase) before injection and this can cause difficulties for hydrophilic compounds. Problems of this kind can, however, be solved by modified extraction techniques.

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BIO-SELECTIVE MEMBRANE ELECTRODES

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In order to prepare membrane electrodes with response and selectivity to biological molecules or species, other than simple ions or gases, it is necessary to modify conventional potentiometric membrane electrodes with additional components selected to "mediate" between biochemical and electrochemical processes. Such mediation can be as simple as pH buffering to ionize a neutral biological substance or extraordinarily complicated as in the case of sequential enzymatic conversions or immunochemical steps.

Although much of the current research involves enzyme electrodes, we should recognize that enzymes are only one class of possible biological materials which could be used as mediators on membrane electrodes. Indeed, it is possible to construct a hierarchy of mediator categories, e.g.:

Single enzymes
↓
Enzymes plus cofactors or activators
↓
Enzyme sequences
↓
Immunoagents (including haptens)
↓
Complement systems
↓
Liposomes and other vesicles
↓
Organelles (Mitochondria)
↓
Intact living organisms

The lecture will consider several approaches to the mediation problem including extrapolations to the future based on high explorative, unpublished work currently in progress in the author's laboratory. Such work includes the use of hapten electrodes for monitoring immunoreactions and the direct employment of intact micro-organisms to develop a "living" membrane electrode.

Key words: Bio-selective membrane electrodes; bio-sensors; enzymes; immunoelectrodes; trace organic electrochemistry.

The decade since the NBS Symposium on "Ion-Selective Electrodes" [1] has brought remarkable advances in the development of potentiometric sensors. Among these has been the rapid evolution of bio-selective membrane electrodes [2] using ion selective electrodes as building blocks in conjunction with biochemical mediators. Such bio-sensors include enzyme electrodes, immunoelectrodes, and most recently, electrodes which employ intact living organisms. Some of these electrodes are of potential interest for various analytical purposes including automated analysis [3].

Perhaps the most successful enzyme electrodes have been those which employ potentiometric gas sensors [4,5] in conjunction with an enzyme which catalyzes the production of an appropriate gaseous product from the desired substrate. This is so because the potentiometric gas sensors are remarkably free from chemical interferences; when this chemical selectivity is combined with the biochemical selectivity of enzymes, an overall electrode system with very high discrimination can result. In favorable cases, selectivity for the desired substrate may be sufficient to permit direct measurement even in complex biological fluids [6].

An example of an electrode consisting of a gas sensor in combination with an enzyme is the adenosine monophosphate sensing electrode shown in Figure 1. In this case, the enzyme AMP deaminase is interposed between the sample solution and an ammonia gas sensing electrode by an appropriate arrangement of membrane layers. A cross section through these layers showing the various chemical and physical processes (Fig. 2) makes clear the origin of selectivity and the sequence of necessary events connecting the substrate to be determined with the ultimate parameter, i.e., a pH change, measured potentiometrically.

The particular configuration of physical layers and chemical steps shown in Figure 2 results in a highly selective response to AMP in the presence of possible interferents. It can be seen from Figure 3 that other nucleotides and related biochemical materials have a negligible influence upon the electrode's response to the primary substrate to be measured.

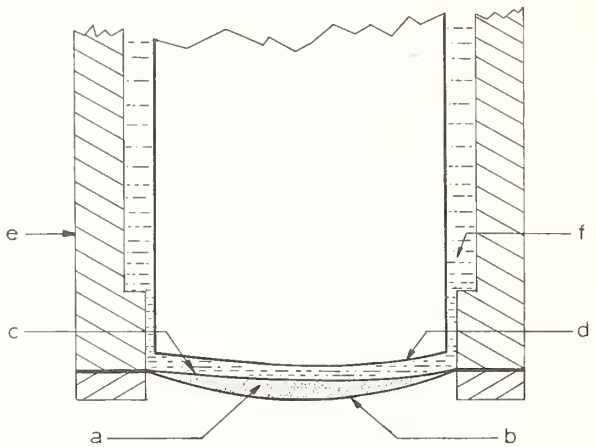

Figure 1. Schematic diagram of AMP electrode. (a) AMP deaminase enzyme layer, (b) cellophane dialysis membrane; (c) NH_3 gas permeable membrane; (d) internal sensing element; (e) plastic electrode body, (f) internal filling solution.

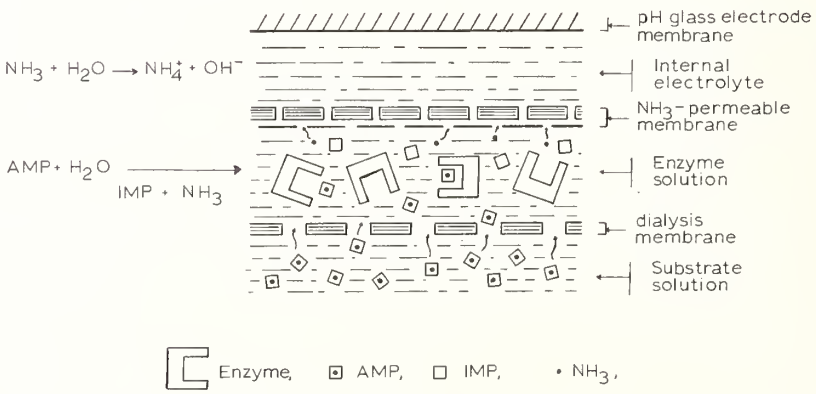

Figure 2. Molecular view of AMP electrode.

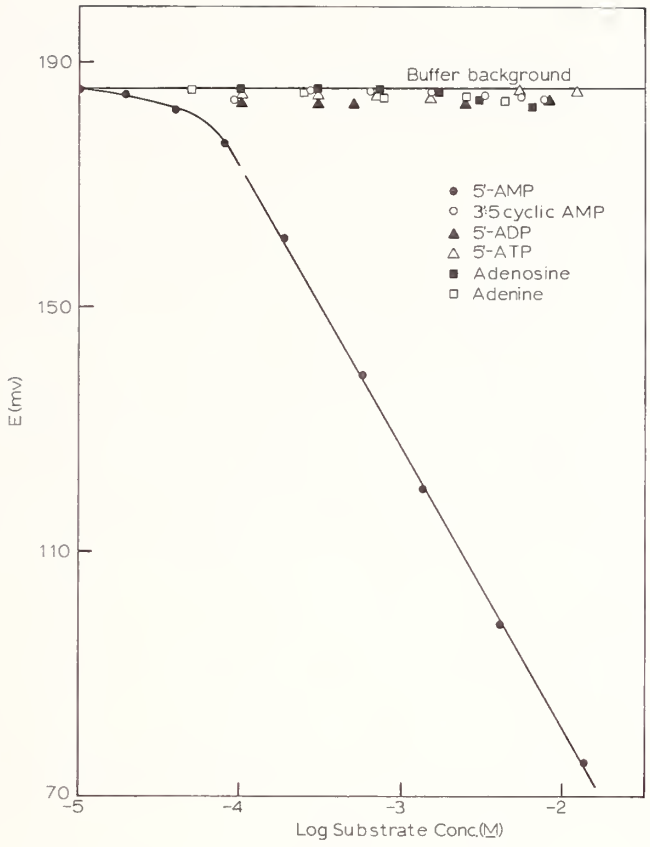

Figure 3. Selectivity study of AMP electrode.

It would be improper, however, to overstate the case with respect to enzyme electrodes, especially insofar as trace analysis is concerned. While selectivity may be highly attractive in favorable cases, the sensitivity of such electrodes is only moderate and is, in any event, ultimately limited by the sensitivity of the internal ion or gas sensing element. Up to the present time, most enzyme electrodes have been designed to function in the millimolar concentration range and few, if any, show an appreciable response below 10^{-5} mol/L substrate concentration. This sensitivity limitation, while real, may not be insurmountable. It may be possible for example, to employ various amplification reactions as part of the electrode mediation scheme either through changes in stoichiometry or via catalytic cycles.

Another possible approach involves the construction of miniature enzyme electrodes suitable for direct probing in very small volumes, perhaps within single cells. As a first step, this would require the construction of externally small potentiometric gas sensors to be used in conjunction with appropriate enzymes. While there is no fundamental reason why conventional gas sensing membrane electrodes should not be drastically scaled down, the experimental and technical obstacles involved can be formidable because of problems of fragility, sealing, and electrical insulation.

One possible design for a microammonia electrode, which has been extensively tested in our laboratory, is shown in Figure 4. The preparation of such electrodes requires a significant investment in micromanipulation equipment as well as considerable practice in testing and fitting together the various components. When everything is working properly, however, such ammonia gas sensors with tip sizes of less than 10 micrometers in diameter yield response characteristics entirely comparable to those of the macro-size electrodes (Fig. 5).

These microelectrodes then can serve as the basis for the construction of highly selective microenzyme electrodes. We have, for example, immobilized small quantities of the enzyme urease just inside the tip of the ammonia electrode illustrated in Figure 4. The resulting microenzyme electrode gave a selective response to urea over the 10^{-2} to 10^{-5} mol/L concentration range, although with a sub-Nernstian response slope (Fig. 6).

In many cases, a suitable enzyme for electrode purposes is not available in isolated form or is lacking in time stability. We have recently made an effort to deal with such limitations through the use of intact living organisms, instead of isolated enzymes, as biochemical mediators at membrane electrodes. Provided that such micro-organisms function as efficient biocatalysts for the conversion of the desired substrate to a product measurable with the internal electrode probe, it is not even necessary to know the details of the internal biochemical steps involved in the bacterial metabolism.

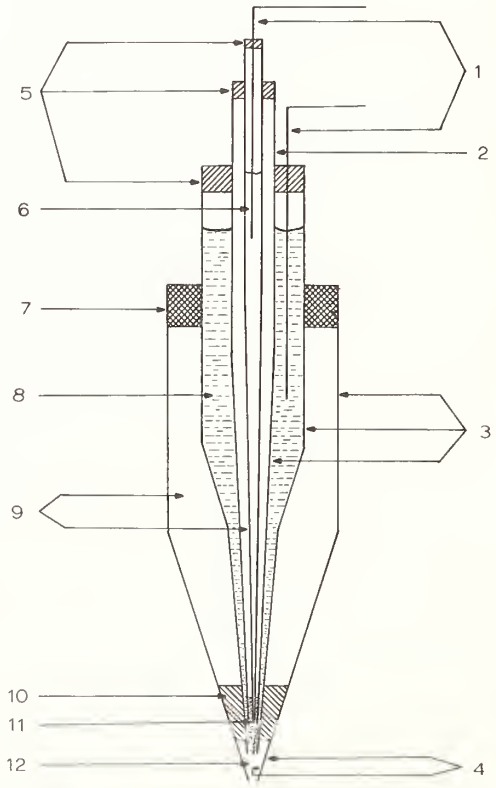

Figure 4. Construction of a micro air-gap gas sensing probe. 1) Ag/AgCl wires, 2) insulated micro pH electrode; 3) ordinary Pyrex glass, 4) hydrophobic surface, 5) glass cover cement, 6) 0.01 M HCl, 7) epoxy resin, 8) 0.005 M NH_4Cl or $NaHCO_3 + 0.1$ M NaCl, 9) air space, 10) high vacuum Apiezon wax, 11) glass cover cement or halocarbon wax, 12) air gap.

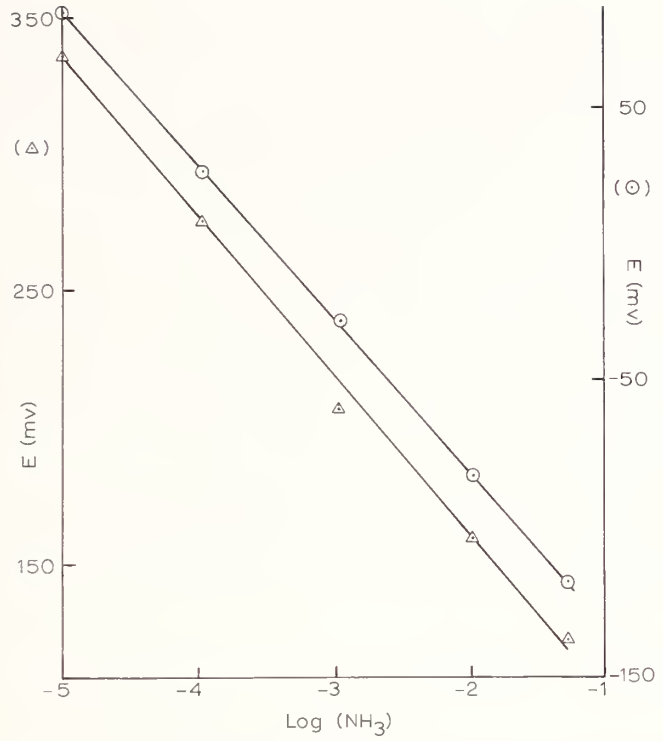

Figure 5. Potential response of a micro air-gap ammonia sensor. (Δ) micro air-gap sensor, (O) Orion ammonia sensor (Model 95-10).

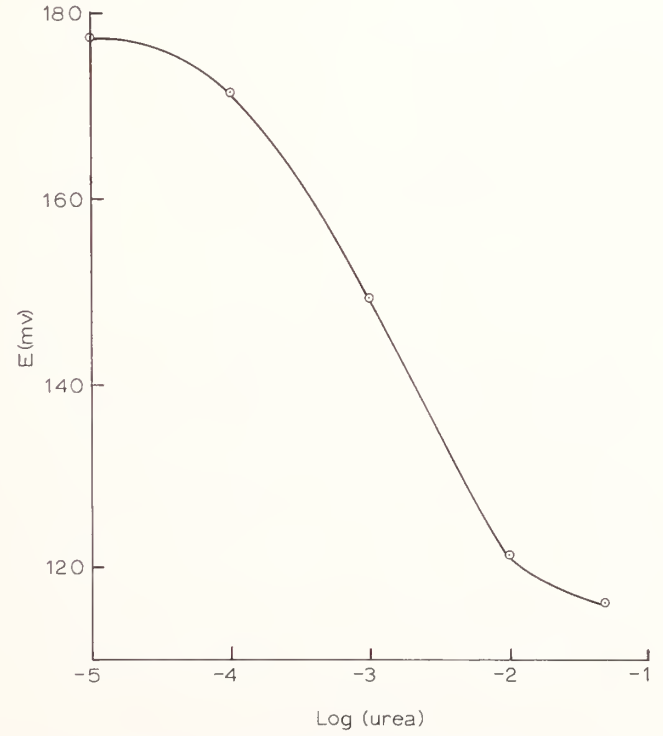

Figure 6. Potential response of a micro urea sensor.

Figure 7 illustrates both the physical construction and sequence of processes involved in such electrodes. Basically, the living bacteria take the place of the isolated enzyme used in conventional electrodes. The key to success is the selection of the proper micro-organism from the many thousands of species and strains available. Desirable goals include high selectivity, sensitivity, good time stability and overall lifetime, fast response, and compatibility of the bacteria with the internal electrode system.

To date, only about 15 possible bacterial-electrode combinations have been evaluated in detail in our laboratory. These have involved not only bacterial strains but also fungi in conjunction with NH_3 and H_2S gas sensing electrodes [7–9].

Of the systems tested, the most successful has been the one using the bacterium Sarcina Flava at an NH_3 gas sensing electrode. This system functions as an excellent sensor for the amino acid glutamine. As can be seen from Figure 8, this bacterial electrode displays good response and high selectivity for glutamine. Although not shown in the figure, the electrode does not respond to the remainder of the 20 essential amino acids or to urea. This electrode has also been successfully employed in biological fluids (Fig. 9).

While the investigation and development of bioelectrodes based on the use of living micro-organisms is still in its early phase, there has already been sufficient success to warrant further efforts in this area. Eventually, such electrodes may offer an attractive alternative for the measurement of biological substances in practical situations.

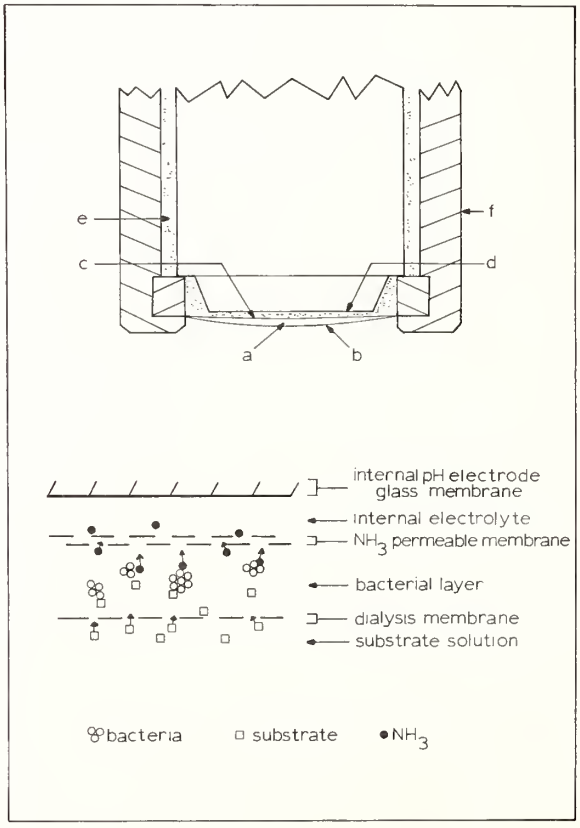

Figure 7. Schematic diagram of bacterial electrode. a) bacterial layers; b) dialysis membrane; c) gas permeable membrane; d) internal sensing element; e) internal filling solution; f) plastic electrode body.

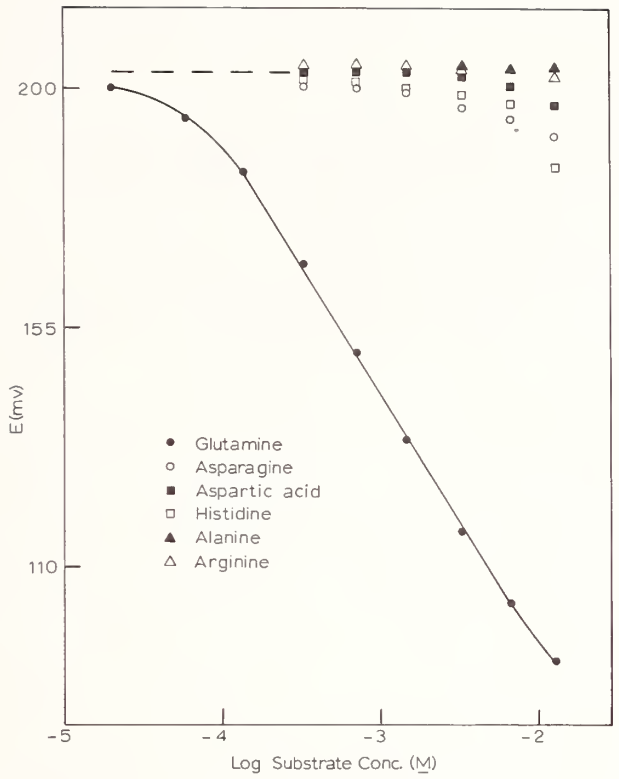

Figure 8. Response and nominal selectivity of the bacterial sensor in pH 7.5 Tris-HCl buffer with 0.01 *M* $MnCl_2$.

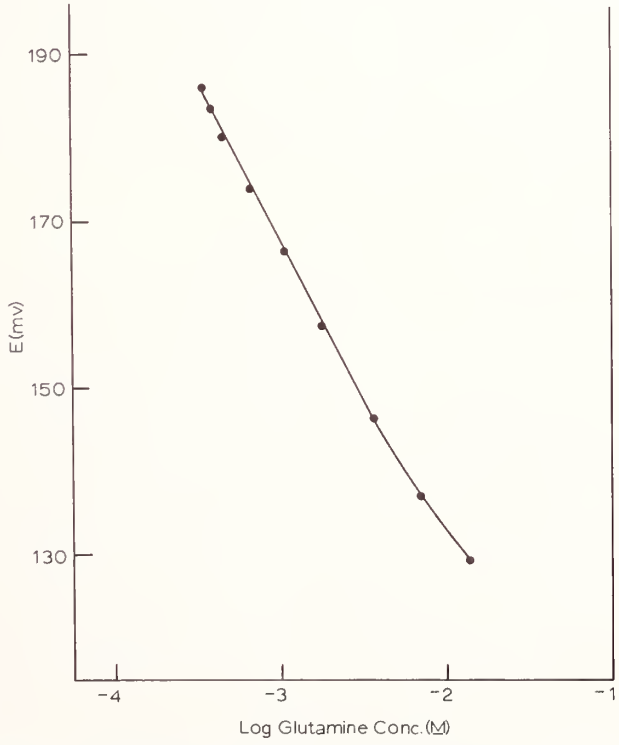

Figure 9. Bacterial glutamine electrode response in control serum samples.

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RECENT DEVELOPMENTS IN HOMOGENEOUS IMMUNOASSAY TECHNIQUES

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Immunoassay techniques, based on the specific recognition of antigens by antibodies, are increasingly used in the measurement of analytes of clinical interest. During the past decade, a number of immunoassays have been developed which do not require physical separation of free and bound labeled analyte prior to quantitation. These homogeneous immunoassay techniques may be further classified according to the type of molecule used as the reaction indicator or label. The review describes assays employing free radicals, fluorescent molecules, chemiluminescent molecules, enzyme cofactors, and enzymes. The EMIT® homogeneous enzyme immunoassay technique, representing the homogeneous immunoassay method in widest use, is described in detail.

Key words: Drug assay; EMIT® fluoroimmunoassay; fluorescence immunoassay; homogeneous enzyme immunoassay; immunoassay.

Increasingly, immunoassay techniques are being used to quantitate many analytes of clinical interest including proteins, viruses, hormones, and exogenous drugs. The specific recognition of antigens by antibodies forms the basis for these methods. Various analytical systems which utilize different monitors of the reaction of immune components have been developed. This paper briefly reviews some recent developments in homogeneous immunoassays and then discusses one such method, the EMIT® homogeneous enzyme immunoassay, and its application to the measurement of drugs in body fluids in more detail.

Immunoassays may be classified according to the method used to detect the reaction between antibody (Ab) and antigen (Ag), described by the basic equation below:

$$\mathrm{Ag} + \mathrm{Ab} \rightarrow \mathrm{Ag{\cdot}Ab} \qquad (1)$$

The analyte of interest is most often the antigen, however, in principle, any of the components may be measured. Two basic classes of immunoassays exist:

Type I—those which employ no labeled reactant; and
Type II—those which employ a labeled reactant.

Assays of Type I have been used exclusively to measure proteins since precipitating complexes formed by proteins with their respective antibodies can be easily detected. The precipitation reaction is the basis of such well established techniques as agglutination and immunodiffusion [1]. More recently nephelometry, or light scattering spectroscopy, has been used to quantitate proteins [2].

Either antigen, antibody, or both, may be labeled with an indicator molecule in Type II immunoassays. In reviewing recent developments in the field of homogeneous immunoassays, attention will be confined to systems in which the antigen is labeled:

$$\mathrm{Ag} + \mathrm{Ag\text{-}L} + \mathrm{Ab} \rightarrow \mathrm{Ag\text{-}L{\cdot}Ab} + \mathrm{Ag{\cdot}Ab} \qquad (2)$$
$$\mathrm{L} = \text{Label}$$

Competition between Ag (the sample) and labeled antigen (Ag-L) for a limited number of antibody binding sites results in a partitioning of the label between free and bound states. Measurement of either the free labeled antigen (Ag-L) or the antibody-bound label (Ag-L·Ab) allows quantitation of the antigen.

Before discussing the types of indicator molecules which have been employed, it is necessary to present one additional conceptual classification of immunoassays: the distinction between homogeneous and heterogeneous assays. This classification was introducted in 1972 by Rubenstein, Schneider, and Ullman [3] in their first paper on homogeneous enzyme immunoassay. As described above, quantitation of the analyte requires measurement of either the free or antibody-bound labeled antigen. If the properties of the free and bound labels are identical, a physical separation is required to distinguish them. An assay which requires separation is termed a heterogeneous assay. If the properties of the indicator molecule can be modulated by the antibody-antigen reaction, no separation of free and bound labels is required and a homogeneous assay can be achieved.

A partial list of the indicators which have been employed as immunochemical labels is found in Table 1. The methods are listed in the table in the order they will be discussed in the text.

TABLE 1. *Immunochemical labels*

1.	Radioisotopes
2.	Free radicals
3.	Fluorescent molecules
4.	Chemiluminescent molecules
5.	Enzyme cofactors
6.	Enzymes

I. Radioisotopes

The familiar method of radioimmunoassay (RIA), which uses radioisotopes as labels, was first developed in 1960 by Berson and Yalow [4]. RIA is undoubtedly the immunoassay method in widest use [5]. Commonly used radionuclides are the β-emitters ^{3}H, ^{14}C and the γ-emitter ^{125}I. The principle advantage of RIA is the sensitivity achievable by use of high specific activity labeled materials. However, there are some serious disadvantages with the method including: the requirement that free and bound labels be separated, health hazards, disposal of radioisotopes, expense of counters, and limited life of reagents labeled with ^{125}I. For this nuclide, the usable lifetime of most reagents is less than 2 months, with constantly changing performance during that period.

Soon after the significance of RIA was recognized, efforts commenced in laboratories around the world to develop quantitative non-isotopic immunoassays which did not require separation steps. During the past 10 years, these efforts have resulted in the development of a variety of homogeneous methods which employ the alternate labels listed in Table 1. Any of the labels listed in Table 1 can also be employed in an heterogeneous assay mode; the emphasis here will be placed on the homogeneous methods.

II. Free Radicals

Free radicals have been used in a method known as FRAT®—Free Radical Assay Technique—initially described by Leute, Ullman, Goldstein, and Herzenberg in 1971 [6,7]. They used stable nitroxide free radicals to spin-label the drug morphine. Since the properties of free radicals, as observed by their electron spin resonance (esr) spectra, are very sensitive to environment and molecular motion, the signals of free and antibody-bound spin-labeled drug are

different. The modulation of the esr signal by the antibody-antigen reaction formed the basis of a homogeneous assay method. FRAT® assays were marketed briefly by Syva Company as the first commercial homogeneous non-isotopic immunoassays. Their main limitations were the complexity of esr instrumentation and lack of availability of such instrumentation in clinical laboratories.

III. Fluorescent Molecules

Fluorescence immunoassays had their origins in the fluorescent antibody techniques for histochemical localization of antigens developed in the late 1950's and early 1960's [8,9]. A discussion of several types of homogeneous immunoassays which rely on a modulation of the fluorescence properties of a labeled antigen follows.

A. Fluorescence Polarization Assays

The principle of fluorescence polarization (FP) was first applied to immunoassay by Dandliker and coworkers [10,11]. The FP of a small fluorescent-labeled molecule tumbling freely in solution is very low. However, when the labeled molecule becomes complexed with antibody, its molecular motion is slowed and the FP increases. This modulation phenomenon provides the means to distinguish between free and bound labels. Recently Landon et al. have described several routine FP assays for the drugs gentamicin and phenytoin in human serum [12,13].

B. Fluorescence Quenching or Enhancement Assay

The effects of protein binding on the fluorescence properties of small molecules have been exploited for some time in biophysical studies of proteins [14,15]. Assays for gentamicin and thyroxine based on the quenching or enhancement of fluorescence intensity of the labeled antigen have recently been reported [16,17]. At present it is difficult to predict the generality and applicability of these methods.

C. Fluorescence Excitation Transfer Assay

Ullman, Schwarzberg, and Rubenstein [18] have described a novel immunoassay technique which uses the principle of fluorescence excitation transfer to detect antibody-antigen complex formation. Several assay modes can be employed. Of most interest, for measurement of low molecular weight drugs, is the mode which uses an antigen labeled with a fluorescent chromophore which acts as an energy donor, and an antibody labeled with a second chromophore which acts as an energy acceptor or quencher. Since excitation energy transfer is distance dependent, transfer occurs only in the complex between labeled components. Therefore, no separation of reactants is required. Assays were described for opiates and for human IgG [18].

D. Reactant-Labeled Fluorescence Assays

Burd and his coworkers have described an homogeneous assay which uses a drug labeled with fluorescent dye [19]. The drug-dye conjugate is a substrate for an enzyme which is added to the assay mixture. The enzyme catalyzes a reaction which results in a change in the fluorescence properties of the drug-dye conjugate. The reaction of antibody with the conjugate reduces the amount of substrate available for enzymatic conversion. The measured fluorescence is proportional to the amount of drug in the sample. A clinical assay of this type for the drug gentamicin has been reported [19].

IV. Chemiluminescent Molecules

Schroeder et al. have also described the use of luminol derivative as labels in competitive immunoassays in which the chemiluminescence of the free labeled antigen is generated by an enzymatic reaction [20,21]. This method is a variant of the reactant-labeled fluorescence assay.

V. Enzyme Cofactors

The enzyme cofactors NAD and ATP have been reported as labels in another variant of the reactant-labeled immunoassay [22–24]. Enzymatic activity can be detected using either spectrophotometry or luminescence measurements. When luminescence detection is used, the product of the initial enzymatic reaction is fed into an enzyme system which catalyzes the production of light.

VI. Enzymes

Enzymes, the most frequently used non-isotopic labels, have been employed in both heterogeneous and homogeneous assay modes. Since the work on heterogeneous enzyme immunoassay has been frequently reviewed [25–26], the only method to be considered here is the EMIT® homogeneous enzyme immunoassay technique [3,28].

The principle of an EMIT® drug assay is depicted in Figure 1. The method employs an enzyme as the label for a drug. A drug derivative is attached to the enzyme to form an enzyme-drug conjugate. The activity of this enzyme-drug conjugate is modulated when anti-drug antibody binds to the conjugate. When the sample (usually a body fluid) is mixed with the antibody and the enzyme-labeled drug, free drug molecules in the sample compete with the enzyme-labeled molecules for a limited number of antibody binding sites. The more free drug in the sample, the more enzyme remains unbound. Enzyme activity can be directly correlated with the concentration of drug in the specimen. Figure 1 shows a representation of inhibition of activity and the mechanism of this inhibition is described as a steric exclusion of the substrate. This concept adequately describes the mechanism when the enzyme lysozyme is used [3,19]. However, when malate dehydrogenase and glucose-6-phosphate dehydrogenase (G6PDH) are employed, enzyme conformational change has been implicated as the mechanism of activity modulation [30,31].

Because enzymes are biochemical amplifiers, the EMIT® technique has potential for a very high level of sensitivity. The sensitivity depends on many factors, among them the choice of enzyme, length of incubation, and method for detection of catalytic activity. The sensitivity limit of current EMIT® assays is in the ng/mL range.

The EMIT® assay procedure is straightforward. The sample, plus antibody/substrate, and enzyme-drug conjugate are measured and mixed using an automatic pipetter-diluter. The assay

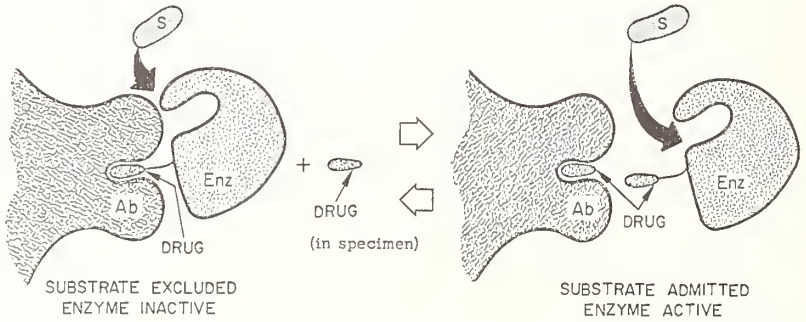

Figure 1. Schematic representation of the EMIT® homogeneous enzyme immunoassay for drug determination.

mixture is then aspirated into the flow cell of a standard laboratory spectrophotometer. The kinetic determination of enzyme activity requires 30 seconds and a single assay is completed in 1 minute.

Qualitative assays of this type have been developed for detection of drugs of abuse in urine. Assays for opiates, barbiturates, amphetamines, benzodiazepines, and benzoyl ecgonine (cocaine metabolite) [29,32,33] which employ lysozyme as the enzyme label are currently available.

Quantitative assays using G6PDH as the enzyme label have also been developed for a variety of therapeutic drugs. The assays which are listed in Table 2 are currently available, or are in advanced stages of development by Syva Company [34–37]. In addition, assays for other classes of drugs, including antimicrobial drugs (gentamicin, tobramycin) and antidepressants (amitryptyline, imipramine) are planned for the near future.

TABLE 2. *EMIT® assays for therapeutic drugs*

Antiepileptic drugs	Cardiovascular drugs	Respiratory drugs	Anticancer drugs
phenytoin	digoxin	theophylline	methotrexate
phenobarbital	lidocaine		
primidone	procainamide		
carbamazepine	N-acetylprocainamide		
ethosuximide	quinidine		
	propranolol		

Performance characteristics (precision, accuracy, and specificity) of EMIT® assays have been validated in clinical trials as well as during the broad use the assays have received [37,38]. Precision, expressed as a concentration coefficient of variation, is generally less than 10%. Accuracy has been demonstrated by comparisons of the EMIT® tests with established methodologies such as RIA, gas liquid chromatrography (GLC), high pressure liquid chromatography (HPLC), fluorometry, or spectrophotometry.

The EMIT® assays are highly specific and are designed to selectively measure a given drug or hormone in the presence of substances of similar structure. Specificity studies for the theophylline (1,3-dimethylxanthine) assay, for example, have shown no clinically significant interference by compounds including caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), other methylated xanthines, and uric acid derivatives [34,38].

A comparison of the features of EMIT® assays with RIA demonstrates that the accuracy, precision, and specificity of the two methods are generally equivalent. The advantages of the homogeneous enzyme method are that assays can be easily performed on existing laboratory equipment, and can be run "stat," rather than in the batch mode typically used for RIA. The detection limit of current EMIT® assays is in the ng/ml range. RIA procedures commonly measure in the pg/ml range. Finally, automation of EMIT® assays is relatively uncomplicated [39] while automation of RIA is difficult and requires expensive specialized instrumentation [40].

It is important to recognize one major limitation of all immunoassays. They are single analyte or family analyte methods in contrast to high resolution chromatographic techniques. Likewise, their use in exploratory analytical work is limited. The methods are, however, extremely useful in routine analytical determinations which require both sensitivity and specificity.

The EMIT® technique is the prime example of both an homogeneous and a non-isotopic immunoassay. Being highly versatile, the technique has already had significant impact on two areas of diagnostic medicine: measurement of drugs of abuse and therapeutic drug monitoring. The fluorescence and reactant-labeled methods discussed here have not yet been widely applied, but appear to hold promise in expanding the analytical capabilities of clinical chemists. The main thrust of the intense activity in the immunoassay area has been in the development of clinical

diagnostic procedures. However, it is easily appreciated that immunochemical assays have potential in areas such as industrial quality and process control, environmental analysis, and forensic analysis. Significant advances in the application of immunoassay techniques in these new areas are predicted.

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ANALYTICAL SYSTEMS FOR TRACE ORGANIC ANALYSIS

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An overview of instrumental systems capable of trace organic analysis will be presented. A simple statement of operating principle will be followed by emphasis on special techniques available for trace work. Factors to be considered in the evaluation include detectivity (M.D.Q.), sample size required, type of detector response (universal or selective) and availability. Advantages and limitations will be presented. Systems which will be discussed will include: MS, GC/MS, GC, LC, AA, NMR, fluorescence, radiochemical and electrochemical systems.

Key words: Analytical systems; atomic absorption; gas chromatography; liquid chromatography; mass spectrometry; trace organic analysis.

I. Introduction

An overview of instrumental methods for trace organic analysis will be presented. No attempt will be made to be comprehensive, but in so far as possible, presently available systems as well as potentially useful systems will be discussed.

The author has ranked these systems in order of usefulness (Table 1) based upon the following criteria:

1. *Detectivity or Minimum Detectable Quantity*—This is probably the single most important specification, but it is linked together with sample size as will be shown shortly. It was difficult to express M.D.Q. values with a single number since in many uses, it will depend upon the individual compound and the special techniques available. In general, the author hopes to be conservative in citing a number, rather than choosing an exceptional value.

2. *Sample*—This information describes the sample state and conditions necessary for analysis. Also stated are typical sample sizes. The basis for consideration in this paper is the ability to measure ppm levels or lower. It should be obvious that larger sample sizes may allow lower concentration levels to be measured, even if the detector used has a larger M.D.Q. A good case in point are the GC and LC detectors. GC detectors in general have much lower M.D.Q. values, but sample size is limited to about 1 milligram. LC systems on the other hand can easily handle hundreds of milligrams and therefore, a lower concentration of a trace component may be detected.

3. *Detector Response*—Each system will be classified as universal, or selective, or very selective. This, of course, refers to the range of organic compounds which will be routinely analyzed.

4. *Availability*—Considered here are the factors of how many systems are working in the world today; how expensive is a system; and, how much expertise is necessary to be able to use the analytical system.

TABLE 1. *Author's ranking of analytical systems for trace[a] organic analysis*

1.	MS	GC/MS
2.	GC	
3.	LC	
4.	AA	AF
5.	NMR	
6.	Miscellaneous: fluorescence, radiochemical/RIA, electrochemical, plasma induced emission spectroscopy, biological assays.	

[a] ppm or smaller.

II. Analytical Systems

A. MASS SPECTROSCOPY—GC/MS

The M.D.Q. available from a MS or GC/MS system depends upon the type of monitoring used. For T.I.C. (see Table 2) the M.D.Q. is about 10^{-9} g. This is, of course, greatly influenced by the type of ionization source used; the specifications of the particular instrument; and, the interference present. This value was chosen to be typical for most systems and samples.

The interesting point to be made here is the great difference in M.D.Q. between T.I.C. (a scanning mode used to identify what compounds are present) and S.I.M. (a quantitative measure of a trace substance whose identity and major m/e values are already known). If the problem is merely quantification, or confirmation of a given compound, S.I.M. can measure down to 10^{-12} g.

Gases, liquids and solids can be handled by MS. The main requirement is that the sample show a vapor pressure of at least 10^{-4} torr by 300 °C. Special techniques such as field desorption does allow spectra to be generated from large non-volatile compounds like nucleic acids. For GC/MS work, the sample must show about 20 torr by 300 °C.

The MS and GC/MS systems are universal; all compound types, organic and inorganic can be analyzed. The only limitation is the vapor pressure requirement listed above. In addition, in the normal scanning mode these systems are specific, that is, they will confirm the structure of a compound by its characteristic fragmentation pattern. For trace organic work, the GC/MS system is preferred as the GC will resolve or remove the interferences or matrix effects of most samples. This greatly simplifies the spectra obtained and aids considerably in positive identification.

Finally, MS and GC/MS systems are expensive; however, they are readily available in most research laboratories and this seems to confirm their position as the most useful system for trace organic analysis. They do require an experienced operator to obtain optimal results.

TABLE 2. *Summary of MS and GC/MS capability for trace organic analysis*

1.	MDQ	T.I.C.[a]	10^{-9} g
		SIM[b]	10^{-12} g
2.	Sample—gases, liquids, or solids; must show vapor pressure of 10^{-4} torr by 300 °C. Can see sub ppm, in special cases ppb.		
3.	Universal and specific—MS gives both qualitative and quantitative information.		
4.	Expensive, but available, expertise required, good quantitative, excellent confirming.		

[a] Total ion monitoring (scanning).
[b] Single ion monitoring.

B. Gas Chromatography

The MDQ's for GC depend greatly upon the detector used and range from 10^{-9} to 10^{-13} g (see Table 3). The detector is the critical component for trace organic analysis and the useable ranges of the common GC detectors are illustrated in Figure 1.

Samples can be gases, liquids or solids, and organic or inorganic. The main limitation is that they must be volatile; they should show about 20 torr by 300 °C. Molecular weights above 800, ionic compounds or highly polar compounds (sugars, amino acids, etc.) cannot be analyzed directly by GC. In some cases derivatization to more volatile compounds, or pyrolysis to smaller molecular weights can extend the useful range of GC. Normal sample sizes are 0.1 to 2.0 mg. The upper limit is determined by both column preference for small sample and detector limits on linear response (Fig. 1). Sub ppm analysis is possible, and with some detectors ppb analysis is possible.

The TCD (thermal conductivity detector) has a universal response; the FID (flame ionization detector) is restricted to organic compounds; the ECD (electron capture detector) is selective for electrophilic compounds. The ECD is most frequently used for analysis of halogenated pesticides

Table 3. *Summary of GC capability*

1. MDQ	TCD	10^{-9} g	NPD: N 10^{-12} g
	FID	10^{-11} g	P 10^{-13} g
	ECD	10^{-13} g	FPD: S 10^{-9} g
			P 10^{-11} g

2. Sample—Gases, liquids or solids; must be volatile (20 torr by 300 °C); 0.1 to 2 mg; sub ppm, with some detectors, ppb.
3. TCD—Universal, all compounds
   FID—Universal for organics
   ECD—Selective
   NPD—Only N or P compounds
   FPD—Only S or P compounds
4. Readily available, not expensive, easy to operate, good quantitative results; poor identifier.

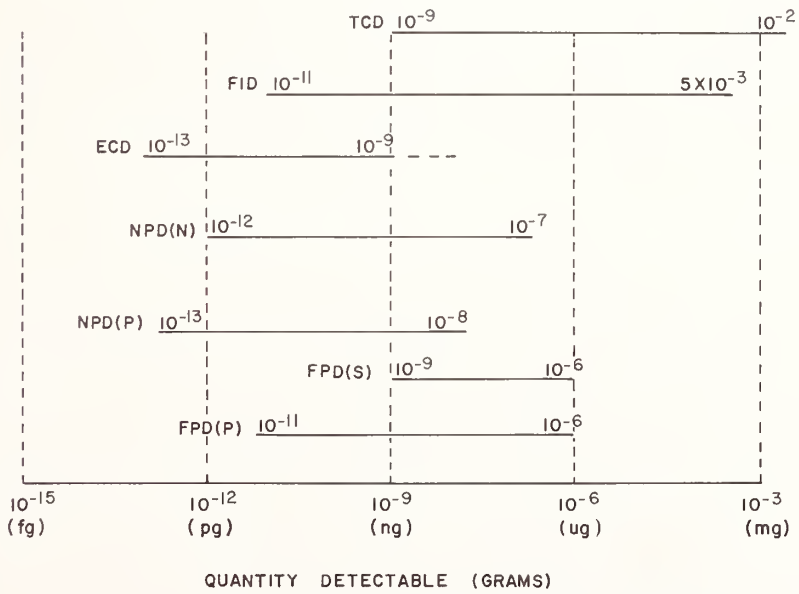

Figure 1. Useable ranges of common GC detectors. Adapted from a similar table by S. P. Cram, ACS course booklet, "Modern Techniques in GC," ACS-1977.

and herbicides, but it will respond to conjugated carbonyls, nitriles, nitrates, polynuclear aromatics, and most organometallic compounds as well.

The NPD (nitrogen phosphorus detector) is very selective, only N or P containing compounds are detected. The FPD (fiame photometric detector) is also very selective, only S or P containing compounds produce a signal.

Finally, gas chromatographs are readily available (more than 175,000 in use worldwide); they are not expensive; they are easy to operate and they give good quantitative results. The major limitation is that GC results alone cannot confirm the identity of a given peak. Chromatographic retention times are characteristic of the sample and chromatographic conditions, but they are not unique; several compounds could have the same retention time.

C. LIQUID CHROMATOGRAPHY

The detectivity is strongly dependent upon sample type and the detector used. The UV spectrophotometers can measure 10^{-9} g of many species, but they do require chromophore groups. Hydrocarbons (except polynuclear aromatics) present problems. The electrochemical detectors can measure 10^{-10} g, but are selective to those groups with electrophores (groups which can be oxidized or reduced). The fluorescence detectors can measure 10^{-12} g and are the most sensitive and most selective LC detectors available today.

The sample can be liquids or solids, organic or inorganic, ionic or covalent and molecular weights from 18 (water) to 6 million (polystyrene) have been analyzed. Best resolution is obtained with low molecular weights (100–2000), but high molecular weight samples can be handled. The sample must be soluble in the mobile phase, and this may require a heated system for many polymeric material. These systems are available commercially. Sample size can range from microgram to gram quantities, even 50 g quantities with dedicated preparative scale systems. Many compounds can be measured at ppm levels, a few at sub ppm levels.

In the author's experience, the UV/VIS detectors are almost universal. Compounds must have a chromophore, but a surprising number of organic compounds do meet this requirement. Lipids, fatty acids, sugars, amines, and hydrocarbons present problems with UV/VIS detectors. Derivatives can help introduce chromophore groups in some cases. The electrochemical detectors are selective and the fluoresence detectors very selective.

LC systems are available world wide; they are moderately expensive (gradient systems run from $10,000 to $25,000) and they do require some expertise to obtain optimum results. LC systems are extremely versatile in the range and type of samples which can be handled (almost anything except gases); good quantitative results are possible, but LC is a poor means of identifying unknown samples (see GC discussion on this same point).

TABLE 4. *Summary of LC capability*

1.	MDQ—UV-10^{-9} g Electrochem-10^{-10} Fluorescence-10^{-12} g
2.	Sample—liquids or solids; sample must be soluble; organic or inorganic; ionic or covalent; sample sizes: micrograms to grams; ppm and sub ppm capability with selected compounds.
3.	UV/VIS—almost universal (requires chromophore) Electrochem—selective Fluorescence—very selective
4.	Available, moderately expensive, require some expertise; very versatile for variety of sample types, good quantitative results, poor identifier.

D. Atomic Absorption (AA); Atomic Fluorescence (AF)

Atomic absorption can measure 10^{-7} g of most metals; atomic fluorescence 10^{-9} g or smaller. Samples are usually solutions of liquids or solids. The classical flame atomization methods require about 1 mL of solution; the electrothermal atomization techniques require only 1–10 μL. Metal atoms must be present for detector response; most organic species will require derivatization. Absorption methods can routinely see 0.1–10 ppm of most metals; fluorescence can measure ppb levels (Table 5).

Both AA and AF are selective in that they can see only metal ions; a further restriction is that only one metal can be measured at a time. This will greatly limit the applicability of AA and AF to true organic analysis, in that metallic derivatives will be required. In this regard AF does not appear to be much more restrictive than AA; a large number of metal atoms do fluoresce and these could be used for derivative formation in most cases.

AA is readily available, easy to use, not expensive and is a standard technique in many laboratories. It will require incorporation of metal atom to be applicable to organic systems. AF is unfortunately not commercially available today. It could become available in the next few years.

It seems appropriate to point out the potential applications for GC/AA and/or LC/AA systems. Neither system is commercially available but interfacing presents no major problems. Literature reports levels of 10^{-9} g of selected organo-metallic compounds [1,2]. These systems would be very selective, and derivatization of most organic compounds would be required, but this does appear to be a promising area for future work.

Table 5. *Summary of atomic absorption and atomic fluorescence capability*

	AA	AF
1. MDQ	10^{-7} g	10^{-9} g
2. Sample	solutions; flame, 1 mL; electrothermal, 1–10 μL; 0.1–10 ppm	same, but ppb
3.	Selective (only metals) (requires derivatization for organics)	same
4.	Available, easy to operate, not expensive, limited organic applications	not available today

E. Nuclear Magnetic Resonance—(NMR)

This technique does not qualify according to our earlier definition of "TRACE" (ppm or less, see Table 1), but it is too important to be neglected.

The minimum detectable quantity is 10^{-3} to 10^{-4} mol/L of liquids or solids in special solvents. A few milligrams of sample are needed for most methods, but micro-cells and time averaging techniques can work on micrograms of samples. Lower limits are about 10–100 ppm at present and these levels demand Fourier Transform techniques.

NMR is a universal system applicable to most organic compounds, and, in addition, provides a wealth of information to elucidate structure and identify the compound. NMR systems are readily available, however they are expensive, and they require expertise primarily in the intrepretation of spectra. These systems do not really perform trace analysis and probably will not do so in the near future (Table 6).

TABLE 6. *Summary of NMR capability*

	Protons (FT)[a]	Fluorine-19 (FT)[a]
1. MDQ	10^{-3}–10^{-4} mol/L	same
2. Sample	solutions of liquids and solids; few milligrams, 10–100 ppm normally	same
3. Universal and specific, provides a wealth of structural information		same
4. Available, expensive, requires expertise, not really trace work at present time		
Micro cells now standard feature.		
Supercons could increase MDQ 10 fold.		
F-19 useful to characterize functional groups.		

[a] Fourier Transform.

F. MISCELLANEOUS TECHNIQUES

1. *Fluorescence*—Fluorescence is an extremely sensitive and useful technique for both trace organic and inorganic analysis. It is not ranked in higher order in this paper because the author feels that it is so selective as to be applicable to only a few organic compounds. In addition, when used without a chromatographic separation step, the possibilities of interferences are so high as to minimize the usefulness of this technique.

2. *Radiochemical methods*—These are also extremely sensitive methods, but are either so selective or require such specialized instruments and techniques as to be considered not of general importance for trace organic analysis.

3. *Electrochemical methods*—Electrochemical methods allow the direct determination of ppm levels of organic compounds which can be oxidized or reduced. Unfortunately, they are not specific enough to be used alone. Their primary applications will be at the end of a gas or liquid chromatograph where interferences have been removed.

4. *Plasma induced emission spectroscopy*—This is an extremely sensitive and useful technique, but has been limited in its organic application. With future commercialization of present research systems, this technique may grow as a useful tool for trace organic analysis.

5. *Biological assay*—No discussion of trace or organic analysis would be complete without mentioning biological assays. These have the sensitivity and specificity to be useful in some cases. In the majority of common organic chemicals biological assays have not been developed and it is not a generally used method at this time.

III. Summary

The author has ranked the commonly available analytical systems in decreasing order of suitability for trace organic analysis. The most useful systems include MS, GC/MS, GC and LC. The discussions and comments limited here would not include concentration steps prior to application of these analytical systems. Concentration steps are, of course, common and a useful means for decreasing the concentration levels which can be measured.

IV. Acknowledgment

The author would like to acknowledge the useful suggestions of Drs. Frank Karasak, John Dillard, Harry Dorn, and Betty Bartschmidt.

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SELECTIVE DETECTION IN CHROMATOGRAPHIC ANALYSIS

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While being the best separation technique, chromatography also excels in sensitivity: traces present in concentrations at the parts-per-billion—1:10^9—level or lower can be detected. However, at such low levels, positive identification is difficult since a large number of compounds may emerge from the column within a short retention time range.

The solution of this problem is to use substance-selective detection, i.e., detectors which respond only to certain types of compounds. In this way, two goals are achieved simultaneously: one has information on the chemical nature of the compound present and, at the same time, interferences of co-emerging compounds of dissimilar chemical nature are eliminated.

Although selective detection is not new in chromatography—after all the colored bands of Tswett already compound identification—its real evolution started in the early 1960s, in *gas chromatography* as an answer to the requirement of detecting trace quantities of halogenated pesticides. The electron capture and coulometric detectors have been developed to fulfill this need. Based on new requirements a number of other detectors were also developed in the last 15 years, providing e.g., selective detection of phosphorus, sulfur and nitrogen containing compounds. Most recently, spectroscopic detectors based on the principles of IR and UV spectroscopy and applying on-the-fly detection techniques further extended our possibilities for selective detection.

In *liquid chromatography* the widely used variable wavelength UV detectors also provide selective detection since the absorptivity of different compounds—even within one class—varies widely. In addition to these, a number of other detectors have been introduced to further enhance selectivity. From these the use of fluorescence is probably the most important: such systems may provide even better sensitivity than the highly selective and sensitive GC detectors.

Key words: Detector design; gas chromatography; liquid chromatography; selective detection.

I. Introduction

The problem of positive identification has always existed in chromatography: after all, our general purpose detectors see everything and respond to everything (or almost everything) emerging from the column. Since the separation power of a column—even the best column—is limited, one always has the possibility of mistaking a peak identified based on its relative retention while it actually corresponds to another compound having the same or very close retention time.

A quick calculation will illustrate this problem (see Table 1). Let us assume that we are using a 50-m long glass open tubular (capillary) column with an HETP of 0.33 mm for *n*-hexadecane. The separation number ("Trennzahl"; see ref. 1) of this column for the C_{15}–C_{16} range is 24.9. This means that we can separate 25 peaks in this time interval between the emergence of these two normal paraffins, in this particular case between 15.1 and 20.6 minutes. Expressed in another way, we can separate in this range peaks the retention index value of which differs by four index units, that means e.g., 1510 and 1514. There is, however, no question that there are more than 25 compounds listed in the 200 volumes of *Bellstein's Handbuch der Organischen Chemie* which would elute from this column between 15 and 20 minutes!

We can go one step further in this example. We can check the total number of compounds we could separate on this column in the first 20 minutes. This can be obtained by calculating the separation number for the C_1–C_{16} range. The result is 97, in other words, we can separate about 100 compounds in the first 20 minutes of the analysis. Again, this is a remarkable performance;

TABLE 1. *Data for a 50-m open tubular column*[a]

	CH_4	n-$C_{15}H_{32}$	n-$C_{16}H_{34}$
Retention time, minutes	5.53	15.1	20.6
Capacity ratio (k)		1.73	2.73
HEPT, mm		0.32	0.33
Separation number (Trennzahl):			
$-C_{15}/C_{16}$		------24.9------	
$-C_1/C_{16}$	------97.3----------		

[a] 50 m×0.27 mm i.d. wall-coated open tubular glass column prepared with SF-96 methylsilicone oil and operated at 165 °C with helium as the carrier gas. Data from ref. [1].

however, the possible compounds which could emerge from this column in 20 minutes is probably at least one order of magnitude higher than this value.

Fortunately, in *industrial* samples the number of components which may be present is usually limited by the chemical process. However, this is not true about natural or biological samples where almost everything which may exist can be present. Thus, positive identification is very difficult and relative retention—retention index—values alone are not sufficient. A good—and probably the only—way out of this dead-end street is to be able to tell the chemical nature of a particular compound. This additional information can be provided by the use of substance-selective detectors. Two words used in the last sentence need further explanation; these are "additional" and "selective."

I use the word "selective" and not "specific." There is a difference between these terms. Except possibly for the mass spectrometer, all detectors used in chromatography are only selective: they tell us whether a peak corresponds to a certain class of compounds but do not inform us about the particular compound. For example, the flame photometric detector used in gas chromatography will tell us that the peak corresponds to a sulfur-containing compound but will not inform us whether it is thiophene or diethyl sulfide. In fact, sometimes it even gives us less information: for example, the electron capture detector will respond to compounds containing a halogen atom in their molecule, but also to nitro compounds. In liquid chromatography the situation is usually better with the variable wavelength UV detectors and particularly with fluorescence detectors but still, one cannot safely call them truly *specific* detectors.

The other word which needs more comments is "additional." Some people say that one of the advantages of selective detectors is that there is no need for highly efficient columns. This is really not true, at least not in the implied sense. Let us not forget that even within the groups of compounds one is detecting with the selective detectors, there are a large number of isomers, sometimes even larger than possible for other compound groups in that particular range. For example, let us consider for a moment a single group: the halogenated hydrocarbons. As listed in Table 2 there are a total of 68 possible chlorocarbons in the C_1-C_3 range (looking only at saturated and olefinic compounds)! If we really want to individually detect them, we continue to need high-resolution columns.

In addition to selectivity, these detectors *usually* have an additional advantage: a higher sensitivity for the particular compounds than that of the usual universal detectors. This is, however, not always the case: e.g., the infrared detector for gas chromatography is less sensitive than a good flame ionization detector, although probably this is the only major exception to this rule.

The higher sensitivity—together with selectivity—is illustrated in Figure 1. If we would analyze a little bird on a universal detector, only a small peak would be obtained, and even this would be on the tailing of a very large peak corresponding to the forest. On the other hand, a "bird-selective detector" would have no response for the trees and the peak for the bird would be

TABLE 2. *Number of possible C_1-C_3 chlorocarbon isomers*

Chlorinated methanes	4
Chlorinated ethanes	9
Chlorinated ethenes	6
Chlorinated propanes	27
Chlorinated propenes	22
	68

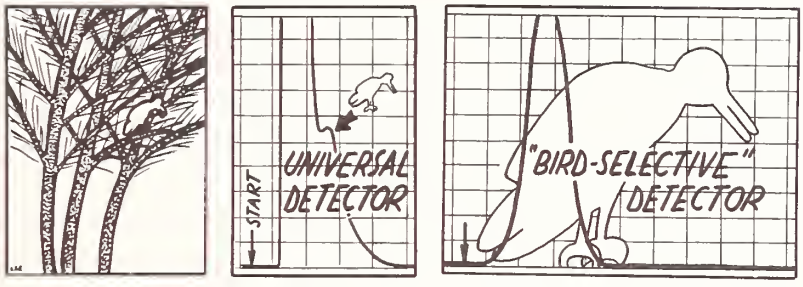

Figure 1. Illustration of the specificity and increased sensitivity of a "bird selective" detector.

orders of magnitude larger than the one obtained with the universal detector. Speaking about real detectors, for example the electron-capture detector can detect substances in the picogram—or even in the sub-picogram range while the flame ionization detector's sensitivity is usually limited to the sub-nanogram range. This means that our most selective detectors can analyze trace compounds present in concentrations as low as parts per billion (i.e., one part in 10^9 parts) or even less. Today, we are using this term as something natural without realizing how formidable the ppb-sensitivity really is. However, let us be honest: our nose, or the "nose" of animals is even more sensitive than some chromatographic detectors.

Actually, the first substance-specific detector (I am now using this expression because this detector is truly specific) used in connection with gas chromatography involved the sensory sensitivity of certain animals: it was related to the small silk moth, *Bombix mori L.* In the early 1950's Butenandt started to investigate the sex attractants (pheromones) of animals and used the silk moth as the model [2]. This was before the advant of gas chromatography and he had to rely primarily on isolation and chemical separation. After much work, extracting the glands of 313,000 female silk moths and doing a lot of chemistry, they finally obtained 5.25 mg of an oily substance. When a glass rod was dipped in this oil and then brought close to the male silk moth, he became excited: it was clear that the pheromone was present in the oil but, naturally, together with other substances: it was not separated but only concentrated.

A few years later Bayer and Anders [3,4] repeated this investigation but in a very ingenious way, now using gas chromatography. They placed a male silk moth in a small box connected to the outlet of the GC column, extracted the gland of *nine* female silk moths and injected the solution of the oil into the column. A number of peaks were obtained and, in most of the time, the poor little male silk moth remained in the corner of his box. However, when one particular peak eluted from the column, he started to wiggle his wings and run around, obviously looking for the female. This peak corresponded to the pheromone.

This example is the best illustration of the powerful combination of a chromatographic separation column with a selective detector.

Today, selective detectors are widely used in both gas and liquid chromatography. It is not the purpose of this paper to discuss them in detail; for this, the readers are referred to a number of specialized books [5–7]. Rather, in addition to a brief summary of the characteristics of the most important detectors, I want to concentrate on a few selected questions related to their application.

II. Gas Chromatography

Table 3 lists the most frequently used selective gas chromatography detectors. In addition to these, spectroscopic detectors are continuously gaining in importance and therefore, I shall also briefly deal with them. However, I will not discuss the combination of gas chromatography with mass spectrometry of Fourier transform spectroscopy: these are too complex systems to call the ancillary instrument simply as a "detector." For details, the readers are referred to specialized textbooks [8,9], or the numerous publications available in the literature.

Table 3 also indicates the minimum detectable limit of the individual detectors i.e., the smallest amount the detector will still "see." These values must be considered as guidelines only and, in actual analysis, the smallest detectable amount might be much more or less. There are basically two reasons for this uncertainty.

First, the data in the literature do not have a common denominator. Some are given as concentrations, others as amount or amount per second, and even as amount of heteroatom per second. Thus, one must be very careful in properly interpreting them.

Let us, for example, consider a flame photometric detector about which we read in the literature that its minimum detectable limit is 4.0 pg/s expressed as sulfur and 1.4 pg/s expressed as phosphorus. Let us consider ethion as the substance determined. Ethion contains 33.4% sulfur and 16.3% phosphorus; thus, if we analyze it with the sulfur filter, the given minimum detectable limit would correspond to 12 pg substance per second, while, if working with the phosphorus filter, the value would be 8.6 pg/s. If we assume a peak width of 5 seconds, the respective absolute substance values are 60 pg and 43 pg; on the other hand, if the peak width is 10 seconds, they are 120 pg and 86 pg. The values will, however, be quite different if we select another substance as the sample. For example, if we would analyze carbon disulfide (S=84.2%) with the same detector under the same conditions, the minimum detectable limit would be 24 pg substance for a 5 s and 47 pg for a 10 s peak width. These examples show that, depending on the substance and conditions, quite different values are obtained.

The second reason for the uncertainty in giving the minimum detectable limits is that the response of some of the detectors is very much substance selective. This is particularly true for the electron capture detector; Table 4 summarizes some literature data [10,11]. As seen structurally very close compounds have orders of magnitude difference in the response. Thus, for example, if

TABLE 3. *The major gas chromatographic selective detectors*

Common symbol	Detector	Minimum detectable limit[a]
ECD	Electron capture detector	10^{-12}–10^{-13} g
FPD	Flame photometric detector	10^{-10} g
	Electrochemical detectors:	
	Microcoulometric detector	10^{-7} g
	Electrolytic conductivity detector	10^{-11} g
NPD	Thermionic detector selective for nitrogen and phosphorus	10^{-12}–10^{-13} g

[a] These values represent approximate minimum detectable limits, based on literature data and personal experience. In many cases, actual data might be quite different.

TABLE 4. *Relative response factors of the electron capture detector*[a]

Compound	Relative response[b]
1-Chlorobutane	1
1-Iodobutane	90,000
1,2-Dichloroethylene, cis	90
1,2-Dichloroethylene, trans	370
1-Chloro-2-methylpropane	1.7
2-Chloro-2-methylpropane	12
Chloroform	60,000
Carbon tetrachloride	400,000
Chlorobenzene	75
Bromobenzene	450
Iodobenzene	27,750
Nitrobenzene	29,250
Acetone	0.5
2,3-Butanedione	50,000
Benzene	0.06
Toluene	0.2
Anthracene	900
Azulene	25,500
Quinone	375,000
Hydroquinone	7.5

[a] Based on data in refs. [10] and [11].
[b] Relative to 1-chlorobutane (= 1).

the detector's minimum detectable limit would be 0.5 pg for carbon tetrachloride, it will only be 3.3 pg for chloroform and 3.3 μg for benzene! Thus, it is also very important to always specify the substance used to establish the minimum detectable level.

A. ELECTRON CAPTURE DETECTOR

The electron capture detector (ECD) [12] was the first truly selective GC detector. It was developed by Lovelock and Lipsky in 1959 [13] but, in the first years, one was not clear about its real potentials. The real application of this detector started only with our changing attitude toward environment. The pesticides found most harmful were chlorinated substances and this detector just happened to have an excellent sensitivity for such substances. Thus, it was natural that the electron capture detector suddenly became very important in environmental protection and control. Early work showed the omnipresence of these pesticides; Figures 2–3, taken from a report published in 1964 [14], illustrate this better than any discussion.

Figure 4 shows the schematic of a typical electron capture detector [15]. Column effluent enters the detector chamber having a radioactive foil usually containing ^{63}Ni. In case of an inert gas flow and a potential sufficient to collect all the electrons liberated by ionizing radiation the ion current measured is constant. However, if molecules which capture free electrons enter the cell this ion current will be reduced creating negative peaks (although we see them as regular positive peaks because of changed recorder polarity). This mode of operation has been used for a long time, however, it has two disadvantages.

First, in order to reduce the electron energy, a gas mixture—usually 5–10% methane in argon—is used as the carrier gas. The methane molecules reduce the energy of free electrons by

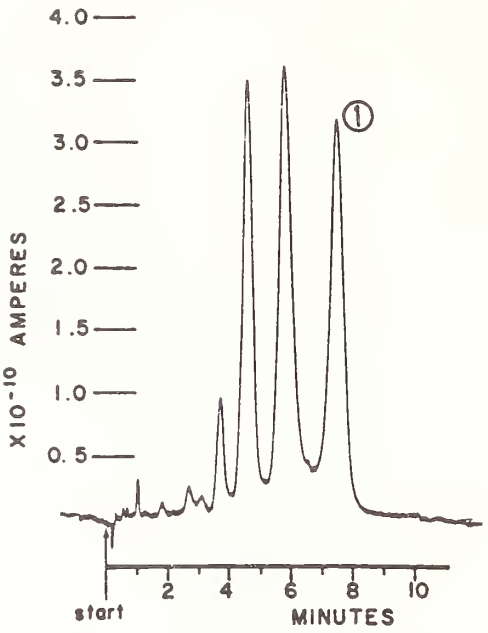

Figure 2. Analysis of an extract of soil (20 g) with the electron capture detector [14]. Column: 6 ft×1.9 mm i.d. glass, packed with 2.5% DC-200 on Chromosorb W AW 80/100 mesh. Carrier gas: argon containing 5% methane. Carrier gas flow rate: 100 mL/min (column)+66 mL/min (scavange). Temperature of injector, column and detector: 290°, 200° and 220 °C. Recorder: 5 mV, attenuation×200 200. Peaks: 1–*p,p'*-DDT.

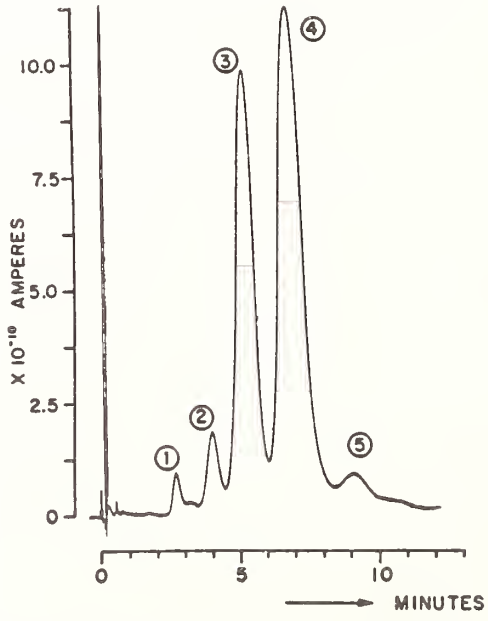

Figure 3. Analysis of an extract of earth worms (2.68 g) with the electron capture detector [14]. Column: 6 ft×1.9 mm i.d. glass, packed with 1.5% SE-30 on HMDS Chromosorb W 80/100 mesh. Carrier gas: argon containing 5% methane. Carrier gas flow rate: 100 mL/min (column)+66 mL/min (scavange). Temperature of injector, column and detector: 275°, 175° and 220 °C. Recorder: 5 mV, attenuation×500. Peaks: 1–4–DDE isomers, 5–*p,p'*-DDT.

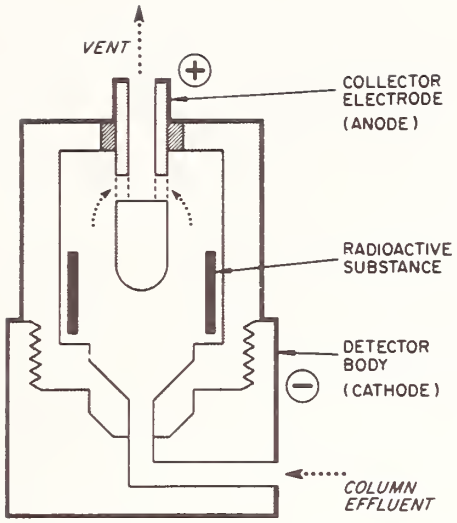

Figure 4. Schematic of a typical electron capture detector [15].

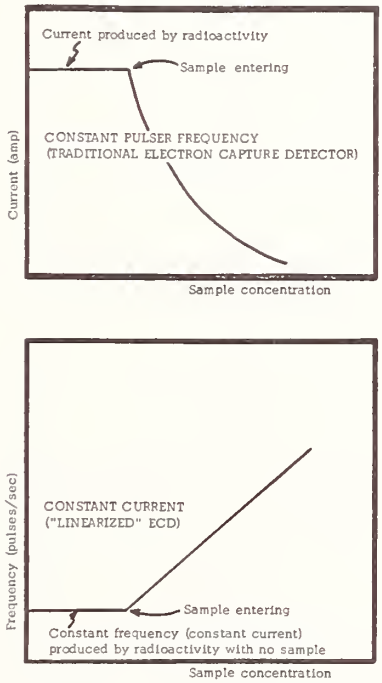

Figure 5. Explanation of the difference between constant pulse frequency and frequency-modulated constant-current electron capture detectors.

non-elastic collisions. Nitrogen is not recommended as the carrier gas because of anomalous effects due to an electron energy far in excess of that required for electron absorption. However, the problem of such a mixed carrier gas is that it is incompatible wi*h the flame ionization detector although one often would like to use simultaneously a selective detector with a detector of fairly universal response.

The second disadvantage was the lack of linearity. This is clear if we investigate the top part of Figure 5. In case of a negative response we really have an asymptotic curve and in this, linearity can be assumed only in a narrow range, maybe two orders of magnitude.

In the last decade the electrical system of the ECD was changed: now, the ion current is kept constant [16–21]. When an electron capturing substance enters the detector cell, the pulse frequency is changed in a closed loop circuit in order to maintain a constant electron current. Here, the basis of quantitative measurement is the relationship between the change of pulse frequency and the concentration of the electron capturing substance. As shown in the lower part of Figure 5, this is a linear relationship; as a conclusion, the linear range of the detector is extended to several (about 5) decades.

A special advantage of the new ECD system is that now, we can use pure nitrogen as the carrier gas and thus, multidetector systems are feasible. Using nitrogen as the carrier gas, the sensitivity of the detector is also increased. This is illustrated in Figure 6 showing the analysis of one picogram of lindane [20]. Here the minimum detectable limit—representing a peak about the size of that shown by an arrow—is as low as 6×10^{-14} g lindane.

The basic problem of the electron capture detector is that it is practically impossible to predict the compound which gives a peak. Table 4 indicated the wide range of compounds which may have response with the ECD. Therefore, already very early in pesticide analysis, other means were sought to permit the absolute identification of chlorine-containing compounds. The microcoulometric and electrolytic conductivity detectors represent such a possibility.

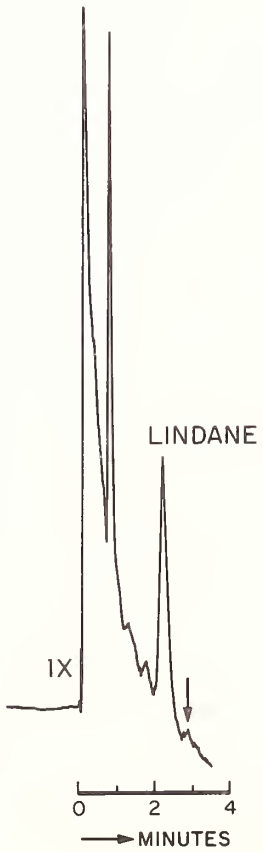

Figure 6. Analysis of 1 pg lindane in toluene solution with the frequency-modulated constant-current electron capture detector using nitrogen as the carrier gas [20]. Column: 3 ft×2 mm i.d. glass packed with 10% OV-1 on Chromosorb G HP 80/100 mesh. Carrier gas flow rate: 60 mL/min. Temperature of column and detector: 200° and 300 °C. Sample volume: 0.25 μL, on-column injection.

B. COULOMETRIC/ELECTROLYTIC CONDUCTIVITY DETECTORS

The microcoulometric detector was first described by Coulson and Cavanagh, in 1960 [22,23]; its block diagram is shown in Figure 7. Here the column effluent is mixed with oxygen, the organic compounds combusted in a furnace and the formed HCl titrated in an automatic titration cell with internally generated silver ions.

A few years later, the principles of the detector were somewhat modified: instead of automatic titration, now the conductivity is measured [24–26]. In this, so-called electrolytic conductivity detector (Fig. 8), column effluent is mixed with a reactant gas—either oxygen or hydrogen—and, in a furnace containing certain catalysts, the organic compounds are either oxidized or reduced. The ionizable species emanating from the combustion zone are contacted with deionized water and the carrier gas is separated from the liquid in a separator. The conductivity of the water is now changed due to the presence of the ionized species; this change can be measured and displayed on a recorder creating the usual peaks.

The electrolytic conductivity detector underwent a number of changes since its inception, e.g., by eliminating water as the liquid. Its presently best known version is the so-called Hall detector [27,28].

Although, in addition to halogen compounds, the electrolytic conductivity detector can also be made selective for nitrogen or sulfur containing compounds, its most important application is still the selective determination of chlorinated compounds. Figure 9 shows a typical chromatogram [29]; it also indicates the sensitivities which are in the sub-nanogram range.

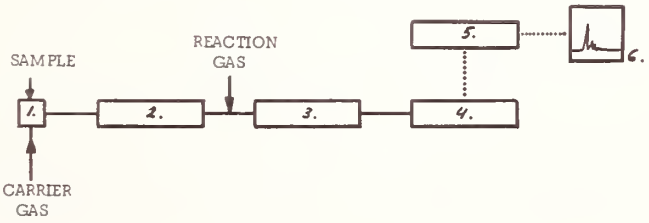

Figure 7. Block diagram of a gas chromatographic system with a microcoulometric detector. 1–Sample introduction, 2–chromatographic separation column, 3–combustion furnace, 4–titration cell, 5–coulometer, 6–recorder.

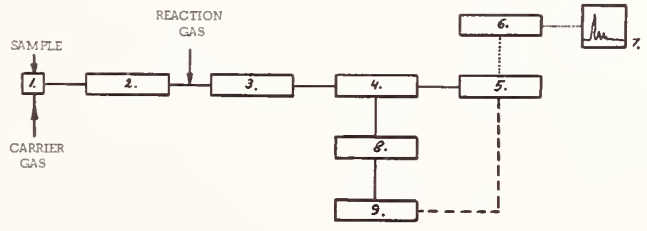

Figure 8. Block diagram of a gas chromatographic system with an electrolytic conductivity detector. 1–Sample introduction, 2–separation column, 3–furnace, 4–gas-liquid contactor, 5–separation cell and conductivity cell, 6–conductivity meter, 7–recorder, 8–pump, 9–solvent reservoir.

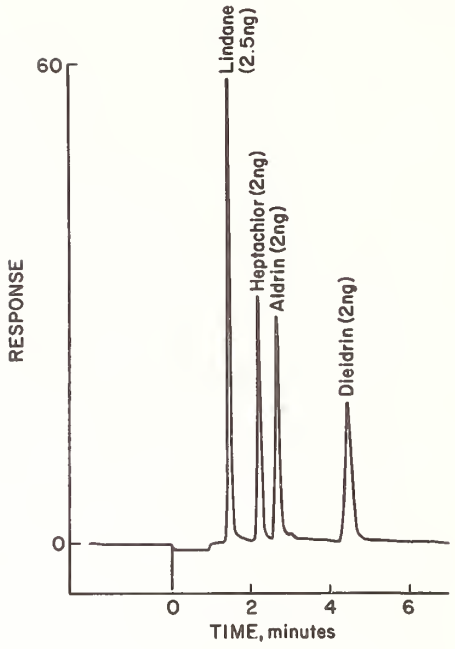

Figure 9. Typical chromatogram obtained with the electrolytic conductivity detector [29].

C. Flame Photometric Detector

Around the middle of the 1960s, a new problem arose from the change in the chemical nature of pesticides and herbicides. The new substances contained phosphorus and sulfur rather than halogens in their molecule and thus, a new way had to be devised for their detection in trace quantities. This led to the development of the flame photometric detector.

The flame photometric detector (FPD) has a number of ancestors but, in general, present-day systems are mostly based on the work of Brody and Chaney [30]. The schematic of a typical commercial detector is shown in Figure 10 [15]; it is really nothing else but a simple hydrogen flame where the emission from the burning flame is viewed through appropriate filters by a photomultiplier which converts it to an electric signal. The maxima of the characteristic filters are 394 nm for sulfur and 526 nm for phosphorus. In some designs, the photomultiplier is next to the body of the detector while in other designs (Fig. 10) the emission is carried by a light-pipe to the photomultiplier. The obvious advantage of this latter design is that the photomultiplier is protected from heat radiation. A further change in the design of some commercial units [31] is the use of two flames, the lower one for combustion and the upper one for viewing. The claimed advantage of this construction is the reduction of the effect of hydrocarbon background quenching the light emission from sulfur or phosphorus compounds [32]. It should be noted, however, that hydrocarbon quenching is only one of the many possible effects: emission signal intensity can also significantly be reduced by the presence of water [33] and carbon dioxide [34] both formed during the combustion process which also will reach the second flame.

The detector's sensitivity is in the sub-nanogram range for sulfur-containing compounds and about one order of magnitude better for phosphorus containing compounds. Figure 11 shows the response for *n*-hexanethiol; the peak corresponds to 2.5 ng of substance or 0.68 ng of sulfur.

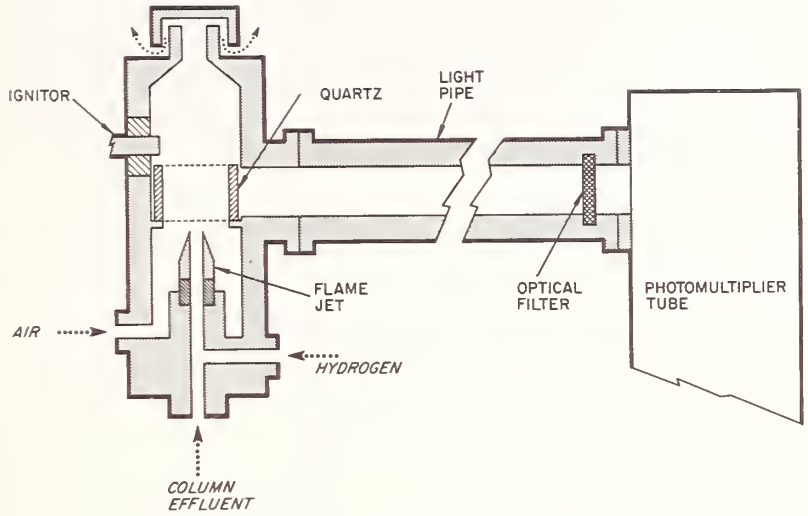

Figure 10. Schematic of a typical flame photometric detector [15].

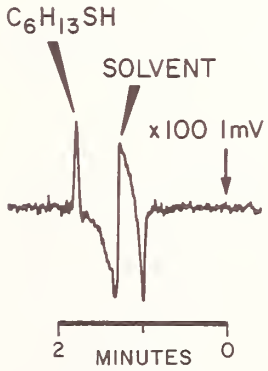

Figure 11. Analysis of *n*-hexanethiol in *n*-octane solution with the flame photometric detector, using the sulfur filter (394 nm) [15]. Column: 6 ft×2 mm i.d. glass, packed with 3% SE-30 on Chromosorb W 80/100 mesh. Carrier gas (He) flow rate: 30 mL/min. Temperature of injector, column and detector: 200°, 60° and 200 °C. Sample: 0.5 μL of the *n*-octane solution containing 2.5 ng of *n*-hexanethiol. Signal-to-noise ratio: 8.8. Minimum detectable limit calculated for twice the noise is 0.1 ng S/s.

Figure 12 shows the chromatogram obtained from 0.5 ng trimethyl phosphate or 0.11 ng of phosphorus.

The special characteristic of the flame photometric detector is the difference in the type of response for the two heteroatoms. In general, if we plot response, measured as peak area, against the amount of substance in a log-log scale then, as long as the detector behaves linearly, the slope of the plot will be one. This can be seen in Figure 13 for trimethyl phosphate. On the other hand, as shown in the same figure, the plot for hexanethiol has a slope of two. This means that response increases quadratically or, that concentration is proportional to the square of response. This is due to the fact that the emitting sulfur-containing species is S_2 and not S. Present-day flame photometric detectors easily overcome this problem, by using so-called linearizing amplifiers, which display the square root of peak area on the recorder.

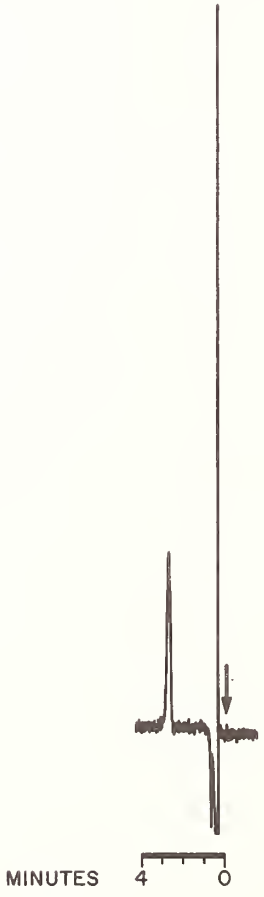

Figure 12. Analysis of trimethyl phosphate in *n*-octane solution with the flame photometric detector, using the phosphorus filter (526 nm) [15]. Column: 6 ft×2 mm i.d. glass, packed with 10% Carbowax 20M and 1% KOH on Chromosorb W 80/100 mesh. Carrier gas (He) flow rate: 30 mL/min. Temperature of injector, column and detector: 250°, 150° and 250 °C. Sample: 0.5 μL of the *n*-octane solution containing 0.5 ng of trimethyl phosphate. Signal-to-noise ratio: 11.6. Minimum detectable limit calculated for twice the noise is 2.6 pg P/s.

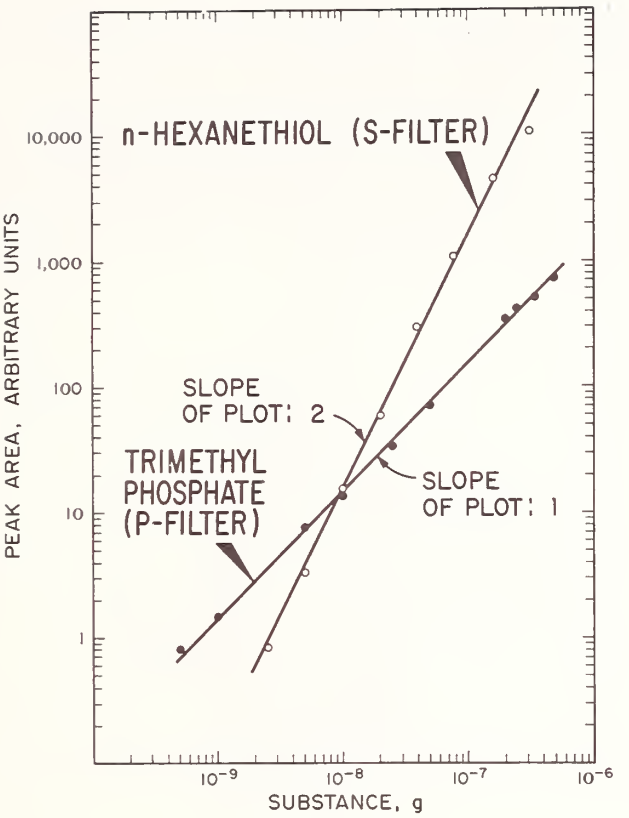

Figure 13. Linearity plots for the flame photometric detector [20].

D. THERMIONIC DETECTOR

In the late 1960s, we have encountered a new social problem: significant increase in the number of cases of drug abuse. This social phenomenon necessitated the quick and reliable determination of small quantities of these drug substances, most of which contain nitrogen in their molecules. The co-called nitrogen-phosphorus detector (NPD) has the special selectivity for these substances.

This detector is a type of thermionic detector originally described by Karmen and Giuffrida [35,36] who, in the early 1960s, found that if we place crystals of an alkali salt over a flame and the collector electrode above this alkali source, then only chlorine and phosphorus containing species will be sensed.

A variety of this type of detector have been constructed and utilized [37–39]. Further modification made the detector sensitive to nitrogen and phosphorus containing compounds [40,41]. Subsequently, new developments in the 1970s—pioneered mainly by Kolb [42–44]—resulted in a more reliable design with extended linear range. The schematic of a typical detector is shown in Figure 14 [45].

The basic difference between this and earlier designs is that now, the alkali salt—in this particular design, rubidium silicate beads are used—is electrically heated and thus, the flame's only function is to ionize the species. In fact, we cannot speak about a flame in the conventional sense; we rather have a hot plasma around the bead. Kolb's design has been adapted in the last years by a number of companies and such types of detectors are now universally available.

The detector shown in Figure 14 has two modes of operation.[1] In the most frequently used mode, shown in the left-hand side of Figure 15, the detector is sensitive to both nitrogen and phosphorus containing substances. In this mode, the detector has a wide linear range—over five orders of magnitude—and a superior sensitivity, in the picogram range. In the other mode of operation (right-hand side of Fig. 15) where a regular flame is maintained and the jet is grounded, the detector is selective to phosphorus-containing substances only. The sensitivity for these substances is still excellent, however, the linear range of the detector is reduced. Therefore, in general, this mode of operation is recommended only for absolute identification; for quantitative analysis the former mode is more convenient.

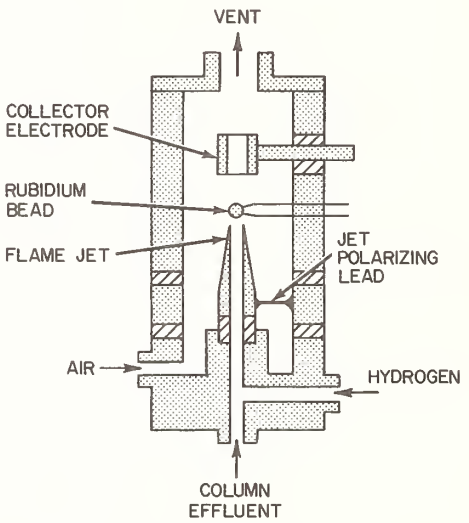

Figure 14. Schematic of a typical thermionic detector sensitive for nitrogen and phosphorus [45]. The electrical heating of the rubidium bead is not indicated.

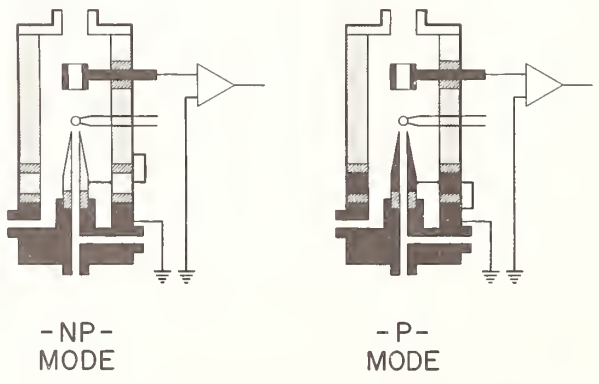

Figure 15. The two possible modes of operation of the detector shown in Figure 14 [20]. Parts with negative polarity are indicated with dotted area and parts with positive polarity with dark area. Hatched area represents insulation. The electrical heating of the rubidium bead is not indicated.

[1] In some designs [43,44], the detector can also be utilized as a regular flame ionization detector.

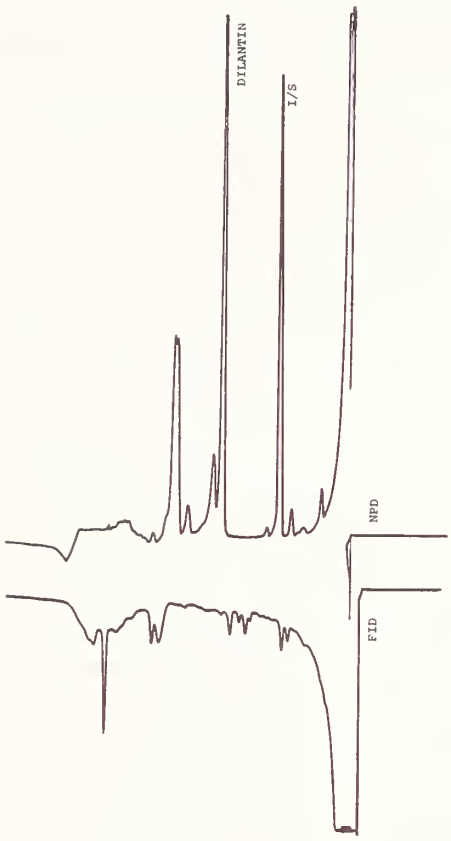

Figure 16. Analysis of a clinical sample for dilantin (diphenylhydantoin) [46]. A 50-μL aliquot of serum was processed and a 2-μL of the final 10-μL ethyl acetate solution was injected into the gas chromatograph. The dilantin peak corresponds to 20 ng substance or 2 mg/liter serum. Internal standard: 5-allyl-5-phenylbarbituric acid. Column: 3 ft×2 mm i.d. glass packed with 3% OV-1 on Chromosorb W HP 100/120 mesh. Carrier gas (He) flow rate: 30 mL/min. Temperature of injector and detector: 260° and 290 °C. Column temperature: 1 minute isothermal at 170 °C then programmed at 16 °C/min to 220 °C, held there for 2 min then programmed at max. rate to 270 °C. Column effluent was split 1:1 to the two detectors. Attenuation: FID×80, NPD×60. Dual-pen recording. Top chromatogram: NPD, bottom chromatogram: FID.

The superior sensitivity and selectivity of this detector can be best illustrated with help of clinical samples. In fact, this detector permitted for the first time the reliable analysis of anticonvulsant drugs at clinical levels in infants' blood utilizing only 50 μL of sample [46]. As an example, Figure 16 shows the analysis of dilantin (diphenyl hydantoin, 5,5-diphenyl-2,4-imidazolidinedione) in such a sample. In the procedure, the free drugs are transformed to the N-methyl derivatives prior to analysis. The high attenuation of the NPD chromatogram clearly demonstrates the availability of additional sensitivity for lower concentrations.

E. Derivative Formation

In chromatography, the sample is modified many times prior to analysis, by forming certain derivatives. In gas chromatography, the principal aim of the manipulations is to make the substance to be analyzed more stable and more volatile. Esterification, methylation, trimethylsilylation are techniques which have been utilized for a long time and various procedures have been developed to facilitate derivative formation in a routine analytical laboratory [47–49].

Derivative formation, however, might also be carried out for an additional purpose: to permit the analysis of a particular compound by a selective detector. In most of cases the aim here

has been to increase the sensitivity of the system, by preparing electron capturing derivatives and substituting an electron capture detector for the flame ionization detector; after all the minimum detectable limit of the former is about two to three orders of magnitude better than that of the FID [50].

Probably Landowne and Lipsky [51] were the first who, in 1963, utilized derivative formation to enhance the system sensitivity for compounds containing hydroxyl groups: they prepared the chloroacetate derivatives of steroids which, in turn, could be analyzed with the electron capture detector. The widely used derivatives, the heptafluorobutyrates were introduced by Clark and Wotiz in the same year [52]. Fluoroacetyl derivatives were also prepared for primary and secondary amines [53–56]. The sensitivity for primary and secondary amines can also be enhanced by preparing the 2,4-dinitrophenyl derivatives [56–58].

Aldehydes and ketones can be sensitized for the electron capture detector by forming the respective Schiff-bases or oximes. The preparation of N,N-dimethylhydrazones [59], methoximes [60,61] or benzyloximes [62,63] of steroids are mentioned here as examples.

As mentioned earlier, the referenced investigations aimed to increase the system's sensitivity, by substituting an electron capture detector for the flame ionization detector. By adding an electrophoric group to the original molecule, a one to three orders of magnitude improvement to the minimum detectable amount can be achieved. This is made clear by the data in Table 5; as seen, while the minimum detectable amount on the FID is practically independent of the type of derivative, there is a significant difference for the electron capture detector.

However, derivative formation in combination with selective detectors can also be utilized to selectively detect compounds belonging to certain classes. This technique is extremely important in liquid chromatography [64]; however, it also has important applications in gas chromatography. For example, acylation with perfluorobutyric group would permit selective detection of mono- and diamines and hydroxyl-containing compounds or oxime formation would permit the selective detection of compounds containing carbonyl groups.

The last example was recently demonstrated by Novotny et al. [65] who utilized the nitrogen-selective detector for the analysis of ketosteroids. The steroids were transformed to their oximes and then analyzed on a short glass open tubular (capillary) column. Figure 17 shows the comparative chromatograms; as seen, the peaks of compounds not having a keto group are missing from the chromatogram obtained with the NPD. Similar investigations were reported by Thenot and Hung [66].

TABLE 5. *Minimum detectable amounts of phenethylamine derivatives*[a]

Substance	Formula	FID	ECD
		g/s	
Phenethylamine (PHEA)	C_6H_5-C_2H_4-NH_2	5.1×10^{-11}	2.5×10^{-10}
Trifluoroacetyl PHEA	C_6H_5-C_2H_4-NH-CO-CF_3	5.6×10^{-11}	1.3×10^{-12}
Pentafluoropropionyl PHEA	C_6H_5-C_2H_4-NH-CO-C_2F_5	3.1×10^{-11}	1.5×10^{-13}
Heptafluorobutyryl PHEA	C_6H_5-C_2H_4-NH-CO-C_3F_7	4.2×10^{-11}	2.7×10^{-14}
N-(2,4-Dinitrophenyl) PHEA	C_6H_5-C_2H_4-NH-$C_6H_3(NO_2)_2$	8.4×10^{-11}	5.1×10^{-14}
Pentafluorobenzaldehyde Schiff base of PHEA	C_6H_5-C_2H_4-N=CH-C_6F_5	2.9×10^{-11}	1.1×10^{-14}

[a] Based on the data of Moffat and Horning [56]. The original values were expressed as mols of *derivative* giving a peak twice as high as the noise, divided by the peak width. For the present table, these values were recalculated as amount of *phenethylamine* divided by the peak width.

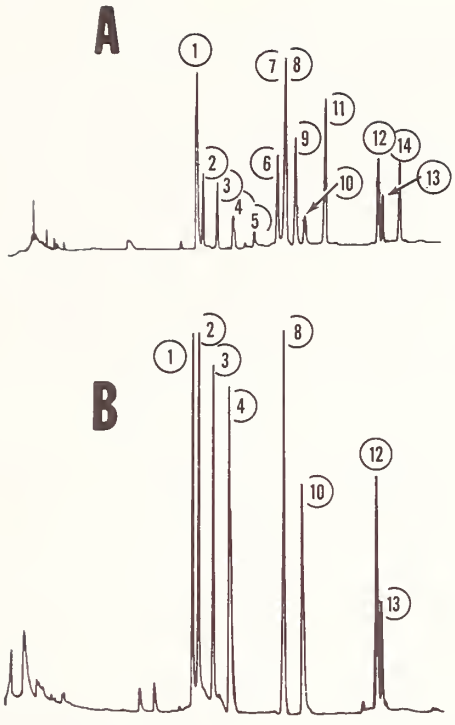

Figure 17. Analysis of a steroid mixture in the form of their oximes on a flame ionization (A) and a nitrogen-sensitive (B) detector [65]. Column: 21 m×0.26 mm i.d. glass wall-coated open tubular, prepared with SE-30 liquid phase. Temperature of injector and detector: 275 °C. Column temperature: programmed from 180° to 255 °C at 1.5 °C/min. The figure represents the 0–50 minutes part of the chromatogram. Peaks: 1–androsterone, 2–etiocholanolone, 3–dehydroepiandrosterone, 4–androstenedione, 5–androstanediol, 6–allopregnanediol, 7–pregnanediol, 8–pregnenolone, 9–pregnanetriol, 10–progesterone, 11–estriol, 12–tetrahydrocortisol, 13–allotetrahydrocortisol, 14–cholesterol.

F. IR/UV Spectroscopic Detectors

All the detectors discussed until now represent classical gas chromatographic detectors, mostly modifications of the basic detectors developed in the second part of the 1950s.[2] They permit the selective detection of a number of compound types, based mainly on heteroatoms in the molecule; the only exception is the electron capture detector where the response is due to the presence of an electron capturing group in the molecule. However, even this brief discussion made it clear that solution to two important problems is missing: they do not permit the analysis of compounds belonging to certain groups, i.e., nitro-compounds or aromatics only, and they do not have special selectivity for oxygen-containing compounds[3] although these represent a very important segment of organic chemistry. Often, it would be very important to selectively identify and analyze e.g., alcohols or ethers only.

The very recently developed IR/UV spectroscopic detectors [67–70] fill this gap: they permit specific group identification, and they can be easily sensitized for the various oxygen-containing groups. Both infrared and ultraviolet are utilized as the principle of detection. The most important difference as compared to earlier extensive work trying to combine molecule spectroscopy with gas chromatography is that now, column effluent is analyzed "on-the-fly," i.e.,

[2] The flame photometric and nitrogen-selective detector represent modifications of the flame ionization detector while the electron capture detector represents modification of the so-called argon ionization detector, a detector fairly popular around 1960.

[3] Selective determination via derivative-forming using the ECD or NPD may be a way to carry out such analysis. However, the problem of having other, unrelated compounds present to which the detector also has a special selectivity still might create problems.

without collection, and instead of scanning it in a given wavelength range, we are looking at it at a preselected wavelength characteristic to certain groups.

Infrared Detector. In the presently available commercial version [71] column effluent is conducted via an electrically heated line to an optical cell with barium fluoride windows, and the desired wavelength (wavenumber) is manually set in the range of 800–4000 cm^{-1}. The ability of this detector to select peaks corresponding to substances having specific molecular structure is illustrated by the example shown in Figure 18 [69]. Here, a peppermint oil sample was analyzed at three different wavelengths; the corresponding flame ionization detector chromatogram is also shown. At 1730 cm^{-1}, the keto-carbonyl frequency, only menthone and iosmenthone are detected; both compounds contain a carbonyl group. In the second chromatogram the frequency was changed to 1250 cm^{-1} where peaks can be obtained for all major components containing a carbon-oxygen linkage. For the third chromatogram, it was further changed to 1060 cm^{-1} to selectively detect menthol.

Selectivity in combination with retention data is a very promising way to identify the individual components of a sample. An excellent example was recently given by Hartigan [70] and his chromatograms are shown in Figure 19. The sample was a chemical sterilizer which smelled "alcoholic." Therefore, a Chromosorb 103 column was selected for the analysis. The FID chromatogram revealed five peaks. The IR detector was first set at 1065 cm^{-1} to look for primary alcohols. The resulting chromatogram revealed three peaks and, based on retention index values, these were tentatively identified as methanol, ethanol and propyl alcohol. It is known that at this wavenumber secondary alcohols have a reduced response as compared to primary alcohols; thus, in order to establish whether n-propanol or isopropanol is present, the sample was rerun at 1120 cm^{-1}, somewhat away of the wavenumber characteristic for primary alcohols. As expected, peak #1 disappeared and peak #2 diminished—after all, both correspond to primary alcohols. On the

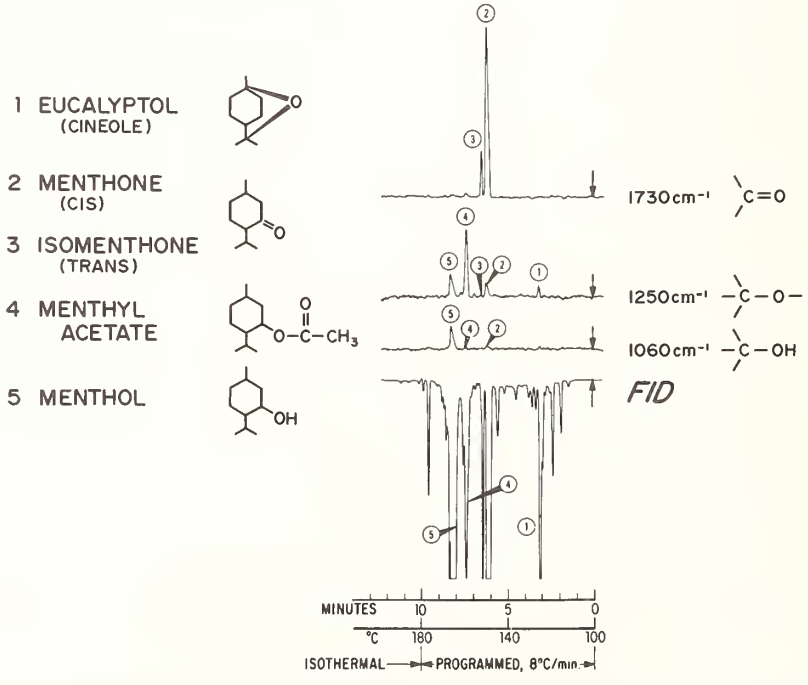

Figure 18. Analysis of a peppermint oil sample with a flame ionization detector and an IR detector, at three wavenumbers [69]. Column: 50 ft×0.50 mm i.d. support-coated open tubular prepared with Carbowax 20M liquid phase. Column temperature: programmed, as indicated. Sample volume: 0.1 μL, no inlet split. Column effluent was split 1:1 to the detectors.

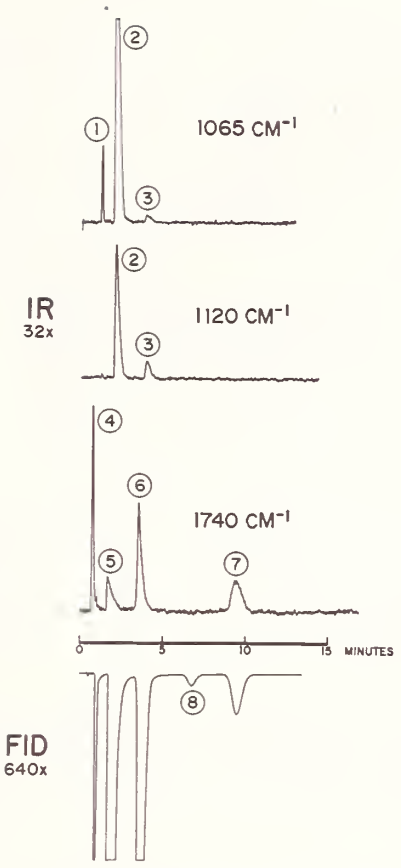

Figure 19. Analysis of a chemical sterilization fluid with a flame ionization detector and an IR detector, at three wavenumbers [70]. Column: 6 ft×2 mm i.d. packed, with Chromosorb 102. Carrier gas (He) flow rate: 30 mL/min. Column temperature: 140 °C. Sample volume: 1 μL. Peaks: 1–methanol, 2–ethanol, 3–propanol-2, 4–water, 5–formaldehyde, 6–acetone, 7–methyl ethyl ketone, 8–2-methyl-propanol-2.

other hand, peak #3 increased in size indicating that it should be the secondary alcohol, isopropanol.

Next, the sample was rerun at 1750 cm^{-1} resulting in a response for four additional peaks. The first of these, peak #4 was assumed to be water; peak #5 buried under peak #2 was formaldehyde; peak #6 coeluting with peak #3 was acetone and peak #7 methyl ethyl ketone. This identification was confirmed by the retention index values.

The only peak which was not yet identified is peak #8 seen in the FID chromatogram. Based on relative retention data this was established as corresponding to tertiary butyl alcohol.

The IR detector, however, is not a "trace" detector: its sensitivity is somewhat limited; it is in the 10^{-7}–10^{-9} g range.

UV Detector. The presently available detector is connected to column exit in a way similar to the IR detector [71]. The optical cell has quartz windows and the wavelength can be manually set in the 190–800 nm range. A good example for its application is shown in Figure 20 [69]. Here, a gasoline sample was analyzed at 250 nm, a wavelength where the detector is essentially insensitive to aliphatic or naphthenic hydrocarbons while providing very strong signals for aromatic substances. This is particularly evident when investigating the front part of the chromatograms. On this column, the low-boiling hydrocarbons are bunched together and unseparated; thus, a very complex "peak" is obtained on the FID in which the benzene peak is obscured. On the other hand, the UV detector chromatogram only shows the benzene and other aromatic peaks.

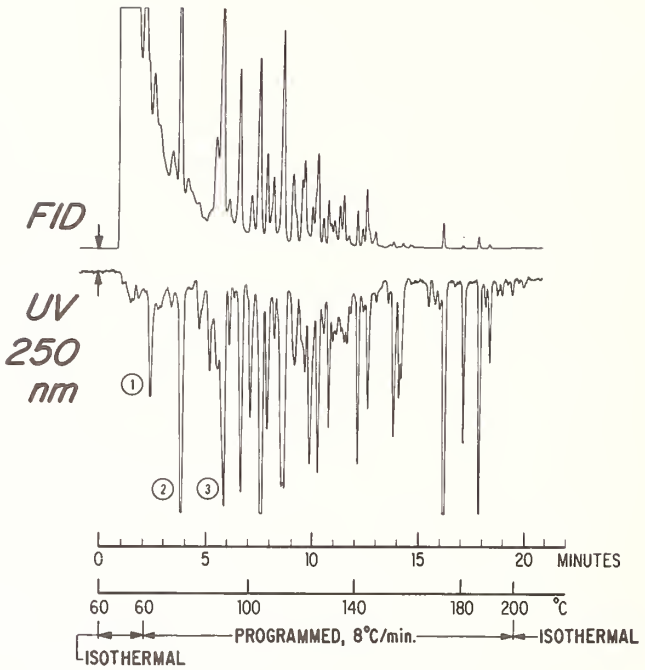

Figure 20. Analysis of a gasoline sample with a flame ionization detector and a UV detector, at 250 nm [69]. Column: 50 ft×0.50 mm i.d. support-coated open tubular prepared with Carbowax 20M liquid phase. Column temperature: programmed, as indicated. Sample volume: 0.2 μL, no inlet split. Column effluent was split 1:1 to the detectors. Identified peaks: 1–benzene, 2–toluene, 3–xylenes.

Depending on the type of compound, the UV detector may show sub-ppm sensitivity.

Both spectroscopic detectors are fairly new and further improvements and applications examples can be expected in the next couple of years.

G. Atomic Absorption Spectrophotometer as a GC Detector

Atomic absorption (AA) spectrophotometry is highly selective and sensitive for metals and thus, it is not surprising that when combined with a gas (or liquid) chromatograph, it provides an excellent way for selective detection of organometallic compounds. A particular advantage is that when analyzing complex samples containing many different chemical species, AA will respond only to the selected metal of interest. Therefore, components which elute simultaneously with the analyte species, but do not contain the metal of interest, will not interfere.

The first paper illustrating the application possibilities of atomic absorption as a metal-specific GC detector was published by Kolb et al., in 1966 [72]. They connected the column exist by a short piece of heated metal tubing directly to the nebulizer of a standard AA system and utilized an air-acetylene flame. In subsequent reports [73–75] column effluent was introduced directly into the burner head using a heated metal transfer tube. Minimum detectable limits were in the sub-microgram range.

In a most recent work [76], the jet of the flame ionization detector was directly used as the burner, to eliminate the effects of dead volumes and improve system sensitivity. However, detailed data are not yet available.

Non-flame atomization systems can improve the analytical sensitivity up to three orders of magnitude. A number of interface systems have been described; generally, a heated metal transfer tube is placed through the sample introduction hole in the graphite tube of the furnace, and the tip of the transfer tube is positioned close to the inner wall of the tube so that the effluent impinges

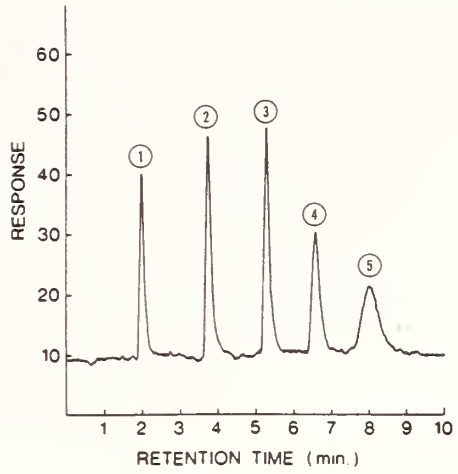

Figure 21. Analysis of alkyllead compounds in gasoline with gas chromatography, using an AA spectrophotometer with a silica furnace as the detector [79]. Column: 6 ft×1/4 in. OD packed with 3% OV-1 on Chromosorb W 80/100 mesh. Carrier gas (N_2) flow rate: 70 mL/min. Injector temperature: 150 °C. Column temperature: 2 minutes isothermal at 50 °C then programmed at 15 °C/min to 150 °C. Peaks: 1–tetramethyl lead, 2–trimethyl ethyl lead, 3–dimethyl diethyl lead, 4–methyl triethyl lead, 5–tetraethyl lead.

directly upon the heated graphite surface [77]. Besides graphite furnaces silica [78,79] and quartz [80] furnaces have also been utilized.

The most typical application of combined GC/AA is the analysis of alkyl lead compounds. Figure 21 shows the chromatogram obtained using a heated silica furnace. Detection limits are in the 0.2–0.5 ng range [79].

A detailed review of the use of AA as selective chromatographic detector has recently been published by Fernandez [81,82].

III. Liquid Chromatography

The second large branch of chromatography is liquid chromatography. Selective detection plays an even greater role here. Actually, even the name of the technique is related to selective detection: Tswett, in his first paper [83], emphasized the fact that the individual zones in the column corresponding to the separated compounds have characteristic colors:

> ...the pigments are resolved, according to the adsorption sequence, from top to bottom, into various colored zones. ... Like light-rays in the spectrum, the different components of a pigment mixture ... are resolved on the calcium carbonate column and then can be qualitatively and quantitatively determined.

This characteristic speciality of a "chromatogram" was also described in detail by the early investigators, e.g., Palmer, E. Lederer and Zechmeister, and many examples can be found in their publications.

Selective detection with help of UV spectroscopy has also been described at a very early stage of liquid chromatography development, pointing out that closely related compounds have differences in the position of their absorption spectra. For example, Winterstein described this for various carotenoids differing from each other only in the position of the double bonds in the two end-groups as early as in 1933 [84].

Today most of the analyses carried out by liquid chromatography are done with the help of selective detectors. There is, however, one basic difference between the selective detectors used in gas chromatography and in liquid chromatography: while practically all detectors used in GC have

been developed for this particular application, in LC the most frequently used selective detectors actually represent existing analytical instruments modified only slightly to permit the direct coupling to the liquid chromatograph. In other words, the theory and practice of these techniques have been developed independently of chromatography and they were only later adapted to this particular usage. For this reason, in the discussion below, I will not deal with the evolution of the individual systems but only illustrate their application possibilities. For detailed information on the basic techniques (UV and fluorescence spectroscopy, atomic absorption spectroscopy) readers are referred to the general textbooks [85–89].

In this paper, only the three spectroscopic detectors—ultraviolet, fluorescence, and atomic absorption—will be discussed. Selective LC detectors based on a number of other principles also exist, such as the electrochemical detectors [90–92] or enzyme detectors [93–95] and naturally, liquid chromatographs can also be connected to mass spectrometers; in fact, this combined technique is very promising and is continuously gaining in importance [96–102].

A. Ultraviolet Detector

Ultraviolet detectors have been used for a number of decades in liquid chromatography. However, for some reason, for long time only the mercury light source with a filter, usually at 254 nm, was generally used. It was only a few years ago that the UV detectors were modified to permit selection of wavelength. It is, of course, well known that almost every compound has a characteristic wavelength where maximum sensitivity can be achieved. This is illustrated in Figure 22 [103]. If only thiamine or pyridoxine is to be determined selectively, work at 324 nm is the best since at shorter wavelengths, thiamine disappears within other unresolved vitamins. However, for ascorbic acid, panthenol, and niacin, one must work at shorter wavelengths.

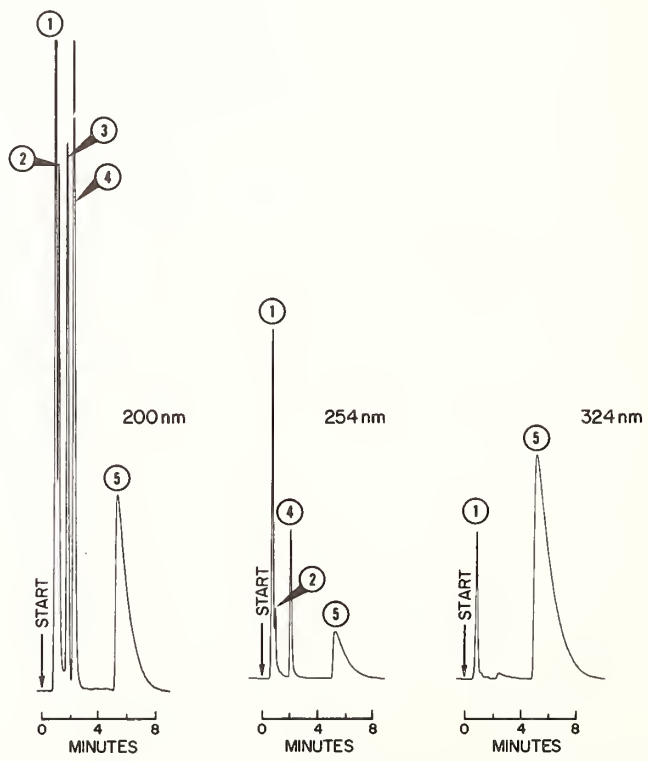

Figure 22. Analysis of vitamins by liquid chromatography, utilizing variable wavelength UV detection [103]. Column: 50 cm×2.6 mm packed with ODS-SIL-X-I. Mobile phase: water, 2 mL/min. Inlet pressure: 500 psig. Column temperature: 65 °C. Peaks: 1–thiamine, 2–ascorbic acid, 3–panthenol, 4–niacin, 5–pyridoxine.

A practical application of the use of variable wavelength UV for selective detection is shown in Figure 23 [104]. Here, a contraceptive pill is analyzed for an estrogenic steroid (steroid #1) and an androgenic steroid (steroid #2). The detectors wavelength is changed during analysis, for two reasons. First, the androgenic steroid does not absorb at 282 nm and thus, a wavelength of 227 nm is needed. Estrogen would have a response at this wavelength and thus, if only the two steroids were present, one could work at one single wavelength only. However, there is also another component extracted out of the glycol matrix which has a high response at 227 nm thereby interfering with the quantitation of the estrogen. Thus, it is convenient to select another wavelength for this compound where interference does not exist.

The other possibility for utilizing variable wavelength UV detection is to use absorbance ratios at several wavelengths [105]. This is particularly helpful in identifying various peaks or investigating whether they represent single compounds or are in fact composite peaks. This technique is particularly useful when stopflow operation is used to permit the determination of absorbance ratios of a peak at several wavelengths.

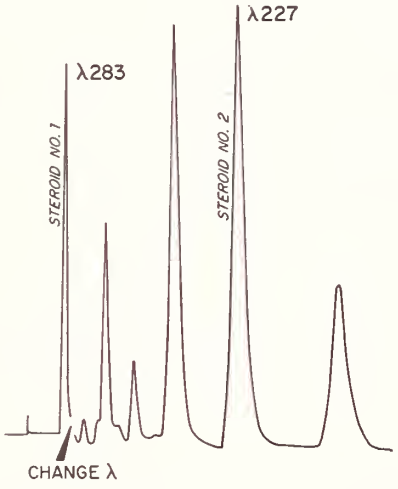

Figure 23. Analysis of a contraceptive pill for steroids, utilizing variable wavelength UV detection [104]. Column: 50 cm×2.6 mm packed with ODS-SIL-X-I. Mobile phase: 94:6 methanol—water, 1 mL/min. Column temperature: 25 °C. Steroids: 1–estrogenic, 2–androgenic.

Table 6 gives the absorbance ratios for a propyl paraben (p-hydroxybenzoic acid propyl ester) peak at four points; the corresponding chromatogram is shown in Figure 24. Three successive sets of ratios were analyzed at three of the points and five sets at point 4. As seen the reproducibility is excellent: the relative standard deviation values are well below 1% making the measurement a practical analytical tool.

How impurities which have a very close retention time and thus, are not separated from the main peak can influence the results is demonstrated in Table 7 and Figure 25 [105]. Here, the peak of niacinamide certainly looks symmetrical and thus, one would assume that it corresponds to a single compound. However, measuring the absorption ratios at four points, different values are obtained clearly indicating the presence of an overlapped peak. In other words, the peak of niacinamide is in fact, a composite peak.

TABLE 6. *Propyl paraben absorbance ratios*[a]

Point	wavelength, nm			absorbance ratio	
	205	210	212	205/210	212/210
	absorbance				
1	0.494	0.443	0.406	1.115	0.916
	0.494	0.443	0.406	1.115	0.916
	0.493	0.442	0.404	1.115	0.914
2	0.932	0.829	0.763	1.124	0.920
	0.929	0.826	0.754	1.125	0.913
	0.926	0.826	0.755	1.121	0.914
3	0.890	0.794	0.725	1.121	0.913
	0.891	0.795	0.727	1.121	0.914
	0.894	0.797	0.728	1.122	0.913
4	0.581	0.519	0.473	1.119	0.911
	0.582	0.521	0.473	1.117	0.908
	0.587	0.519	0.472	1.131	0.909
	0.585	0.522	0.474	1.121	0.908
	0.586	0.520	0.473	1.127	0.910
Mean	0.584	0.520	0.473	1.121	0.913
s.d.[b]	0.0025	0.0013	0.0007	0.047	0.0034
s.d.%[c]	0.44	0.25	0.15	0.42	0.37

[a] For the actual peak, see Fig. 24. From ref. [105].
[b] Standard deviation.
[c] Relative standard deviation (coefficient of variation).

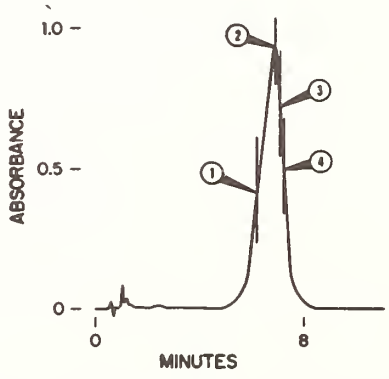

Figure 24. Absorbance ratio measurements to test the purity of a propyl paraben peak [105]. Column: 50 cm×2.6 mm packed with ODS-SIL-X-I. See Table 6 for the measured values.

TABLE 7. *Niacinamide standard absorbance ratios*[a]

Point	Absorbance ratio	
	210/240	260/240
A	2.84	0.90
B	3.11	1.10
C	2.58	0.93
D	1.83	0.70

[a] For the chromatogram, see Fig. 25. From ref. [105].

Figure 25. Absorbance ratio measurements to determine an impurity eluting with a niacinamide standard [105]. Column: 50 cm×2.6 mm packed with ODS-SIL-X-I. Mobile phase: water, 1 mL/min. Column temperature: 40 °C. Detection was done at 240 nm except where absorbance at other wavelengths was measured. See Table 7 for the measured values.

TABLE 8. *Absorbance ratios for drugs in urine vs. values obtained for pure substances*[a]

Drug	Absorbance ratio 205/210	220/210	225/210
Methadone standard	1.32	0.49	0.31
Methadone in urine	1.32	0.49	0.30
Morphine standard	0.90	0.75	0.42
Morphine in urine	0.94	0.67	0.53

[a] For the chromatogram, see Fig. 26. From ref. [105].

A further illustration of this technique is shown in Table 8 and Figure 26 referring to the identification of drugs of abuse in urine [105]. In the table the absorbance ratios of standards—pure substances—are compared with those of the peaks identified in the chromatogram. These data show that the morphine peak is not "clean": some other compound is also eluting at the same time from the urine sample and thus, the morphine peak represents a composite peak.

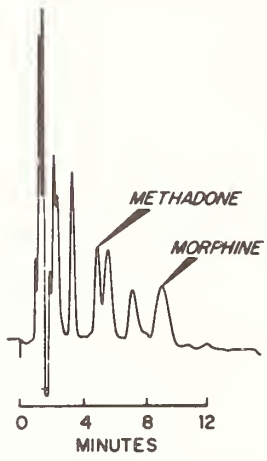

Figure 26. Determination of methadone and morphine in urine by liquid chromatography, utilizing absorbance ratio measurements [105]. Column: 50 cm × 2.6 mm packed with fluoroether SIL-X-I. Mobile phase: 8:92 water—acetonitrile, containing 0.3% H_3PO_4. Flow rate: 2 mL/min. Temperature: 70 °C. Detection was done at 210 nm except where absorbance at other wavelengths was measured. See Table 8 for the measured values.

B. Fluorescence Detector

The second, and probably the most interesting new type of selective detection system for liquid chromatography is utilizing the principles of fluorescence.[4] Fluorescence is often more specific than UV absorption because the appropriate selection of both absorption and emission wavelengths permits similar compounds to be differentiated by a larger variety of physical properties [108,109].

The increased sensitivity of fluorescence detection over UV detection is clear from Figure 27 showing the analysis of polynuclear aromatics [109]. The lower trace is the chromatogram obtained with the UV detector at 280 nm while the upper trace is the chromatogram obtained with the fluorescence detector at the given conditions. By changing the excitation and emission wavelengths some of these compounds can be enhanced while others suppressed resulting in an analysis almost specific for certain compounds; this is illustrated in Figure 28 [109]. By changing the emission wavelength from 395 to 450 nm, coronene is made to show considerable emission while at the same time, the signal for pyrene is considerably reduced. On the other hand, by increasing the excitation wavelength from 338 to 368 nm, signals for pyrene and coronene are suppressed while the signal for perylene is enhanced.

The specificity of fluorescence detection is also demonstrated in Figure 29 showing the analysis of grape juice products for methyl anthranilate, the principal flavorant of Concord grape beverages [110]. The results are compared to UV detection in order to illustrate the inadequate sensitivity and specificity of the latter. The detection limit for methyl anthranilate with the fluorescence detector is about 300 pg, or, assuming a 10-μL sample, 0.03 μg/mL.

Up to now the examples referred to compounds with natural fluorescence. Naturally, one can achieve high selectivity and sensitivity by derivatizing nonfluorescent compounds using fluorescent fluorophors [64]. One of the most frequently used derivatization agent is dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride). A good example of its application is the analysis of amino acids, e.g., the determination of phenylalanine in the serum of newborns in the case of phenylketonuria (PKU). Figure 30 compares the chromatograms of three samples, a blank serum, an actual serum, and a mixture of standards [111]. As seen, the analysis could be finished in less than 12 minutes.

[4] Reports on the use of fluorescence detection in gas chromatography can also be found in the literature [106,107]. The preliminary results in the direct analysis of polynuclear aromatics in air particulate samples are impressive; however, there is not yet enough information available to permit the evaluation of the practical usefulness of such a system.

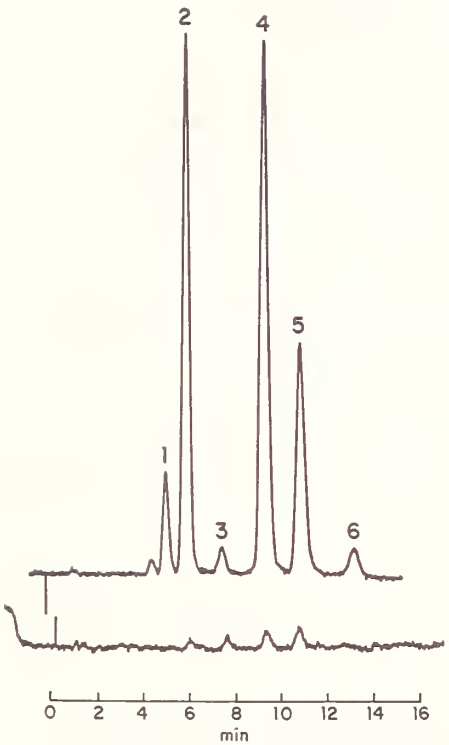

Figure 27. Analysis of a mixture of polynuclear aromatics by liquid chromatography, with UV and fluorescence detection [109]. Column: 25 cm×2.6 mm. Mobile phase: acetonitrile—water, linear gradient from 45:55 to 0:100., in 100 min. Flow rate: 1 mL/min. Temperature: 60 °C. Detector: UV at 280 nm, 0.02 a.u.f.s.; fluorescence at excitation of 338 nm and emission of 285 nm. Peaks: 1–anthracene, 2–pyrene, 3–chrysene, 4–benzo[a]pyrene, 5–benzoperylene, 6–coronene. The amounts present range from less than 5 ng for pyrene to about 0.7 ng for coronene.

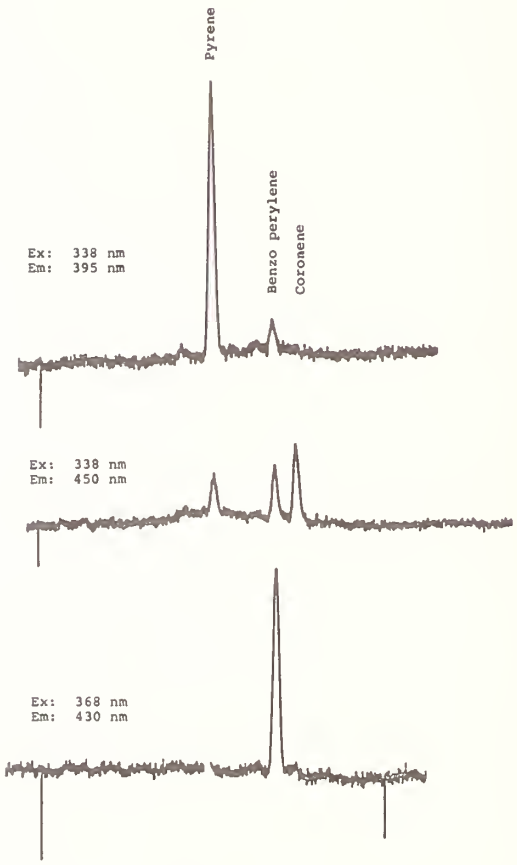

Figure 28. Selectivity for polynuclear aromatics in liquid chromatography, using fluorescence detection [109]. By changing the fluorescence conditions, different compounds in a mixture can be enhanced and others suppressed. Amounts present: perylene 364 pg, pyrene 876 pg and coronene 528 pg. For conditions, see Figure 27.

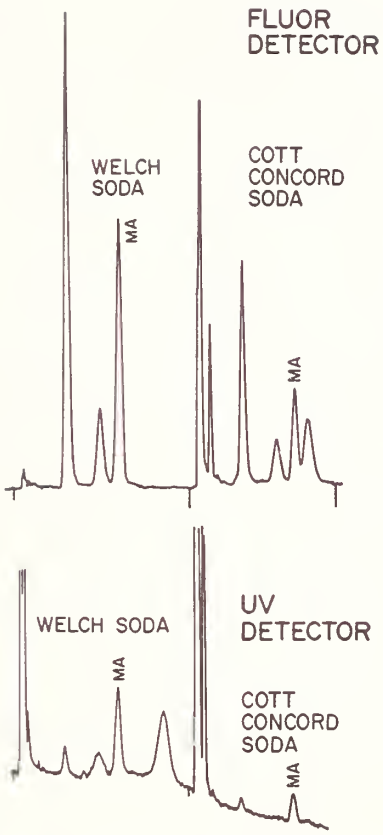

Figure 29. Analysis of grape beverages with liquid chromatography using UV and fluorescence detectors, for methyl anthranilate (MA) [110]. Column: 25 cm×2.6 mm ODS-SIL-X-I. Mobile phase: 3:97 acetonitrile—water, buffered to pH 6. Low rate: 2 mL/min Temperature: 60 °C. Detector: UV at 217 nm, 0.02 a.u.f.s.; fluorescence at excitation of 330 nm and emission of 430 nm. Sample volume: 6 μL. Actual MA concentration values: Welch grape soda, 1.1 μg/mL; Cott Concord grape soda: 3.0 μg/mL.

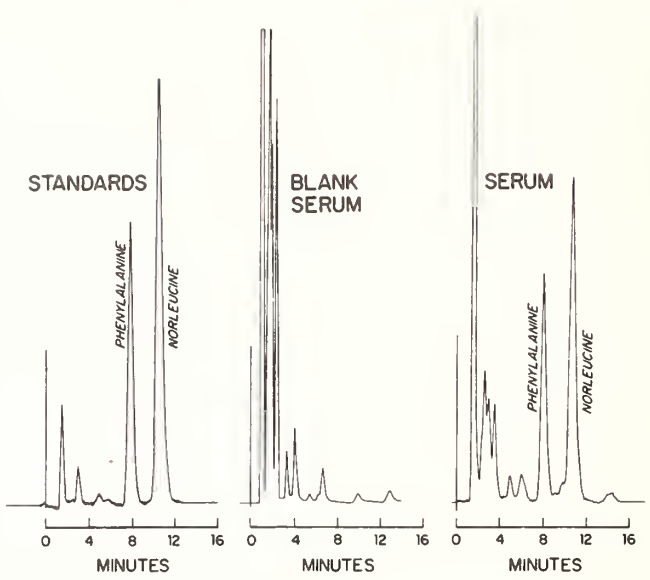

Figure 30. Determination of phenylalanine by liquid chromatography with fluorescence detection, as the dansyl chloride derivative [111]. Column: 25 cm×2.6 mm packed with 10 μm-sized RP-8 (octyl silane). Mobile phase: 50:50 methanol—water containing 0.1% H_3PO_4. Flow rate: 1 mL/min. Fluorescence at excitation of 345 nm and emission of 545 nm. Norleucine was used as the internal standard. Standards: 20 ng each of phenylalanine and norleucine in aqueous solution. Blank: 10 μL of a normal serum, carried through the procedure. Serum: 10 μL aliquot of a PKU serum; calculated amount of phenylalanine: 233 μg/mL.

C. Atomic Absorption Spectrophotometer as a LC Detector

As already discussed in connection with gas chromatography, atomic absorption is a highly selective and sensitive method for the analysis of metals. Naturally, it can also be combined with liquid chromatography, for the analysis of metalorganic compounds.

By attaching a short piece of Teflon tubing to the outlet of the chromatographic column, it is very easy to interface it directly to the nebulizer. It is advisable to approximately match the flow rate of the column effluent to the nebulizer uptake rate; generally, flow rates in the 2–4 mL/min range represent the optimum [112–114]. Figure 31 shows a typical example, the analysis of alkyl and aryl zinc compounds in lubricating oils. In this particular case column effluent was first passed to a UV detector and then directly to the nebulizer of the AA spectrophotometer. It is clear that on the UV detector, other compounds present produce a very large background signal which starts to elute immediately after the alkyl zinc peak. In contrast, when combining LC with AA (left-hand side), the aryl zinc peak can be obtained with no background interference signal.

Although a stopped-flow procedure has been described [113] to interface high-pressure liquid chromatography to a graphite furnace, it is safe to say that the interfacing of a liquid chromatograph with any type of non-flame atomization device is very difficult. The particular limitations are that the total analysis time is long and one does not obtain a continuous chromatogram.

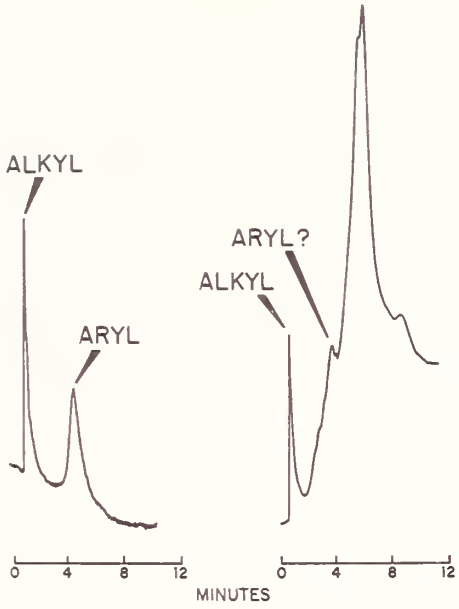

Figure 31. Analysis of alkyllead and arylzinc compounds in lubricating oils by liquid chromatography, with UV (right) and AA (left) detection [114]. Column: 25 cm×2.6 mm packed with ODS-SIL-X-I. Mobile phase: methanol—water, linear gradient from 50:50 to 0:100.

IV. Questions Related to the Use of Selective Detectors

After discussing the various selective detectors utilized in gas and liquid chromatography, I would like to deal briefly with a few selected questions related to the use of such systems. All the examples were selected from the field of gas chromatography; however, the conclusions are equally applicable to liquid chromatography.

A. Multidetector Usage

In many cases, the use of a single detector might give ambiguous or even incorrect results. This is particularly true for complex, natural samples.

The following example (Fig. 32) illustrates the possibility of incorrect interpretation when using a single detector [20]. Let us consider a sample consisting of six pesticides to which a small amount of a polychlorinated biphenyl (PCB) mixture was added. If we use an electron capture detector (which is usually the first choice in pesticide analysis), the resulting chromatogram will show a complex pattern. In the figure, the positions of the six pesticides are indicated, but it is obvious that one could not "find" these peaks in the chromatogram: each is overlapped by at least a dozen PCBs. Thus, we could not determine the six pesticides in the PCB background and we might easily believe that the large number of PCB isomers alone produced this messy chromatogram.

All six pesticides contain in their molecules a phosphorus atom and, in the case of parathion and methyl parathion, also a nitrogen atom. Thus, the thermionic detector selective for N and P containing compounds (NPD) is an ideal choice for their analysis. As shown in Figure 32, the chromatogram obtained is very clear, there is absolutely no response for the PCBs. Therefore, using the NPD alone, we would have no idea about their presence. We need *both* detectors to have a fair idea about the complexity of the sample.

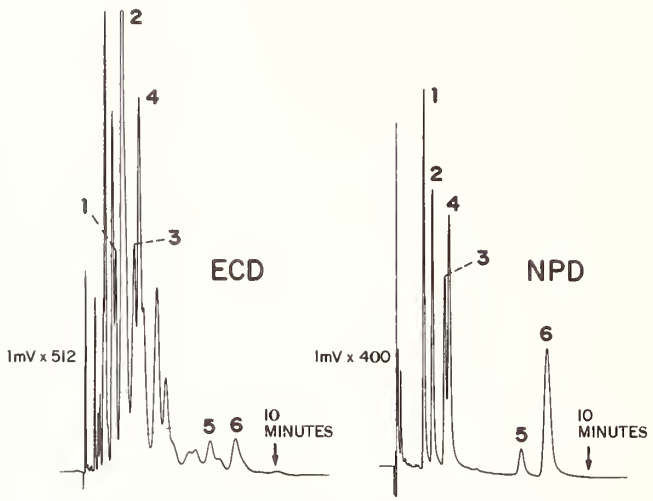

Figure 32. Analysis of a pesticide mixture in isooctane solution to which a small amount of a polychlorinated biphenyl mixture was added, by gas chromatography with two different detectors [20]. Column: 6 ft×2 mm i.d. glass, packed with 3% OV-101 on Gas Chrom Q 80/100 mesh. Column temperature: 190 °C. Sample volume: 1 μL; each of the six pesticides was present in the amount of 5 ng. Peaks: 1–di-syston, 2–methyl parathion, 3–malathion, 4–parathion, 5–methyl trithion, 6–ethion.

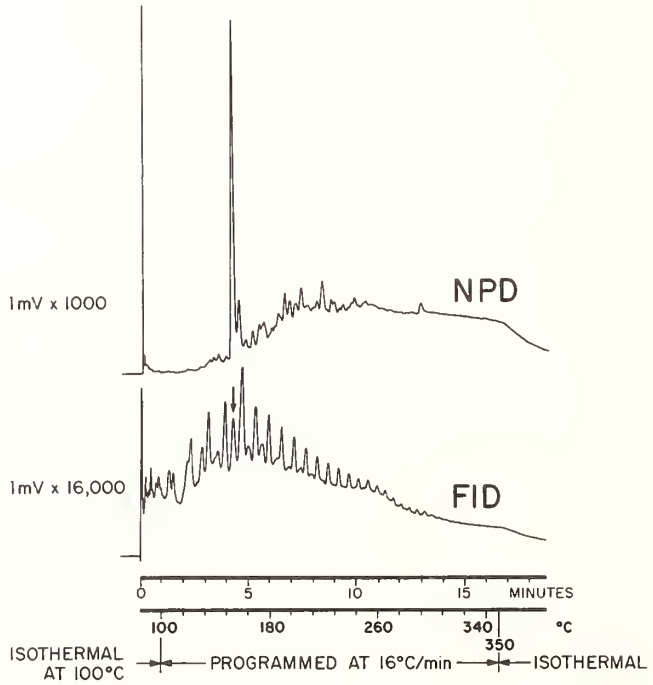

Figure 33. Analysis of a crude oil sample by gas chromatography with two different detectors [20]. Columns: 3 ft×2 mm i.d. packed with 3.8% W-98 silicone oil on Chromosorb W 80/100 mesh. Carrier gas (He) flow rate: 30 mL/min. Column temperature: programmed, as indicated. Sample volume: 0.5 μL.

A very useful combination is represented by the use of a universal detector in combination with a selective detector. In this way, the "fingerprint" of the sample is obtained on the universal detector. Figure 33, for example shows the analysis of a crude oil sample by gas chromatography

[20]. The FID chromatogram is not characteristic but given an idea on the general boiling point distribution while the NPD chromatogram is highly characteristic for the particular sample.

An important reason to use a universal detector together with a selective detector is related to the definition of selectivity. These detectors are not entirely blind to the other components: they only have a much reduced sensitivity for them. For example, if the selectivity of a nitrogen-sensitive detector is given as 1:30,000 for nitrogen compounds vs. hydrocarbons, this means that if the minimum detectable limit is 1 pg, we would need 30 ng of a hydrocarbon to give the same peak size as the 1-pg compound. This naturally means that in trace analysis, it is highly desirable to also analyze the sample under the same conditions with a flame ionization detector, in order to see whether the small peak on the NPD is not the result of some other substance present in a much higher concentration and emerging at the same time. Such an operation can be done easily with most modern gas chromatographs, splitting the flow at column exit one-to-one and connecting the two detectors parallel to each other.

Dual detector use is also advantageous in liquid chromatography; here, however, the detectors might be in series. The previous example on the use of AA in combination with a liquid chromatograph already illustrated this: here the column effluent was first passed through the cell of a UV detector.

B. Dual Column—Dual Detector Systems

Sometimes even with two different detectors one cannot be absolutely sure in identification. The analysis of drugs of abuse is a good example for this: after all, practically all compounds of interest contain nitrogen in their molecules. In such a case selective detection—that means positive identification—can be further enhanced by using columns with different polarities. Figure 34 illustrates the differences in the relative retention values for various nitrogen-containing drugs on a non-polar (OV-1) and a polar (OV-17) liquid phase.[5] Such dual-column systems have been in use at many places for a number of years for the routine analysis of drugs of abuse [115–117].

The use of two columns with different polarities is often prescribed in methods used for pollution studies, in order that a credible, qualitative identification of the individual peaks can be made [118,119]. Such an analysis can easily be carried out by splitting the mobile phase flow 1:1 after sample introduction and using two parallel columns each having a detector at its outlet [19]. Figure 35 shows an example for such a system [120].

C. Selection of Liquid Phase

Every liquid stationary phase used in GC has a vapor pressure and thus, the carrier gas flow is continuously carrying out of the column vapors of the liquid phase; this is the so-called column bleeding. It is obvious that the detector cannot distinguish whether the organic molecules entering it are from the sample or the liquid phase. Since the dynamic range of the detectors is a fixed characteristic, an increased background due to excessive column bleeding will result in poorer minimum detectability.

"Column bleeding" in the sense we speak about it in gas chromatography is much less likely in liquid chromatography; however, it may exist in a different form, e.g., as bleeding of residual monomers from polymeric resin column packings.

Since selective detectors have a higher sensitivity for certain compounds groups they will show an excessive background if the liquid phase (the substance which bleeds off) belongs to such a group. This is, for example, true for the nitrogen-selective GC detector if used together with β,β'-oxydipropionitrile liquid phase. Thus, one should always consider this question when selecting the column to be used in a system incorporating a selective detector.

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[5] Based on data tabulated in ref. [117].

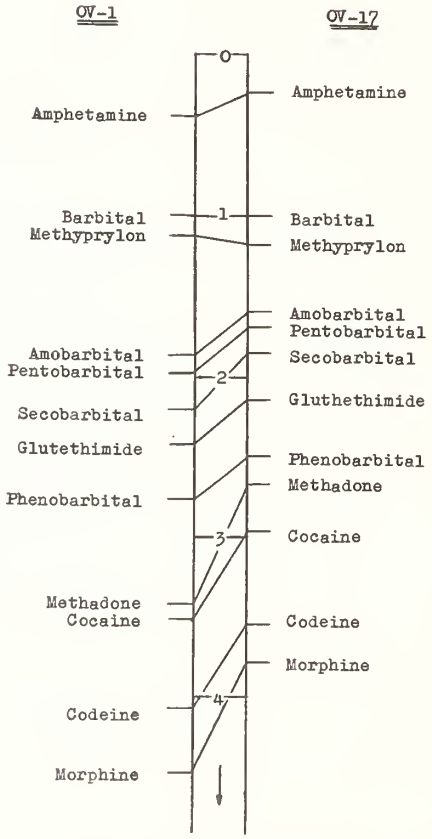

Figure 34. Relative retention values of various drugs on two liquid phases. Standard: barbital. Column temperature: isothermal for 1 min then programmed at 24 °C/min to 280 °C and held there until the end of the analysis.

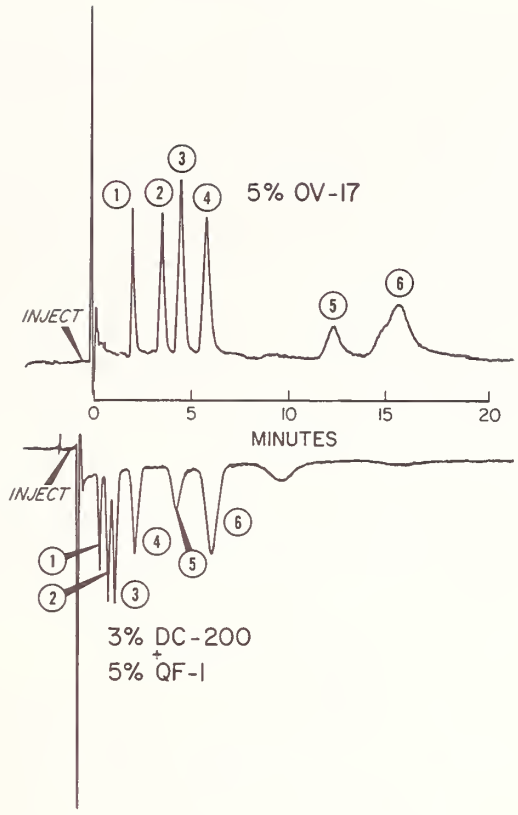

Figure 35. Analysis of a standard pesticide mixture by gas chromatography, with two parallel columns and electron capture detectors [120]. Columns: 6 ft×2 mm i.d. packed with (a) 5% OV-17 and (b) 3% SC-200+5% QF-1, both using Gas Chrom Q 80/100 mesh as the support. Carrier gas: 5% methane in argon, 50 mL/min through each column. Column temperature: 200 °C. Recorder: 1 mV, attenuation×16. Peaks: 1–lindane, 2–heptachlor, 3–aldrin, 4–heptachlor epoxide, 5–*o,p'*-DDT, 6–*p,p'*-DDT.

D. Selection of Solvent

Solvents are used for two purposes in chromatography.[6] The most obvious use is to dissolve the sample prior to introduction into the chromatographic system. It is obvious that one should not use a solvent for which the particular detector has an enhanced response, e.g., a chlorocarbon when using an electron capture detector. However, it is not always obvious that the same rule should also be applied when selecting the solvent used in column preparation, to dissolve the liquid phase prior to coating. Trace amounts of this solvent will remain in the final column packing and it will take a long time until the high background caused by this residual solvent can be eliminated.

[6] In liquid chromatography, the mobile phase is often called the "solvent." This is, however, an incorrect usage.

E. CONTAMINATIONS

The last question to be mentioned is the possibility of introducing unexpected impurities during the process of sample handling. Obviously, impurities in the reagents or reaction byproducts might cause spurious peaks; however, such peaks can also be introduced quite unexpectedly. The following example illustrates this.

In connection with the nitrogen-selective detector the analysis of anticonvulsant drugs in the blood of infants has already been mentioned. During this application development, an unexpected large peak has been observed many times in the samples which clearly could not correspond to any administered drug. This peak was even present in standard serum samples which definitely contained no drug (Fig. 36). It took some time until it could be established that this peak corresponded to tri(2-butoxyethyl) phosphate, a common pasticizer used in the rubber stoppers of some blood collecting tubes which are common in the clinical laboratories of hospitals. Since the nitrogen-selective detector is also sensitive to phosphorus-containing compounds, it responded to this contamination [46].

Figure 36. Analysis of a drug-free serum sample, collected in the standard blood-collecting tube, with rubber stopper, by gas chromatography using two detectors [46]. A 50-μL aliquot was processed and a 2-μL aliquot of the final 10-μL ethyl acetate solution was injected into the gas chromatograph. Attenuations: FID×80, NPD×160. For additional conditions see the caption of Figure 16. The large peak in the NPD chromatogram indicated with an arrow corresponds to tri(2-butoxyethyl) phosphate.

V. Conclusions

The aim of this paper was to give a brief guided tour in the field of selective detectors used in gas and liquid chromatography.

I believe it is safe to say that the most important developments in chromatography in the next couple of years can be expected in selective detection, representing improvements in the existing detectors, further utilization of spectroscopic detectors and also, the introduction of detectors and techniques based on new principles. This is particularly true for liquid chromatography where e.g., further development in fluorescence detection may actually approach the ultimate goal: to be able to specifically detect certain compounds in a sample.

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AUTOMATION OF TRACE ORGANIC ANALYSIS

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As analysts' workloads rise in response to increasing regulation, and as minimum detectable levels of organic substances decrease with improvements in detectors, the need for automation becomes more apparent. Although start-np costs may seem appreciable, the ultimate benefits of automation generally include lower cost per sample and increased precision as human resources (expensive and subjective) are diminished.

The technique of choice depends upon the structure of the analyte and the accompanying interferants. For low molecular weight substances (especially those containing halogen, nitrogen, or sulfur) GC is a likely candidate. And gaining rapidly in popularity is HPLC with its potential for pre- or post-column derivatization to greatly enhance sensitivity via UV or fluorescent tags. Where levels are especially low, instruments are available to perform continuous sample concentration. For larger molecules, the principle of the immune reaction can lead to biological amplification as in radioimmunoassay (RIA) and enzyme-labeled antibody (ELA) schemes, and automated instruments currently exist for both of these very sensitive techniques.

Applications for the automation of trace organic analyses are found in clinical chemistry (e.g., trace drugs in biological fluids), in the food industry (e.g., aflatoxins in peanuts), and in environmental area (e.g., pesticides in water). Examples of such automation will be cited, along with factors which should be considered before undertaking any program to introduce total or partial automation into an analytical facility.

Key words: Automation; clinical chemistry; environmental chemistry; gas chromatography; liquid chromatography.

At first glance, it might seem desirable for all analytical chemists to have an instrument which would accept untreated, unmeasured samples at one end and provide final answers at the other end, without human intervention. Such instruments do exist (for certain analyses) and some will be described herein.

Let us begin with a definition of "trace": it is a minute or *barely detectable* amount. But what was "barely detectable" yesterday may be easily detectable tomorrow, as we see improvements in methodology and/or instrumentation. Substances which were "absent" yesterday (because we couldn't detect them with yesterday's instruments) are present today—which is what Zweig predicted several years ago in his essay "The Vanishing Zero..." [1].

There are several reasons why we may want to automate an analysis or process: the saving of money (by saving time and/or materials, or by reducing or eliminating personnel), or a desire to increase accuracy and precision. Many questions must be answered, however, before automation can be justified:

- Is the work load large enough?
- Will automation be faster? ...more economical? ...more accurate?
- Does "automation" mean "computerization"? If so, computation only, or computation and control?
- Should one automate *all* operations, or leave some for the operator?

Assuming that one has answered all these questions satisfactorily and is ready to undertake a program of automation, he should be mindful of some real disadvantages associated with such a venture:

- It may be very expensive to implement.
- It may be very time-consuming to develop.
- It may be too rigid and non-versatile.
- One faulty part may put the entire system down (if there is no over-ride provision).
- Intermediate results may not be readily available.
- It may not comply with "standard" methods.
- Personnel attitude is frequently somewhat negative.
- Suppliers' quality control must be excellent (expendables cannot be changed significantly).

Now that a long list of questions and inherent disadvantages has been posed, you are perhaps left with the impression that virtually nothing is worth automating. But let's look at it from another viewpoint. We are being confronted with ever-increasing regulations and controls, which means that more testing will be required (with its accompanying higher costs). More tests can be performed by hiring more employees, or by purchasing more instruments in an effort to make each employee more productive. Alternately, one can incorporate automation, and more than likely realize such spin-offs as higher precision in analytical results and a lower cost per test.

What is involved, then, in this trace organic analysis which lends itself to automation? Hertz, et al. [2] have defined seven steps: collection, storage, extraction, concentration, isolation, identification, and quantitation. In this report we'll ignore the first two steps and discuss the *total* automation of the remaining five. The last three (isolation, identification, and quantitation) can often be accomplished by a single techinque: chromatography.

A list [3] of the 32 most popular instruments and techniques representing the responses of over 2500 users, has been summarized in Table 1. It shows liquid chromatography (LC) to be in the number one spot for the third year in a row, and the projected growth rate for LC (nearly 50% for the coming year) suggests its continuing utility. But the other two types of chromatography—thin layer chromatography (TLC) and gas chromatography (GC)—have their place in analytical chemistry, too, though perhaps with less potential for full automation.

TABLE 1. *Instrument survey*

Rank				% who	Growth rate (%)	
78	77	76	Instrument**	have	Total	New
1	1	1	LC	28	49	30
2	2	2	ISE	33	30	13
3	6		Particle size anal	16	30	20
5	3	7	Mass spec	18	25	15
6	10	6	GC	49	24	7
8	5	4	Auto wet chem	14	22	12
11	19	13	Polarography	10	20	11
15	11	17	NMR	14	17	8
19	16	14	Atomic abs	30	15	7
28	28	27	TLC	26	10	6
32	32	30	Spectro (vis)	51	7	2

*From 2570 respondents.

**From 32 different types.

Reference: *Res/Dev* (Feb.) 24 (1978).

Several years ago the J. T. Baker Company introduced an automated instrument [4] to do continuous TLC on a moving strip of 35 mm film. Apparently the instrument tried to perform too many operations at once, because it failed to gain a place for itself in the market. Automatic plate spotters [5] which could load several samples at once, with precision, handled only a single step; being far simpler units, some made it to the marketplace.

The latest in TLC automation is the Iatroscan [6], introduced in the United States at the 1977 FASEB meeting in Chicago. This device handles ten quartz rods coated with a refractory material which can be treated much like a TLC plate. After development, these rods are moved singly thru a flame where the separated bands are pyrolyzed and the trace organics quantitated via a flame ionization detector. Again, partial automation seems to be more successful than total automation, in the case of TLC.

Let us now examine GC for trace organic analysis. Several years ago it was recognized [7] that GC could be far more useful if (a) sample preparation were automated, (b) it were *much* faster, and (c) a dynamic interface existed. Ideally, one would begin with his unmeasured, untreated sample and have an automated sample preparation module feed a high-speed GC thru an on-line, dynamic interface. This doesn't mean merely automatic injection, such as can be accomplished by several available attachments, but rather some apparatus which precludes any manual intervention—some collection of modules which is completely automated.

Such a fully automated instrument is shown in Figure 1, and was constructed for the analysis of drugs of abuse in the urine of addicts. The general requirements for an instrument to analyze for traces of amphetamines, barbiturates, and narcotic alkaloids were (a) separate GC columns for each drug group, so no compromises would have to be made, (b) isothermal operation for fastest operation, and (c) a clean sample, so columns would have an acceptable lifetime.

In operation, urine samples (unmeasured and untreated) were aspirated from a single sampler and divided into two streams, where appropriate clean-up cartridges handled acidic and basic drugs separately. The stream from the acidic drug cartridge went to one GC column, whereas the stream from the basic drug cartridge was further divided for injection into two different GC columns. All three columns ran in parallel.

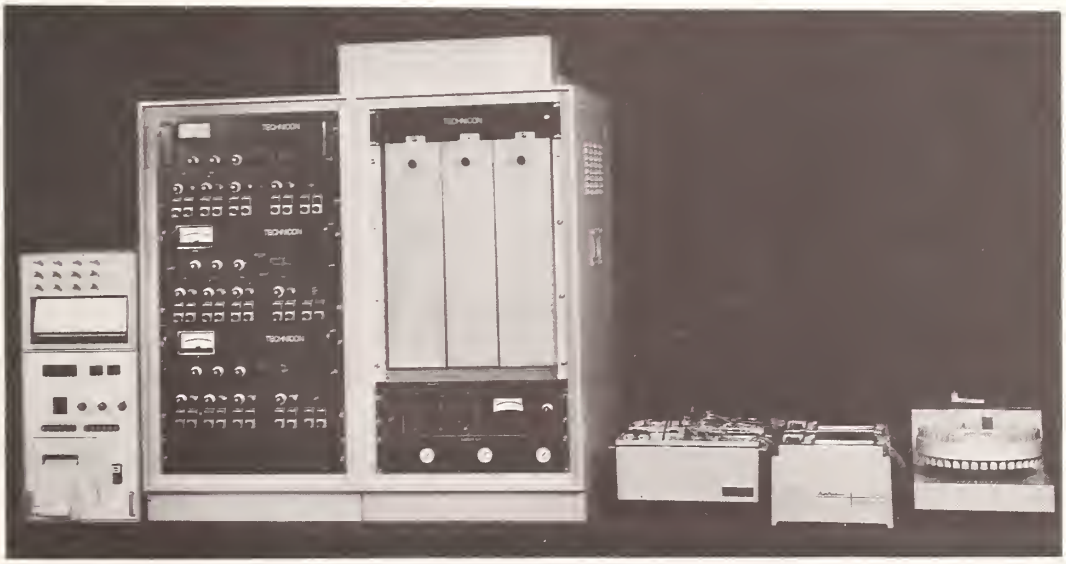

Figure 1. AA/GC (AutoAnalyzer/Gas Chromatograph) combination.

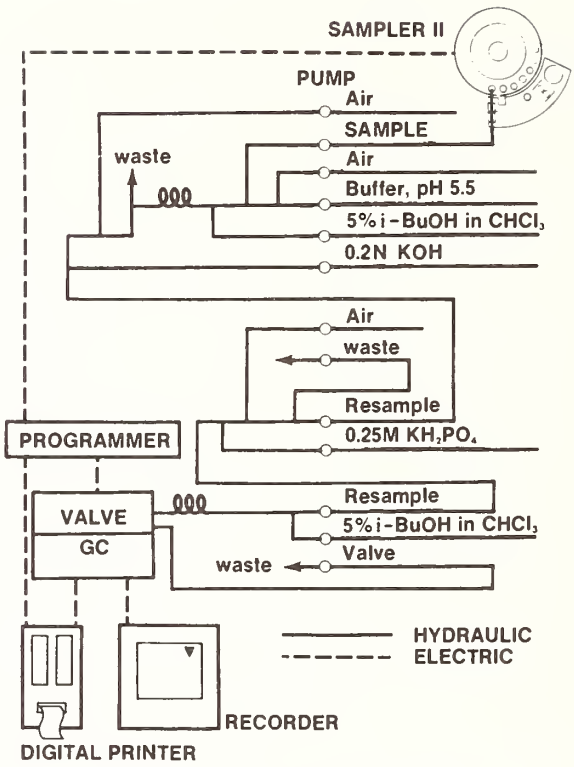

Figure 2. Barbiturate cartridge for AA/GC.

The two clean-up cartridges were identical, except for pH's. The barbiturate cartridge is shown in Figure 2, wherein a multi-channel peristaltic pump directs fluids thru mixing coils, extraction coils, and phase separators. Detection was possible at a few micrograms per milliliter. Further details can be obtained from the reference; suffice it to say here that three extractions were accomplished, serially, at different pH's, to get rid of interfering substances, and the cleaned up sampled was pumped thru a special syringe which permitted programmed injection into the appropriate GC column.

Now let's take a look at LC, or more specifically high performance LC (HPLC) where total automation has also been achieved. Again, we're not talking about instruments with sophisticated electronics for data reduction and/or control, which still require manual sample preparation, but rather instruments which accept samples which haven't been subjected to labor-intensive pre-treatment.

To start at the beginning, chromatography can be broken down into its five essentials:

- Sample,
- Pre-treatment, to make sample chromatographable,
- Injection into column of choice,
- Separation of sample components on column, and
- Detection & readout (quantitation).

Let's elaborate briefly on the first two. The sample may be a liquid (such as a biological fluid, a beverage, or waste water) or it may be a solid (such as pharmaceutical tablets, pieces of tissue, samples of soil, or food). Depending upon what the sample is, the pre-treatment may be as simple as diluting it, mixing, and taking an aliquot. If it's a solid, it may require disintegration and dissolution. If there are insoluble particulates, they may be removed by filtration; soluble

excipients may be removed by dialysis. Sometimes we must extract the material of interest out of its aqueous matrix into an organic solvent. Extractions may be single, double, triple—whatever is needed to rid ourselves of those interfering components which can prolong a chromatogram. Sometimes it is even necessary to evaporate a solution to dryness and take up the residue in another solvent. *Every one of these steps has been automated.*

The simplest HPLC system must contain a column, followed by some sort of detector and readout, and preceded by a sample valve and pumping means for the mobile phase. By adding a sampler, an actuator for the valve, a pump to aspirate samples thru the valve's loop, and a timer to synchronize sampler and valve actuator, we have the elements of a fully automatic system. But it has limitations:

- The sample must be a liquid,
- The liquid must be aqueous (or high-boiling organic), and
- The sample must require no pre-treatment.

It is well known that sample preparation represents about 75% of the chromatographer's effort. This problem has been addressed in several ways: Waters [8] has introduced Sep-Pak™—a cartridge approach to sample cleanup, and ChemResearch [9] offers a sample processor which can perform many of the usual cleanup operations. Samples resulting from either of these cleanup procedures can then be placed in special turnable [10] for automatic transfer to an HPLC system.

An alternate approach avoids these manual steps by performing the cleanup continuously, on-line, as diagramed in Figure 3. The use of a multi-channel peristaltic pump permits such operations as (a) dilution of the sample to the proper concentration range, (b) addition of a buffer for the optimum pH for greatest extraction efficiency, (c) use of an internal standard for peak height rationing, (d) on-line reactions to form derivatives, or any combination of these operations.

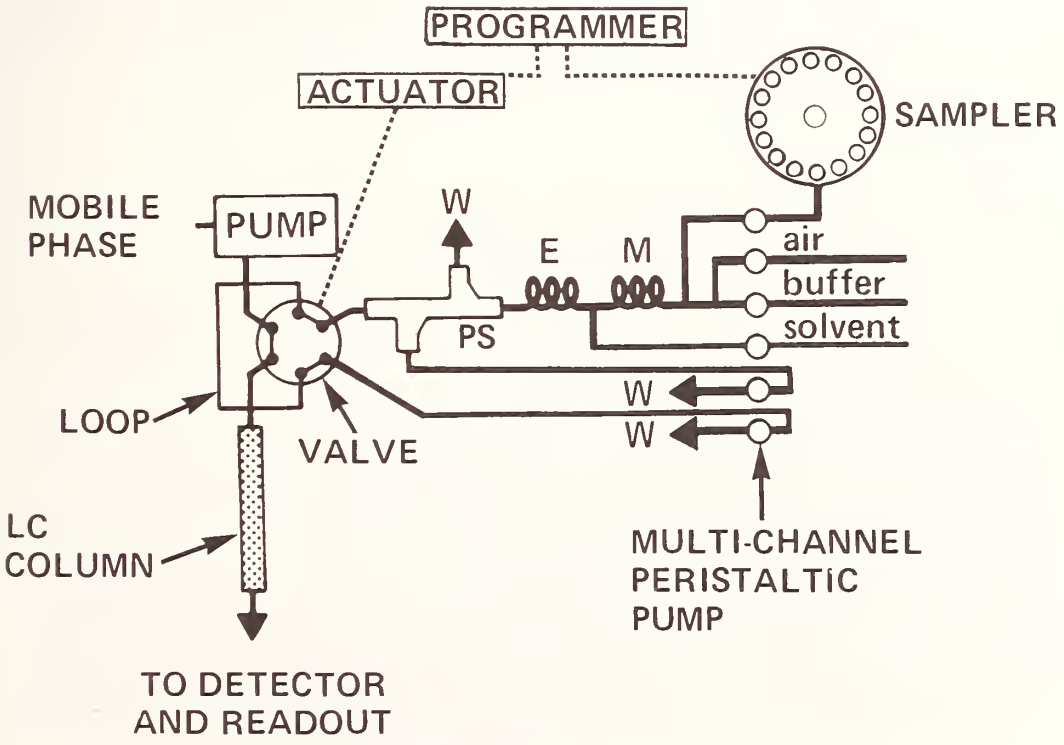

Figure 3. Simplest automated HPLC for solvent extraction front-end.

When the best solvent for extracting a material from its native matrix is not the best solvent for introducing the sample into a column, a solvent exchange can be performed on an Evaporation to Dryness Module (EDM). Diagramed in Figure 4, the EDM consists of an inert matrix of circular cross section, wound around two pulleys, and threaded thru a glass evaporator tube. The evaporator tube has a side-arm going to vacuum, so there is a continuous flow of air thru the tube, over the surface of the inert matrix. The sample (in the original solvent) is pumped onto the matrix at a point where the fast-moving air causes it to flow in a sheath stream over the surface of the matrix. About halfway down the evaporator tube, all the solvent is gone, and the residue is carried out of the tube to the take-up station where it is re-dissolved in a second solvent.

The EDM can also be used to concentrate trace levels of substances so they can be analyzed without special detectors. By stopping the matrix for 3 minutes and letting the solution evaporate on the right-hand end of the matrix, one can build up a sizeable residue. The matrix can then be restarted and passed through the take-up station in 0.3 minute. If the solvent flow rates are the same, then a tenfold concentration improvement is obtained. By combining higher solvent ratios with the stopping of the matrix, samples can be concentrated even more. This should prove particularly useful to pesticide analysts where trace levels are generally encountered.

Provision has been made on the EDM to heat the air which is drawn into the evaporator tube, to substitute an inert gas for air, to filter the air to remove UV-absorbing components, and to protect the sample from light by using its opaque cover.

Many of the modules described so far have been combined into a working system for the anlysis of trace organics in biological fluids. Figure 5 shows an analyzer for determining therapeutic levels of theophylline in serum at the rate of 20 samples per hour. Only 50 μL of serum is required, and concentrations as low as 1.0 μg/mL can be detected with CV's less than 2 percent.

The flow diagram for this system is depicted in Figure 6. On the left are the modules for automated sample preparation: sampler; pump for adding buffer, internal standard, and organic solvent; cartridge for extraction and phase separation following deproteinization; and EDM for solvent exchange and concentration. The right-hand portion shows the HPLC pump, automatic injection valve, column, UV detector, and data handler. A typical chromatogram is reproduced in

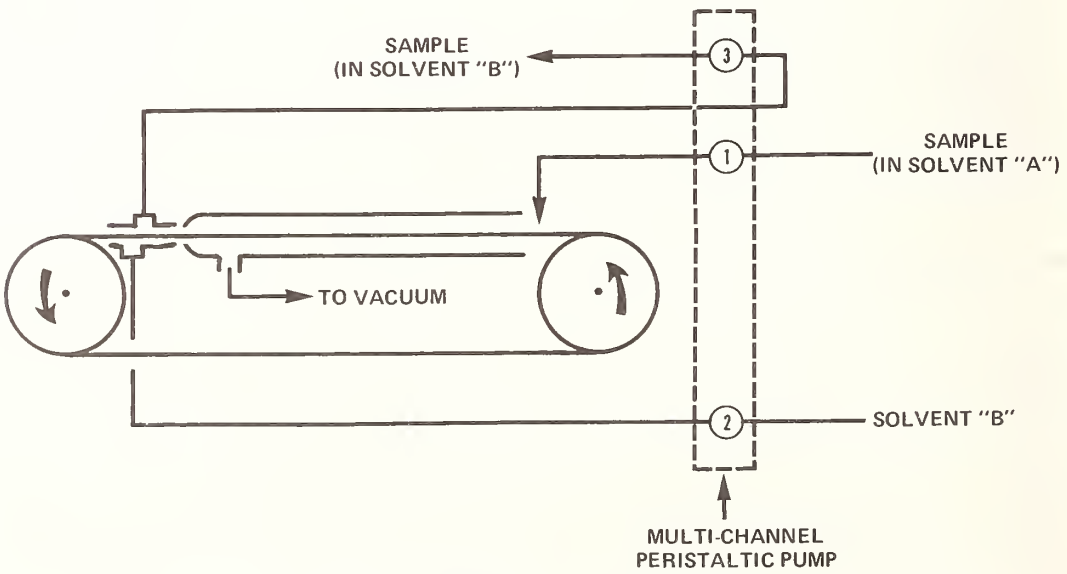

Figure 4. EDM (Evaporation to Dryness Module).

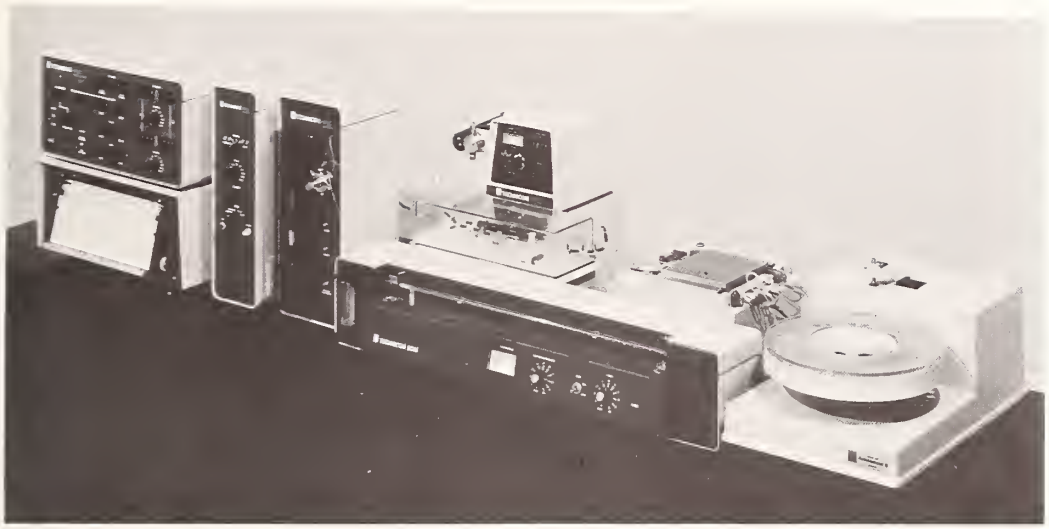

Figure 5. Theophylline analyzer.

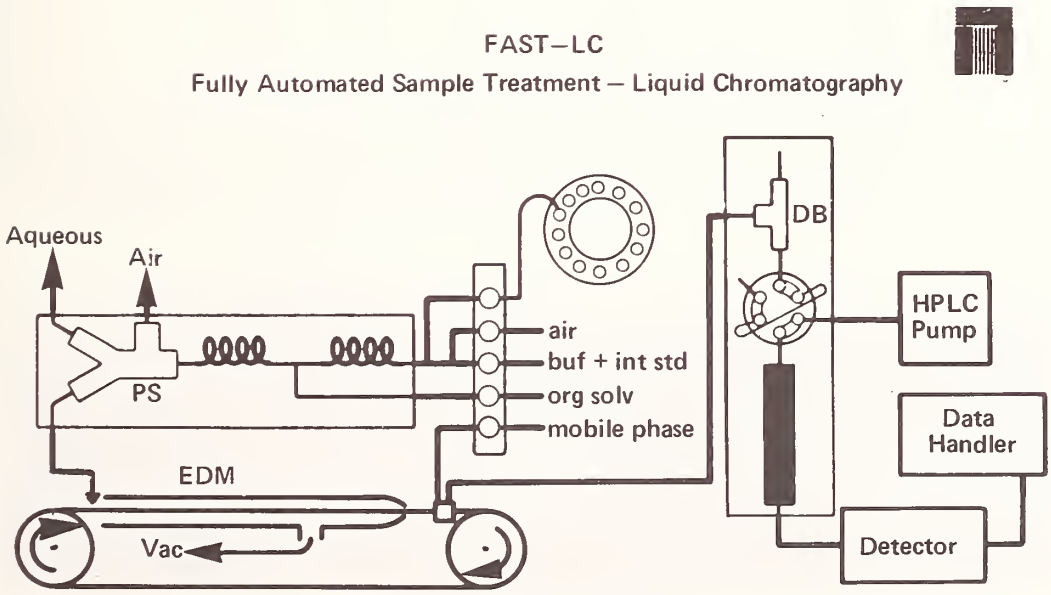

Figure 6. Theophylline analyzer.

Figure 7, and shows both the drug peak and its internal standard, the ratio of which is a measure of theophylline concentration.

When solid samples are to be analyzed, a different front-end is required for HPLC analysis. Vitamin tablets, for example, must be disintegrated and suspended in a liquid prior to extraction. The fat-soluble viamins A, D, and E present a real analytical problem, partly because of the very low level of vitamin D (10 μg per tablet, dispersed in 35 mL of solvent, provides less than 300 ng/mL) in the presence of the other two vitamins at levels which are higher by three orders of magnitude.

A fully-automated vitamin system is shown in Figure 8, with a SOLIDprep™ sampler at the right for handling a tray of 20 tablets without operator involvement. The programmer (at the left, on top of the strip chart recorder) controls all timing functions, including range changes to permit readable displays of all three chromatographic peaks, as reproduced in Figure 9. This system has

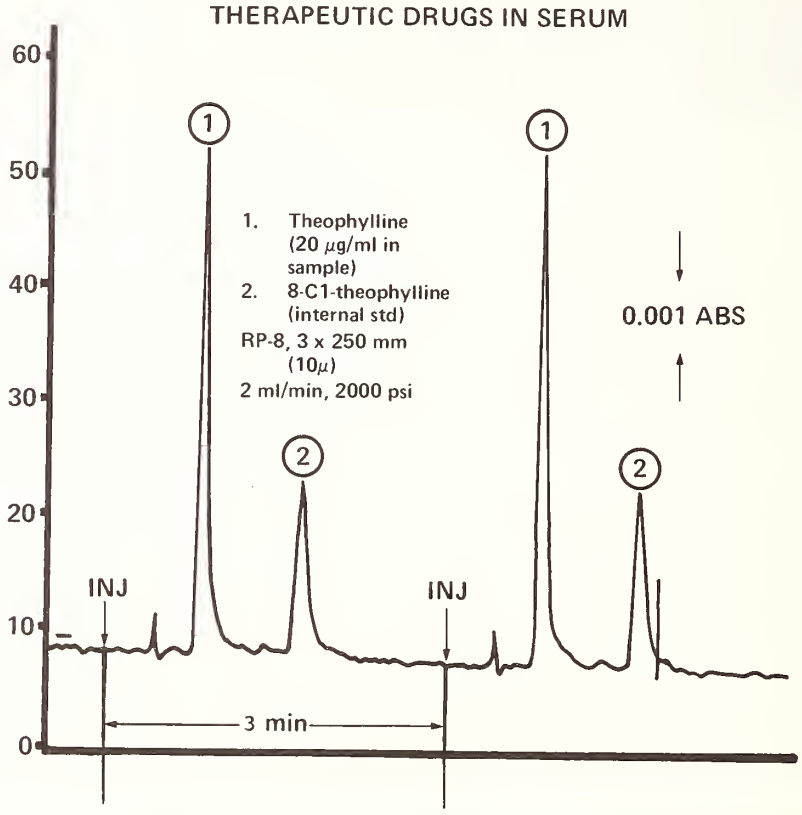

Figure 7. Theophylline chromatogram.

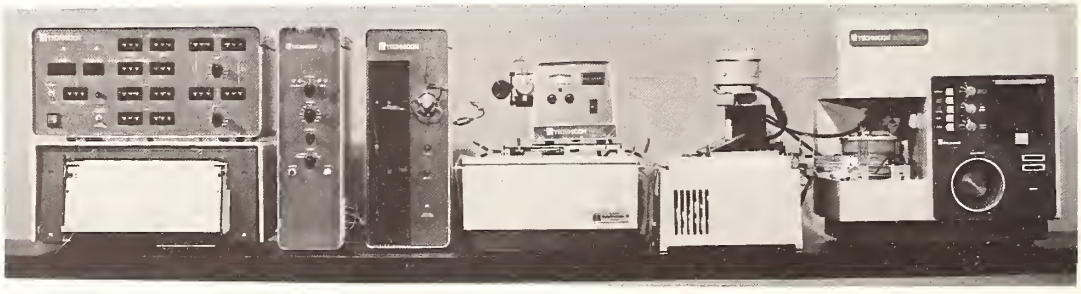

Figure 8. Fat-soluble vitamin analyzer.

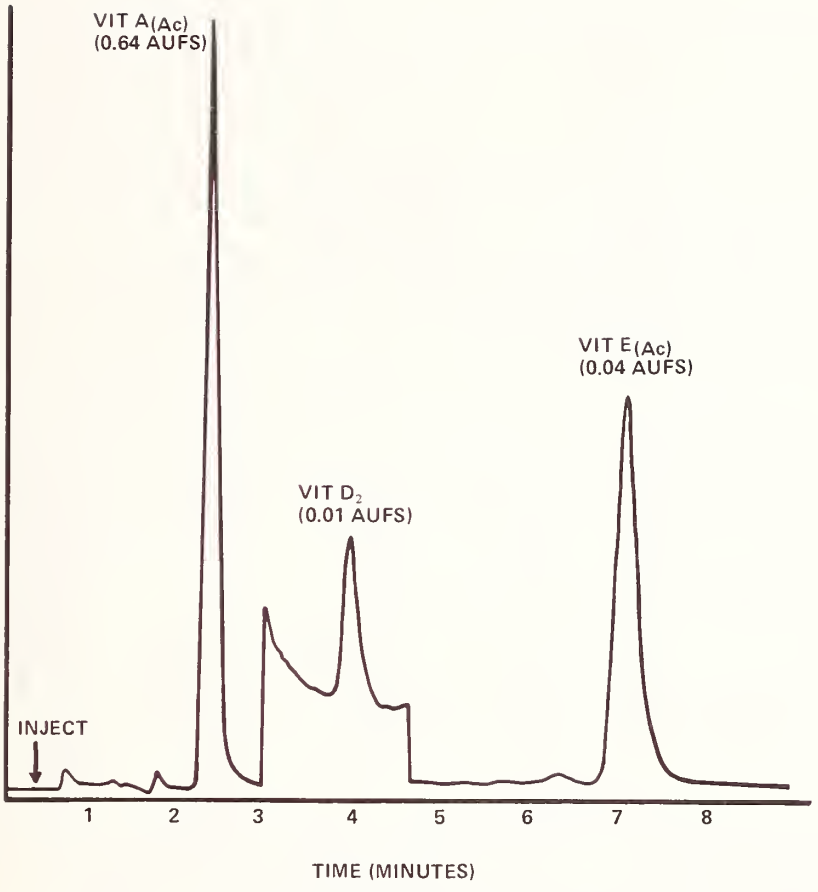

Figure 9. Vitamins A, D, and E chromatogram.

been described in more detail elsewhere [11,12], and Karger, et al. [13] has addressed the avoidance of interferences associated with the low levels of vitamin D_2.

Derivatization has always played an important role in analytical chemistry. There are at least three reasons why one might wish to make derivatives:

- To allow chromatography,
- To improve separability, or
- To enhance detectability.

Users of GC are well aware of the first reason, since many analytes aren't chromatographable otherwise. And except for optical isomers, the second reason isn't generally applicable. But the trace levels of organics encountered today often require us to use every trick in the book to lower the limits of detection. Depending upon what functional groups are available, there are many reagents which can be used to add UV absorbance (as shown in Table 2), fluorescent tags, electrochemical handles, etc. Many of these reactions lend themselves to the continuous-flow concept often employed in automated sample pre-treatment, and we are likely to see ever increasing numbers of on-line derivatizations, both before and after the column. (Frei [14] is a leader in this technique, and his book is highly recommended.)

TABLE 2.

Functional group	UV-absorbing derivative
carboxyl	p-nitrobenzyl ester
	p-bromophenacyl ester*
hydroxyl	3,5-dinitrobenzoate
amino	p-nitrophenylacetamide
carbonyl	p-nitrobenzyloxime

*With crown ether catalyst.

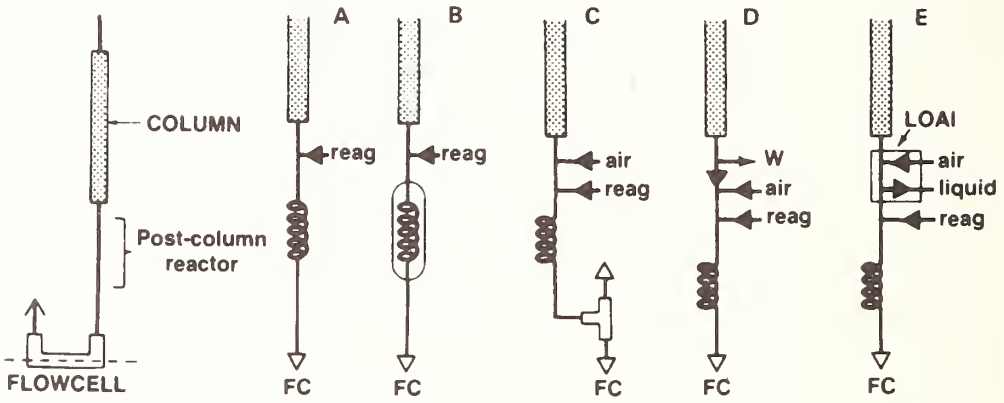

Figure 10. Post-column reactors.

Post-column reactors, as the term implies, are somewhere between the column and the flowcell (or whatever detector is used). There are many embodiments of post-column reactors, and a few configurations are shown in Figure 10. The first is merely a coil long enough to permit a reagent to react sufficiently. The second version is identical to the first, except the coil is placed in a jacket, either to raise the temperature to expedite a reaction or to control the temperature for temperature-sensitive reactions.

If a reaction is going to last any appreciable time, one can avoid loss of resolution by inserting bubbles before the reagent addition, as shown in the third version. Once bubbles are in, they must be removed with a debubbler (as shown), or electronically ignored as they pass thru the detector. A fourth version permits part of the column effluent to go to waste, or to another parallel detector, or to a fraction collector for other analytical purposes. And for the purist who doesn't want the addition of air to perturb his system, there is a LOAI filling (LOAI=Liquid Out–Air In) which permits one to exchange some of the liquid for the air bubble without changing the overall volume or flowrate.

Let us now consider another approach to trace organic analysis. The immune reaction is an extremely sensitive one in which antigens (A_g) and antibodies (A_b) combine in a manner which permits the measurement of either one or the other. One way of detecting trace levels of an A_g, is to dilute the unknown level with a known level of a radio-labeled A_g, let both compete for an A_b,

then separate the free from the bound form. This is called Radio Immuno Assay (RIA), and these are the steps which must be performed:

- Pipet sample, standard, radiolabel, and A_b,
- Incubate,
- Separate free and bound forms,
- Count radioactive bound A_g with scintillation detector, and
- Calculate isotope dilution (permitting the analysis of *very* low levels of the unlabeled A_g).

Manually, this would be accomplished batch-wise in test tubes, and the separation step could be very time-consuming. However, automation can be achieved by wrapping magnetic particles in a material to which the A_b can be attached. One can then add this magnetic A_b solid phase to a mixture of unknown sample and known label, allowing sufficient time in mixing coils for the required incubation of the competing A_g's.

The actual separation can then be accomplished by electromagnets placed around the transmission tubing, as shown in Figure 11. The solid phase (after combining with the competing A_g's) is retained by the magnetic field, while unbound A_g passes to waste (Fig. 11-A). After separation has taken place, the bound portion is released and flows thru the scintillation detector for counting (Fig. 11-B).

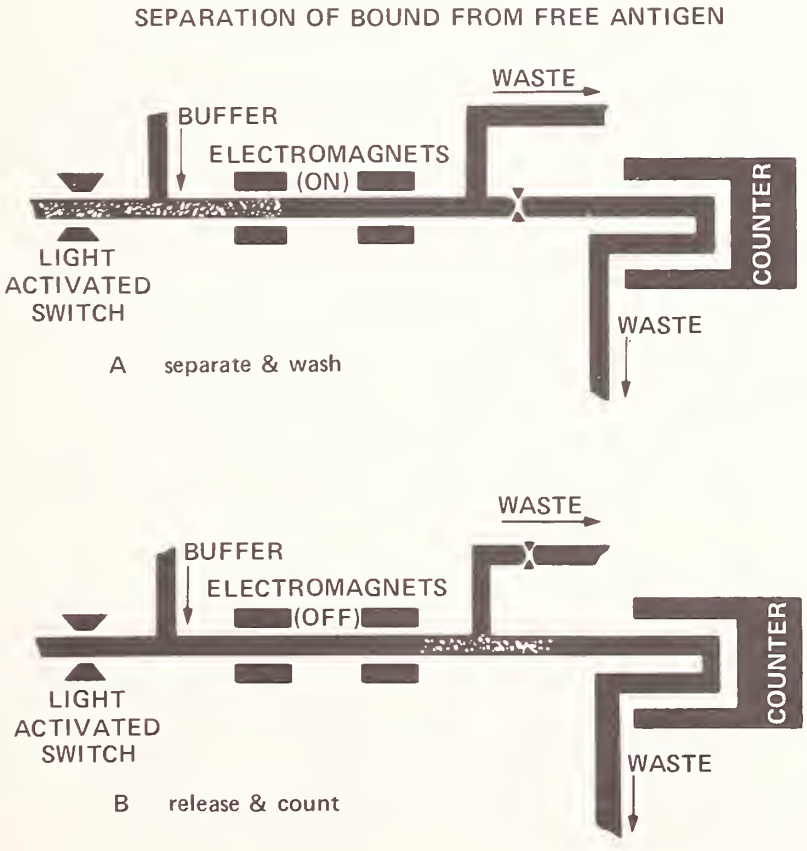

Figure 11. RIA (Radio-Immuno Assay) system diagram.

The automated RIA can operate at 30 samples/hour, without human intervention. Cohen and Stern [15] have described its use for thyroxine (T_4) and a host of other immunoassays such as triiodothyronine, cortisol, and digoxin. The system is capable of recoveries averaging 100%, with CV's under 8 percent. With its microprocessor to analyze the data produced, this is another fully-automated instrument for trace organic analysis.

A final example involves an enzyme reaction. The classical way to depict such a reaction is

$$E + S \leftrightarrow ES \rightarrow E + P$$

where E=Enzyme, S=Substrate, ES=Enx/Sub complex, and P=Product. This can be combined with the immune reaction as follows:

$$\begin{array}{ccc} A_g + A_b \rightarrow A_g/A_b & & \\ \quad + & & \\ \quad \text{Enz} & & \\ \quad \downarrow & & \\ \text{Substrate} & A_g/A_b/\text{Enz} & \text{Product} \\ \text{(colorless)} & \rightarrow & \text{(colored)} \end{array}$$

where the A_g/A_b/Enz complex is capable of catalyzing the conversion of some colorless substrate to a highly colored product.

In the automation of such a series of reactions, we begin with a batch of test tubes to which we've attached an A_g to the inner wall (see Fig. 12). We can then pump in a test serum and let it incubate for several minutes. If there are A_b's present, they will bind to the A_g and will not be carried away during the subsequent washing step. The enzyme is then added (labeled "conjugate" in the figure), and it likewise binds to the A_b, building up a sort of sandwich—test tube wall/A_g/A_b/Enz. After another wash, the substrate is pumped in, and it will be enzymatically

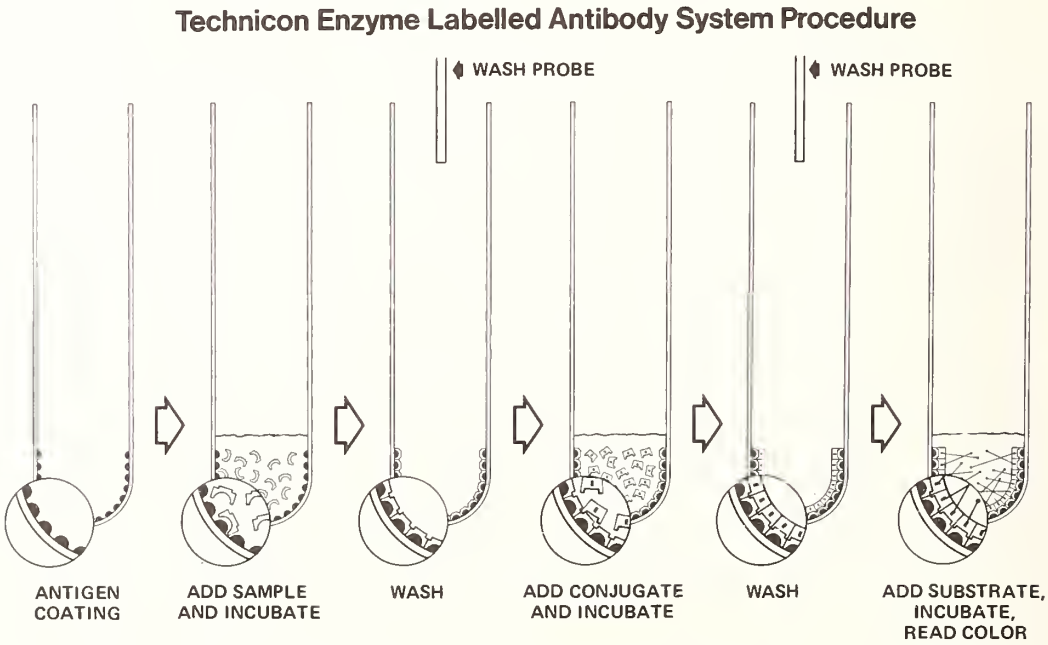

Figure 12. ELA (Enzyme-Labeled Antibody) principle.

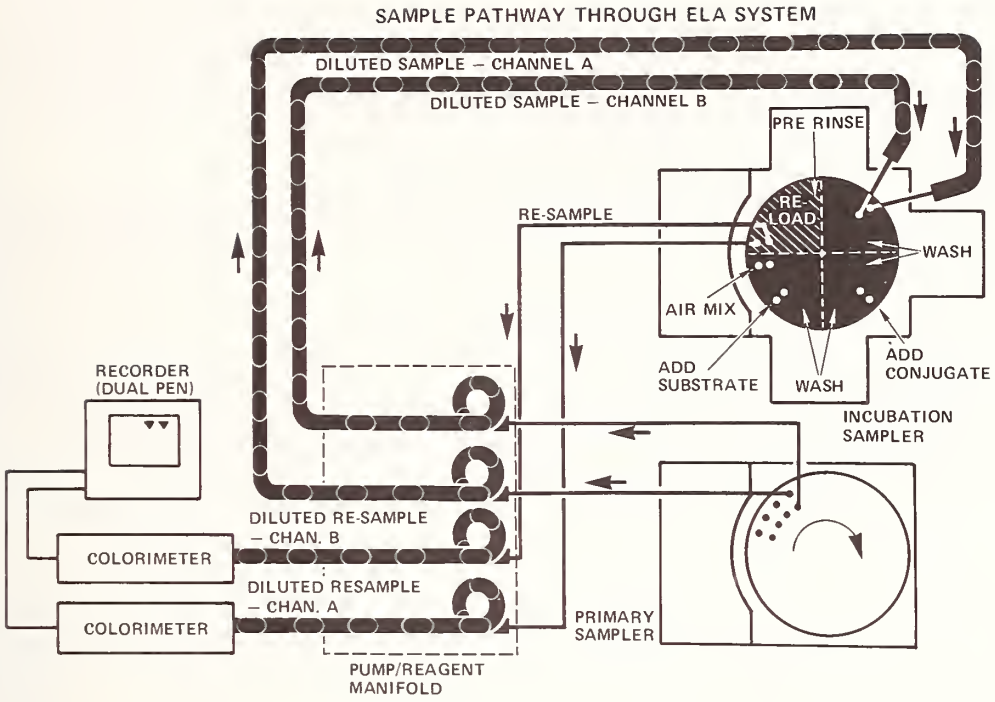

Figure 13. ELA flow diagram.

converted to a colored product if there is any Enz, which is dependent upon there being some A_b, which is what we're testing for in the first place.

This scheme, known as Enzyme-Labeled Antibody (ELA), has been used successfully for the early detection of hog cholera, trichinosis, and (to a limited extent) brucellosis. Outbreaks of these diseases have caused serious economic losses to the swine and cattle industries in recent years.

Figure 13 is a diagram of the special incubation turntable with its four stations (to handle the four operations of sample addition to A_g-coated tubes, washing and addition of Enz, washing and addition of substrate, and removal of product for colorimetric analysis). Double probes, operating on two rows of A_g-coated tubes, permit analytical rates up to 300 samples/hour. With its microprocessor and printer, the instrument can give on-site answers regarding the disease state of test animals 20–45 minutes after sample addition—a rate which is faster than manual methods by more than an order of magnitude. Other configurations of the instrument employ complement fixation or hemagglutination (or its inhibition) as biological amplifiers in the detection of trace organics.

Automation of trace organic analysis has been achieved, using well-established techniques such as GC, HPLC, RIA, and ELA. And instruments do exist which can accept unmeasured, untreated samples and provide results without operator involvement. As detection limits are pushed lower and lower by advances in technology, automation will help the analyst obtain valid data by maintaining precision and accuracy while avoiding the effects of operator subjectivity.

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[9] Bulletin #1560-2 ChemResearch, Instrumentation Specialties Co., Lincoln, NE.

[10] For example, see those offered by MicroMeritics, Perkin-Elmer, Dupont, etc.

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ELECTRON CAPTURE NEGATIVE ION CHEMICAL IONIZATION MASS SPECTROMETRY

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The utility of the electron capture negative ion chemical ionization technique for quantitating organic compounds by conventional GC-MS selected ion monitoring methodology is discussed. Detection of dopamine, amphetamine, and Δ^9 tetrahydrocannabinol derivatives at the attomol (10^{-18} g) level is reported.

Key words: Chemical ionization; electron capture; gas chromatography-mass spectrometry; negative ions.

In an earlier paper we described new methodology, pulsed positive ion-negative ion CI (PPINICI) [1], which facilitates simultaneous recording of positive and negative ion CI spectra on a quadrupole mass spectrometer. Also discussed were unique analytical applications of several negative ion chemical ionization (NICI) reagent gases and the capability of NICI to provide conformation of sample molecular weight, structural information complementary to that obtained in the positive ion mode of operation, and sample ion currents 100 to 1000 times greater than that available from positive ion methodology. It was also suggested that electron capture NICI would facilitate detection and quantitation of many organics at the 10^{-12}–10^{-13} g level and therefore would find widespread use as a technique for the quantitation of trace level mixture components by combined GC-MS.

Here we discuss the analytical potential of electron capture negative ion chemical ionization in greater detail and provide data which defines the lowest level of sample detection achieved using our present instrument configuration. Additional information concerning analytical applications of positive and negative ion CIMS can be found in a recent review [2].

Formation of negative ions by interaction of electrons and sample molecules can occur by three different mechanisms (eq. 1–3), each of which is dependent on electron energy [3,4]. Ions resulting from the latter two processes are usually produced with low efficiency from most molecules and frequently dominate negative ion spectra generated with high energy electrons. In addition many of these fragment ions occur at the low mass end of the spectrum and are therefore not uniquely characteristic of sample molecule structure.

$$AB + e^- \xrightarrow{\text{ca. 0 eV}} AB^{\cdot -} \quad \text{resonance capture} \tag{1}$$

$$AB + e^- \xrightarrow{\text{0–15 eV}} A + B^- \quad \text{dissociative resonance capture} \tag{2}$$

$$AB + e^- \xrightarrow{> 10 \text{ eV}} A^+ + B^- + e \quad \text{ion pair production}$$

In contrast to the above situation, many sample molecules capture near thermal energy electrons and are converted to either stable molecular anions, $M^{\cdot -}$, or high molecular weight

fragment ions [5]. When this process proceeds with high efficiency it becomes ideally suited for use in the quantitation of organic molecules.

Bombardment of methane at 1 torr with 100 eV electrons generates CH_5^+ and $C_2H_5^+$ ions in high abundance [6]. As indicated in eq. 4–6, formation of each positive reagent ion is accompanied by the production of a low energy electron. Each ionizing event removes about 30 eV from the bombarding electron [7] and the energy of the incident electron beam is further reduced by additional nonionizing collisions with neutral methane molecules [8]. Thus, operation of a mass spectrometer under methane CI conditions should afford a mixture of both positive reagent ions and a population of electrons with near thermal energies.

$$2\ CH_4 + 2\ e \rightarrow CH_4^+ + CH_3^+ + H + 2\ e^* + 2\ e \tag{4}$$

$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3 \tag{5}$$

$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2 \tag{6}$$

Electron Energy Distribution. Qualitative evidence concerning the electron energy distribution in the ion source under CI conditions was obtained by comparing the maximum signal generated by the positive methane reagent ions with that produced by several negatively charged sample molecules. Results are summarized in Table 1.

TABLE 1. *Energy distribution of electrons in the CI ion source*

Sample	Methane positive ion current (nA)	Sample negative ion current (nA)	Electron energy window (eV)	% total electron population consumed[a]
SF_6	2.51	1.21 (SF_6^-)	0.0 ±0.03	48%
		0.12 (SF_5^-)	0.0 -1.0	5%
CH_2Cl_2	2.85	1.85 (Cl^-)	0.035–0.45	65%
H_2O	2.77	0.075 (OH^-)	≈6.4	3%

[a] Obtained by dividing the sample negative ion current by the methane positive ion current.

Interaction of sulfur hexafluoride with low energy electrons affords two ions SF_6^- and SF_5^-. Generation of SF_6^- occurs over a narrow electron energy range, 0.0 ± 0.03 eV [9]. Production of SF_5^- peaks at an electron energy of 0.15 eV and falls to a near zero level for electron energies in excess of 1.0 eV [9]. When SF_6 is introduced into the CI source with methane as the reagent gas, the negative ion current increases with increasing sample concentration until a signal corresponding to 1.21 nA and 0.12 nA is obtained for SF_6^- and SF_5^- respectively. Addition of more sample fails to increase the negative ion current. Assuming (1) that the number of positive ions and the number of negatively charged particles (electrons and negative ions) are equal in the area of formation of collectable sample ions, and (2) that extraction, transport, and mass analysis of positive and negative ions occur with equal facility, the above results suggest that, of the useable electrons in the CI ion source, 48% have energies near 0.0 eV and 53% have energies less than 0.15 eV. Experiments with methylene chloride which affords Cl^- on capture of electrons in the energy range 0.035–0.45 eV [9] indicate that 65% of the available electron population possess energies less than the latter value.

Production of H^- from water by dissociative electron capture requires electrons having energies near 6.4 eV [9]. Formation of O^- from water occurs at several electron energies, 6.5, 8.6, and 11.4 eV. Both of these ions react with water to produce OH^-. Ion current measurements on the OH^- ion indicate

$$H^- + H_2O \rightarrow H_2 + OH^- \quad (7)$$

$$O^- + H_2O \rightarrow HO + OH^- \quad (8)$$

that only 3% of the electron population has the necessary energy to facilitate dissociation of the water molecule. Much larger quantities of OH^- ions are probably generated near the electron beam entrance port of the ion source but the above experimental data indicate that few if any of these OH^- ions successfully diffuse to and through the ion exit slit. These results provide support for the thesis that the detected ions are formed very close to the ion exit aperture of a CI source.

Projected Resonance Electron Capture Sensitivity. Gas phase positive and negative ion molecule reactions which proceed at the diffusion controlled limit exhibit rate constants near 1×10^{-9} cm^3 s^{-1} [10]. In contrast the rate constant for formation of a negative ion by resonance electron capture can be as high as 4×10^{-7} cm^3 s^{-1} [11] or *ca.* 400 times greater than that for an ion molecule reaction. Accordingly, if sample molecules are converted to stable positive and negative ions on every encounter with CH_5^+ and thermal electrons respectively, the negative sample ion current should exceed the positive ion sample current by a factor of *ca.* 400. Ionization by resonance electron capture methodology should therefore facilitate detection and quantitation of many organic molecules at levels between 2 and 3 orders of magnitude lower than that accessible by positive ion EI or CI methodology. At present quantitation of many organics can be accomplished at the low picogram level using GC-MS in combination with the selected ion monitoring (SIM) technique. Operation of the mass spectrometer under CI conditions in the negative ion mode should extend the sensitivity of the GC-MS SIM technique to the low femtogram (10^{-15} g) or attomol (10^{-18} mol) level.

For the detection of organic samples at the femtogram level, it is necessary that the sample form a molecular anion on nearly every encounter with a thermal electron. This is only the case when resonance electron capture is exothermic, i.e., the molecule has a positive electron affinity. Due to the exothermicity of the electron capture process, the resulting anion is formed in an excited state. Excess internal energy in the molecular anion can be dissipated by several processes as shown in Figure 1 [9]. Dissociation via simple bond cleavage produces a radical and an even electron anion whereas elimination of a neutral molecule from $M^{-\cdot}$ affords a new radical anion. Since the internal energy of most molecular anions is low (0–2.5 eV) fragmentation pathways

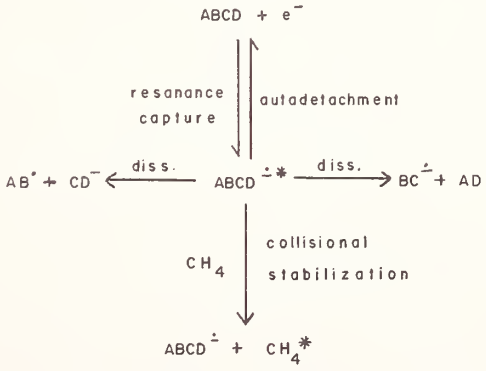


Figure 1. Energy dissipation modes available to excited molecular anions, $M^{-\cdot *}$.

involving both bond formation and cleavage, and therefore low activation energies, tend to predominant in many electron capture negative ion CI spectra. The reverse of the ionization step, expulsion of an electron or autodetachment, is also significant for many sample anions but becomes unimportant when the molecular anion can disperse the excess internal energy over a large number of accessible overlapping electronic and vibrational states. Under CI conditions (1 torr pressure of reagent gas) if a molecular anion fails to undergo autodetachment in 10^{-7}–10^{-8} s, it will experience a stabilizing collision with a neutral reagent gas molecule. In the absence of collisional stabilization, few if any excited molecular anions would survive the period of milliseconds required for an ion to traverse the distance between the ion source and the detector in a quadrupole mass spectrometer.

While many organic samples afford negative ion currents comparable to that observed in the positive ion mode, very few organic structures capture thermal electrons and form either stable molecular ions or high mass fragment ions with anything near unit efficiency. Despite this fact, ionization by resonance electron capture will undoubtedly become the method of choice for quantitating many trace level mixture components by combined gas chromatography-mass spectrometry. Most organic samples have to be derivatized to increase their volatility and to facilitate passage through the gas chromatograph. Realization of the enhanced sensitivity inherent to the resonance electron capture technique can be achieved by employing derivatives which facilitate high efficiency electron capture to produce sample anions which have a low probability of undergoing either dissociation or autodetachment.

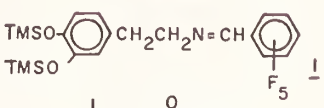
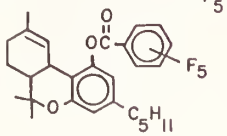
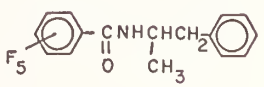
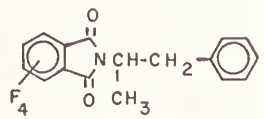
To illustrate the analytical potential of the above methodology, we report positive and negative ion CI mass spectra as well as negative ion detection limits for several derivatives of phenols and amines under GC-MS conditions. As shown in Table 2, the silylated Schiff base derivative formed from pentafluorobenzaldehyde and dopamine affords a negative ion CI mass spectra in which the molecular anion carries 95% of the negative ion sample current. At low sample concentration in the ion source, the current carried by this ion exceeds that of the most abundant positive ion by two orders of magnitude (N/P=102). The tetrafluorophthaloyl derivative of amphetamine and the perfluorobenzoyl derivatives of amphetamine and Δ^9-tetrahydrocannabinol behave in a similar manner and afford electron capture negative ion spectra in which the molecular anion or an $M^{\dot{-}}$ -HF ion carries most of the sample ion current. In each case the ratio of negative sample ion current to positive sample ion current exceeds 10^2.

Previously we reported that the N/P ratio obtained at low levels of perfluorobenzoyl amphetamine also held at the detection limit; 500 pg (S/N=4/1) in the positive ion mode and 5 pg (SN=4/1) in the negative ion mode [1]. The negative ion detection limits reported in Table 3 were obtained using the newly developed conversion dynode electron multiplier [12]. In this device negative ions are accelerated into a metallic surface held at a high positive potential (+2500 V) and placed several millimeters away from the face of a Galileo Model #4770 continuous dynode electron multiplier (Galileo Electrooptics Corporation, Sturbridge, Mass.). Positively charged sample and/or metal ions generated as a result of negative ion impact on the conversion dynode are then collected on the first dynode (–2000 V) of the nearby conventional positive ion electron multiplier.

With the above detection system the noise on both the positive and negative ion signal is equal and a factor of 10 less than that observed on our earlier negative ion detector [1]. By improving the S/N ratio we now find that the limit of detection is between 10–25 fg (20–50 attomols) for each of the four compounds, 1-4 (Table 2). Tracings of the actual data recorded in the SIM mode for the dopamine derivative 1 are shown in Figure 2.

It is important to note that the enhanced sensitivity reported above is achieved by simply switching the sample ionization mode from positive ion CI to electron capture NICI. All other instrumental parameters remain unchanged. We wish also to emphasize the significance of the N/P ratio obtained at low sample concentration. This ratio is a direct measure of the ionization efficiency in the positive ion and negative ion mode of operation and should remain constant when

TABLE 2. *Methane PPINICI mass spectra*[a]

	MW	N/P[b]	% total negative sample ion current M$^{-\cdot}$	% total negative sample ion current (M-HF)$^{-}$	% total negative sample ion current Other m/e (%)	% total positive sample ion current (M+1)$^{+}$	% total positive sample ion current Other m/e (%)
 1	475	102	95.0	--	385(1.4),[e] 167(3.7)[f]	56.7	504(4.6),[c] 460(35.4), 267(3.3)
 2	508	328	90.1	--	167(9.9)[f]	48.6	537(14.3),[c] 296(7.1),[g] 195(11.4),[h] 135(18.6)
 3	329	100	--	83.3	289(11.3),[i] 212(2.7), 198(2.7)	45.4	370(1.1),[d] 3.58(7.0),[c] 238(30.4),[j] 118(16.2)
 4	337	678	100	--	--	34.7	378(0.8),[d] 366(2.8),[c] 246(30.2),[j] 245(25.0), 147(6.7)

[a] Ion source temperature, 100 °C; ion source pressure: 6×10^{-4} torr.
[b] Ratio of the most abundant negative and positive sample ions at low sample concentration.
[c,d] Ions resulting from the attachment of $C_2H_5^+$ and $C_3H_5^+$ respectively, to the sample.
[e] $(M^-(CH_3)_3SiF)^{-\cdot}$.
[f] $C_6F_5^-$.
[g] $(M+1-C_6F_5COOH)^+$.
[h] $C_6F_5CO^+$.
[i] $(M-2HF)^{-\cdot}$.
[j] $(M+1-C_6H_5(CH_3)^+$.

TABLE 3. *Lowest levels of detection achieved using electron capture negative ion CIMS*

Compound	Sample quantity injected onto GC column	S/N[a]
1[d]	25 fg[b] (53 attomol)[c]	4
2[d]	10 fg (20 attomol)	1
3[d]	10 fg (30 attomol)	4
4[d]	10 fg (30 attomol)	12

[a] Signal to noise ratio.
[b] 1 fg = 10^{-15} g.
[c] 1 attomol = 10^{-18} mol.
[d] See Table 2 for structures.

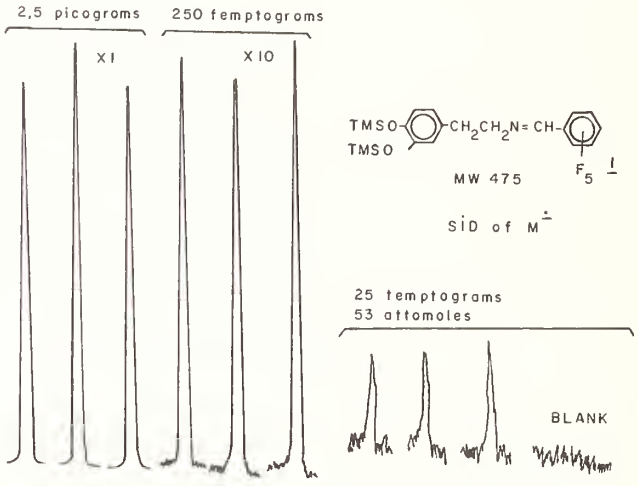


Figure 2. Response obtained by monitoring the M^{-} ion (m/e 475) of the dopamine derivative under GC-MS conditions with the instrument operating in the SIM mode. Signals correspond to three successive injections of 2.5 pg, 250 fg, and 25 fg samples respectively.

determined on different spectrometers. Thus, if the lowest level of detection in the positive ion mode is known on a given instrument, the N/P ratio reported here can be employed to calculate the lowest level of detection achievable on the same equipment using EC-NICI methodology. In contrast, absolute lowest levels of detection reported under either positive ion CI or EC-NICI conditions are dependent on many experimental parameters other than ionization efficiency and therefore can be expected to vary from laboratory to laboratory.

In addition to providing enhanced sample ion current, the use of EC-NICI for quantitation of organics may also result in significantly reduced background noise and a lower probability that a sample ion at a particular m/e ratio will be obscured by the presence of a fragment ion derived from a high molecular weight contaminant unresolved from the sample by the GC conditions employed in the analysis. We pointed out earlier that most organic molecules do not suffer efficient ionization on interaction with thermal electrons in the gas phase. Through proper choice of derivatizing agents it is possible to selectively enhance the ion current derived from the analyte without increasing the ion current due to other molecules in the sample mixture. Although this aspect of the EC-NICI method may be important in many quantitation studies, it must also be mentioned that trace quantities of impurities with high electron affinities (i.e., molecules containing halogen) can seriously deplete the population of electrons in the ion source available for

sample ionization. A sharp drop in sample sensitivity results. For this reason halocarbons are undesirable solvents for use in electron capture GC-NICIMS studies.

Comparison of EC-GC and EC-NICIMS. Since the mechanism of sample ionization is the same under both EC-GC and EC-NICI conditions, it is perhaps appropriate to comment on the analytical potential of the two techniques. Detection of organics under EC-GC conditions is accomplished by passing the sample through a chamber containing a population of thermal electrons and by monitoring the electron current passing through two electrodes placed within the chamber [8]. A decrease in the standing current occurs when sample molecules capture electrons and are converted to negative ions which in turn suffer neutralization by the three body recombination of positive and negative ions mediated by the carrier gas. The theoretical detection limit of the EC-GC detector is estimated to be 330 attomols (10^{-18} mol) [8]. Because the EC-NICI technique measures negative ion abundance rather than variations in the standing electron current, it is expected that the mass spectrometric technique will be at least 10–100 times more sensitive than present EC-GC detectors. The experimental EC-NICI detection limits reported in this paper already exceed the above theoretical limits of the EC-GC detector by a factor of 10. Information concerning sample molecular weight and structure furnished by the mass spectrometer further enhance the value of the EC-NICI technique. Of course, if the dominant sample ionization mechanism is dissociative electron capture to produce small anions such as Cl^-, no advantage is gained by employing the more expensive EC-NICI methodology.

Biological Assay. The first biological assay using the GC-MS electron capture NICI technique has recently been developed by Markey and coworkers [13]. Quantitative analysis of Melatonin, (N-acetyl-s′-methoxytryptamine), in the form of a perfluoropropionyl derivative has been accomplished at the 100 fg level in human plasma using the GC-MS SIM technique with the spectrometer operating in the EC-NICI mode.

Acknowledgment

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithershurg, Md. (Issued April 1979)

MIXTURE ANALYSIS BY MASS SPECTROMETRY

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Trace analysis is considered from general principles with emphasis on the contrasting procedures of chromatography/spectroscopy and mass spectroscopy/spectroscopy. The latter, embodied in the MIKES technique, uses the mass spectrometer in both its mass transport and its compound identification roles. The effects of chemical noise on detection limits in chromatographic and non-chromatographic techniques are emphasized. Alternatives to mass-analyzed ion kinetic energy spectrometry (MIKES) for direct analysis of mixtures hy sequential ion separation and identification are systematized. Studies using MIKES in conjunction with a variety of methods of ion formation and various ion dissociation processes are reviewed. The technique of single reaction monitoring is presented and examples of its use are covered.

The use of negative chemical ionization in conjunction with MIKES for mixture analysis is explored. It is shown that both positive and negative secondary ions may usefully be detected, and that a systematic ion chemistry underlies these MIKE spectra. Applications of CI/MIKES to metabolites in urine, preservatives in foodstuffs, drug preparations and to natural products are covered. Examples of isomer identification are given, including some which employ high energy ion/molecule reactions other than the usual collision-induced dissociation process. New developments involving pyrolysis/MIKES, improvements in the quantitative accuracy of the methodology, alternative instrumentation, and extensions of the method to the study of hitherto intractahle hiological materials are foreseen.

Key words: Mass-analyzed ion kinetic energy spectrometry; MIKES; mixture analysis; trace analysis.

I. Principles

Mass spectrometry has for many years provided a particularly sensitive and specific method for the identification of organic compounds [1]. The universal applicability of the technique, however, has limited mixture analysis to the determination of major constituents since the signals for minor constituents are obscured by the numerous intense peaks due to the major species. Minor components of mixtures have sometimes been determined using alternative ionization techniques, particularly field ionization [2], negative ionization [3] and chemical ionization [4]. Metastable peaks, which relate parent and daughter ions, have also found occasional use in mixture analysis [5]. Dramatic progress in analyzing mixtures for trace components, however, came only when separation of the mixture and analysis of the separated components were carried out in sequence in a combined chromatograph-mass spectrometer [6]. The gas chromatograph-mass spectrometer (gc/ms) and the liquid chromatograph-mass spectrometer (lc/ms) utilize this principle. This paper will show that there are advantages in returning to the use of mass spectrometers alone for trace mixture analysis. What can be achieved is a better integrated instrument—the functions of mixture separation and component identification remain separate but they are more smoothly coordinated.

To introduce the principles behind this concept consider the established alternative, gc/ms. The interface between the chromatograph and the spectrometer has always been the weak point in this instrumentation (Table 1). Furthermore, the contrasting physical properties which exist on each side of the interface in gc/ms are accentuated in lc/ms.

In the alternative concept [7], developed here, there is no interface. The mixture to be analyzed is converted into ions immediately upon introduction into the instrument and the ions are

TABLE 1. *gc/ms interface*

Property	gc	ms
pressure (atm)	~ 1	$\sim 10^{-9}$
charge state	neutrals	ions
rate of passage (cm/s)	$\leqslant 1$	$\sim 10^{7}$

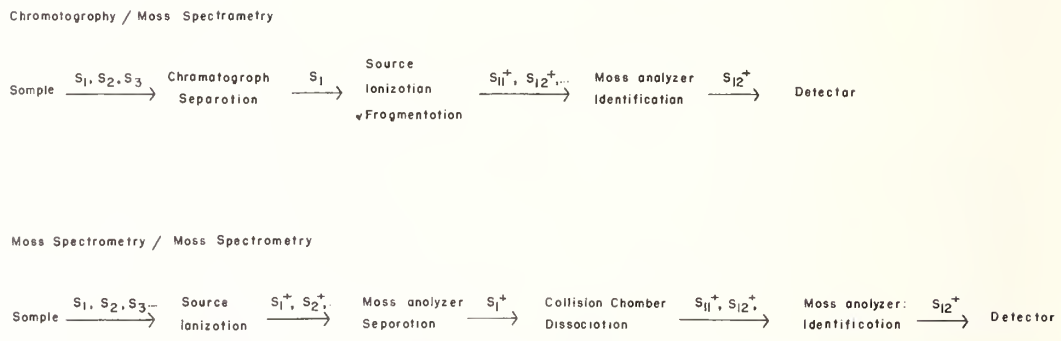

Figure 1. Comparison of the sequence of steps used in mixture analysis by chromatography/mass spectrometry with that in a tandem mass spectrometer such as the MIKES. Note in particular the reversal in the ionization/separation sequence.

subject to physical probes which affect separation and identification on the fly. This technique is currently being practiced using the mass-analyzed ion kinetic energy (MIKE) spectrometer [7b], a form of double-focusing mass spectrometer in which the usual order of analyzing fields is reversed. The sequence of events in this method is contrasted to that in the chromatography/spectroscopy techniques in Figure 1.

The MIKES technique would merit close attention even if the factors just noted were the only ones of significance. In fact, there are very substantial gains in sensitivity, especially relevant to trace analysis, which also follow. With continuing improvements in electronic detection, the barrier faced increasingly in trace analysis is chemical noise [8]. It may not be an accepted concern of the analyst that the world at large is becoming more impure (chemically mixed). It is, however, an explicit concern, once a sample has been received for analysis, that it be subject to the minimum of contamination. In this, the chromatographic methods fail *in principle.* The sample is treated with large excesses of foreign compounds, *viz.* support, stationary phase and carrier gas in gc, and column packing and solvent in lc. The amounts of these compounds used relative to a trace component being analyzed may be staggering. For example, when analyzing a 10 mg sample at even the rather modest ppm level using a typical gc column containing ~ 1 g of stationary phase, this ratio is 10^{8}.

Interference by this extraneous material or one of its constituents—previously adsorbed and retained sample, impurities, pyrolysis or chemical transformation products—thus becomes likely. Furthermore, aside from inadequate background subtraction, there is no simple characteristic which distinguishes sample from chemical noise—both together are recorded as ions making up a mass spectrum.

One shortcoming of gc/ms and lc/ms in trace analysis is, therefore, that they subject the sample to a contact phase in which both the amount of foreign material and the possibility of retention of sample or a transformation product is high. Column bleed in gc is perhaps the major limitation on gc/ms sensitivities in advanced instruments [9]. In addition to *introducing* chemical noise the chromatography/spectroscopy methodologies are also inferior to the spectroscopy/spectroscopy procedure in *discriminating* against chemical noise. Such discrimination

can be achieved by following the contact stage of analysis (in which contamination can occur) by one or more clean stages. These clean stages should employ physical rather than chemical methods of analysis and should be carried out under conditions such as high vacuum, which minimize sample carryover. The above concepts are shown in schematic form in Figure 2. The methodology advanced in this paper avoids the contact stage and employs two clean stages. The only phase interface occurs on introduction of the sample into the instrument. The result should be a significant improvement in detection limits, other things being equal. The present detection limits in MIKES, with a number of features far from optimum, are in fact comparable to those in gc/ms. Direct evidence for improved usable sensitivity after two as opposed to one stage of analysis (in spite of sample losses of $>90\%$ in the low efficiency fragmentation step) is given in Figure 3.

It is of note that in regard to chemical noise as well as other properties, the characteristics of gc/ms extend to other chromatography/spectroscopy methodologies such as gc/ir. It is because mass spectrometry includes a mass *transport* role that it alone of all present forms of spectrometry can be substituted for chromatography.

One further conceptual point which deserves mention is the operation in MIKES of an advantage relating to the quality of the information obtainable at a given level of sensitivity. The

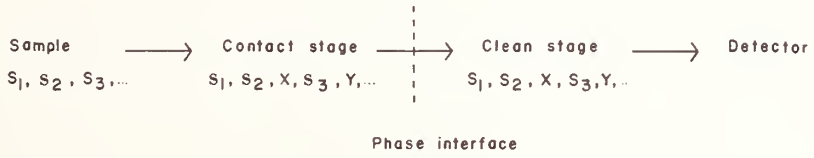

Figure 2. Effect of exposure to extraneous material. In general, chemical methods of analysis, including chromatography, will pollute the sample. Methods which employ physical probes in a high vacuum environment can be classed as clean methods. The sequence of contact and clean stages shown here is that encountered in gc/ms.

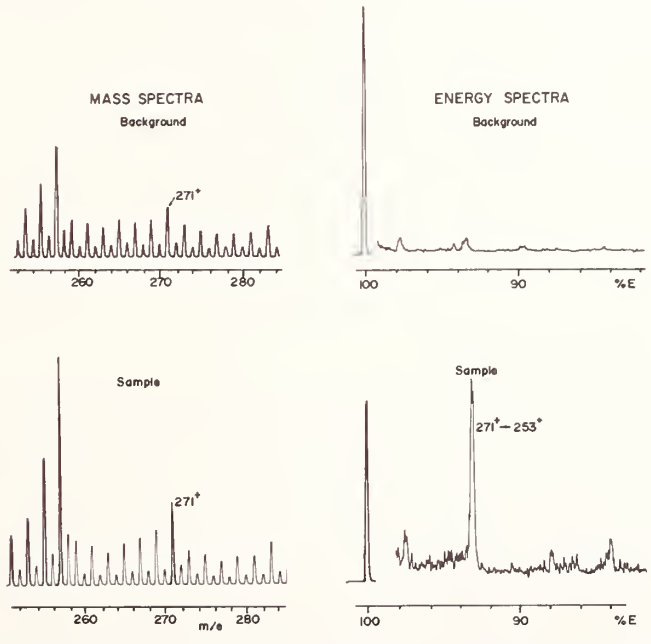

Figure 3. Immunity to chemical noise achieved by employing two vs. one stage of analysis. The MIKE spectrum (lower right) contains usable information whereas the mass spectrum (lower left) does not, even though total ion intensities are much higher. From ref. 13b.

point can be made by comparing the sensitivity and specificity of the MIKES method to that of high resolution mass spectrometry (HRMS). While elemental composition is more informative than nominal mass it is often less unique than a single transition in a MIKE spectrum, especially for trace analysis. (Compare use of MIKE spectra for structural studies on *isomeric* ions [10]. Note also that, while there are many elemental compositions associated with a particular nominal mass, in practice most organic compounds are found distributed among a very small fraction of these compositions). What is most striking is that the increased specificity of MIKES is obtained at sensitivity comparable to that of HRMS. Ignoring instrumental losses and inefficiencies in transmission or dissociation, the resolutions used in MIKES and HRMS are consistent with this. For mass 100 both analyzers in MIKES should have 1 in 100 resolving powers, for a net transmission factor of 1 in 10^4. This same value represents what would be necessary in an exact mass measurement. The advantages of sequential low resolution analyzers over a single higher resolution device can thus include more specificity at comparable signal strengths as well as greater immunity from chemical noise.

II. Trace Analysis

Some characteristics of MIKES which are advantageous in trace analysis are evident from the preceding discussion; others will now be presented. Prominent among these is the fact that small total samples are required. The technique is suited to direct sample introduction into the ion source from a probe. Solids or small volumes of solution can be analyzed in this way. Requirements regarding volatility and thermal stability are relaxed *vis-a-vis* gc/ms. As a consequence of the foregoing features and of the immunity to chemical noise it has proven possible in many cases to examine complex samples for particular components without prior chemical or physical treatment. This feature is exemplified in the detection—with high specificity and at high sensitivity—of particular alkaloids in intact leaf fragments [11]. An example is provided by the identification of cocaine in coca leaf, Figure 4.

In trace organic analysis there is a reciprocal relationship between sensitivity and the degree of specificity which can be attached to the measurement. When specificity can be relaxed—as when testing for a particular compound with known spectral properties—then sensitivity can be increased. This is effected in gc/ms by techniques in which only a limited number of ions are detected. In the extreme, the technique of single ion monitoring results. A comparable situation exists in MIKES where a single product ion can be monitored in association with the selected reactant ion. Single (or selected) reaction monitoring is thus the highest sensitivity variant in MIKES. It is worth noting that both the gc/ms and the MIKES single monitoring procedures base compound recognition on two independent parameters as Table 2 indicates. In the coca leaf example just cited, a complete MIKE spectrum was not necessary to characterize cocaine; any of the intense fragment ions would have sufficed. In such circumstances use of the high sensitivity single reaction monitoring method is warranted. This is done by monitoring ion current as a function of time as illustrated in Figure 5.

It is a simple step from single monitoring to multiple monitoring methods. If various fragment ions associated with a single compound are to be monitored, this is readily achieved in both MIKES and in gc/ms. In both situations of course the advantage of increased specificity is offset by a loss in sensitivity. We expect this procedure to be most useful as providing an extra margin of safety in characterizing a constituent when a single reaction ($m_1^+ \rightarrow m_2^+$) does not suffice due to the complexity of the sample.

There is another and quite distinct use to which multiple reaction monitoring methods can be put. This involves the monitoring of several different molecular species, effectively simultaneously. This might be done when the sample is changing in composition with time, as in industrial processes or during the course of chemical reactions, or it might be done to avoid instrument fluctuations when accurate quantitation of two or more species is sought. Chlorine

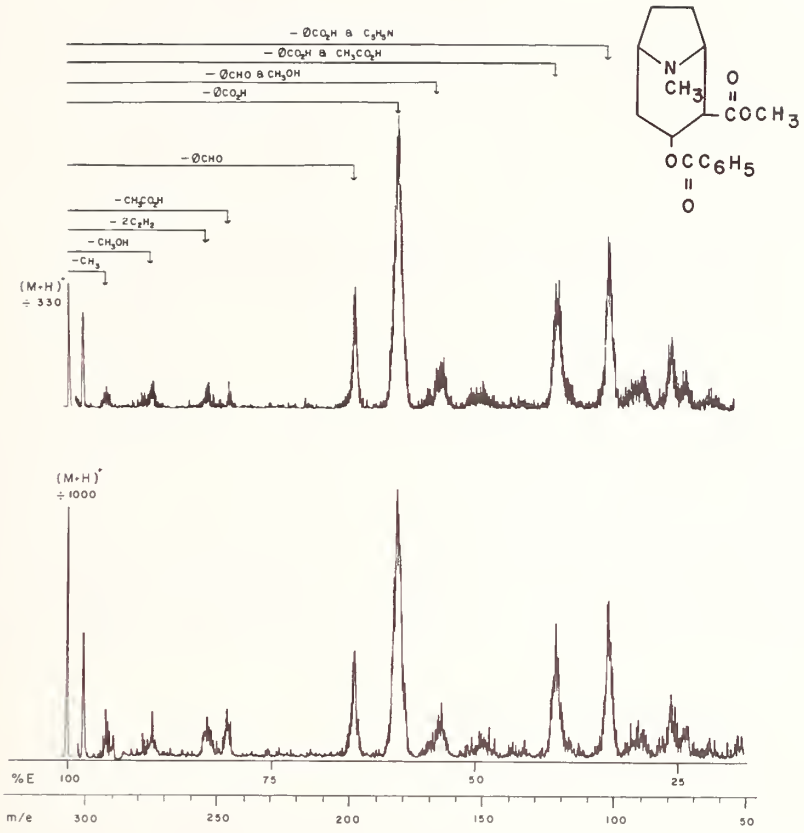

Figure 4. Identification of cocaine in untreated coca leaf. MIKE spectra of 304^+ in the authentic alkaloid (upper) and a leaf fragment (lower) match exactly. Reactions leading to fragment ions are indicated. From ref. 8.

Table 2. *Single ion monitoring (gc/ms) and single reaction monitoring (MIKES)*

	gc/ms	MIKES
Criteria:	Retention time	Reactant ion mass
	Fragment ion mass	Fragment ion mass

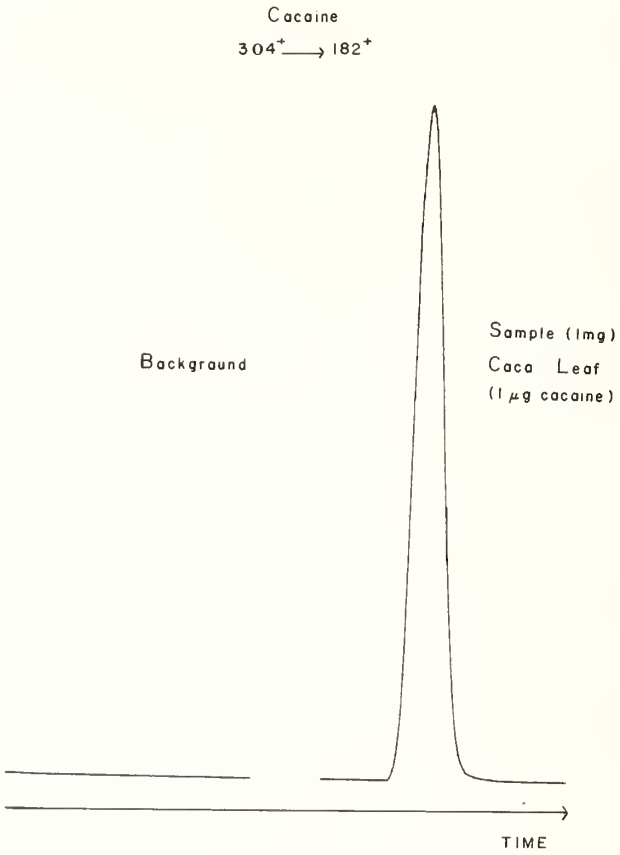

Figure 5. Identification of cocaine in coca leaf by monitoring as a function of time the reaction $304^+ \rightarrow 182^+$. This single reaction method maximizes the sensitivity of analysis.

isotope effects have been determined in this way [12]. Clearly chromatographic methods cannot compete with the purely mass spectrometric methods here. It is inherent in chromatography that time resolution of individual constituents is achieved. The duty cycle in chromatography is typically many minutes. By contrast in MIKES even with continuous flow introduction and particularly when monitoring the effects of chemical or physical stress on the sample within the instrument, a repetition rate of below a second is feasible.

III. Implementation

We turn now from the general principles underlying the methodology to the particulars of its implementation. There are a variety of different methods applicable to each of the required operations of ionization, mass-selection of the primary ion, dissociation, mass-analysis of the secondary ions and detection. Some of these options are given in Table 3 and the number of possible combinations can be seen to be quite large. Several of these have been put into practice with most use being made of chemical ionization in a reversed sector mass-analyzed ion kinetic energy (MIKE) spectrometer [13].

Since the ionization method can be considered an accessory to the instrument, the basic instrument depends on the types of mass analyzers chosen. Thus, using nomenclature of Table 3, we have for example, Q/B, Q/Q, B/Q and B/E spectrometers. Among these options, preliminary results have been obtained with a Q/Q instrument [14] but otherwise all studies have utilized the MIKES or B/E configuration. Techniques also exist [15] in which MIKE spectra are simulated on

Table 3. *Systems for direct mixture analysis by mass spectrometry*

Ionization	Mass-selection	Dissociation	Mass-analysis
CI	B	CID	B
EI	Q	m*	Q
FI		SID	E

CI=chemical ionization; EI=electron ionization; FI-field ionization; B=magnetic analyzer; Q=quadrupole mass filter; CID=collision-induced dissociation; m*= metastable ion dissociation; SID=surface induced dissociation; E=electrostatic analyzer.

E/B instruments, that is on conventional double focusing mass spectrometers, by having dissociation occur prior to either sector and maintaining a fixed relationship between the sector potentials.

The fact that an energy analyzer can be used for the second stage of mass analysis (in B/E or in Q/E instruments) deserves comment. It depends on momentum conservation in a high energy collision; the energy of the reactant m_1^+ is partitioned according to the masses of the fragments m_2^+ and m_3. A measurement of the kinetic energy of m_2^+ thus represents a measurement of its mass since

$$KE(m_2^+) = \frac{m_2}{m_1} KE(m_1^+).$$

The abscissa in a MIKE spectrum can, therefore, be calibrated either in terms of the ion kinetic energy, for example as a fraction of the energy of the ion m_1^+ being studied, or the calibration may be made directly in terms of the mass of the fragment ion m_2^+.

In the remainder of this section examples of results for various experimental conditions not covered elsewhere are given. Results on barbiturates [16] are used to illustrate the effects of varying the ionization method and the fragmentation method. The charge state of both the ions selected and of those sampled can also be varied but this development is covered in the section on negative ions.

There are advantages in employing ionization methods which (i) do not cause molecular rearrangement and (ii) give the minimum number of ions for each neutral molecule. These are best satisfied by chemical ionization (although the field ionization techniques may satisfy (ii) in giving less complex mass spectra). Nevertheless, very useful results can be obtained by electron ionization (EI). Figure 6 shows the characteristic MIKE spectrum of the molecular ion of aprobarbital (alurate). The losses of 42 and 43 mass units characterize the isopropyl group, that of 41 the allyl group and the very abundant loss of 15 apparently arises because of the interaction of these substituents as shown. It is of note that mass spectra taken by EI are not subject to matrix effects so that mixtures can be analyzed if the EI MIKE spectra of the pure components are known [13a,b]. The analysis of barbiturates in mixtures follows directly from these results.

In MIKES the mass-selected ion can either undergo spontaneous dissociation or dissociation may be induced [13]. The advantages of using collision-induced dissociation (CID) are shown in Figure 7. Protonated secobarbital $(M+H)^+$ undergoes just one metastable ion reaction, a McLafferty rearrangement with loss of C_5H_{10} (70); in the presence of a target gas this process increases in relative intensity and many more reactions occur. This behavior, including the tendency for CID to favor simple bond cleavages over rearrangements is characteristic of positive ions [17]; one of the rare exceptions is also provided by the barbiturates where the molecular ions $(M^{+\cdot})$ do not show appreciably enhanced dissociations in the presence of target gases [16]. This uncharacteristic behavior is not seen for the protonated or the ethylated compounds.

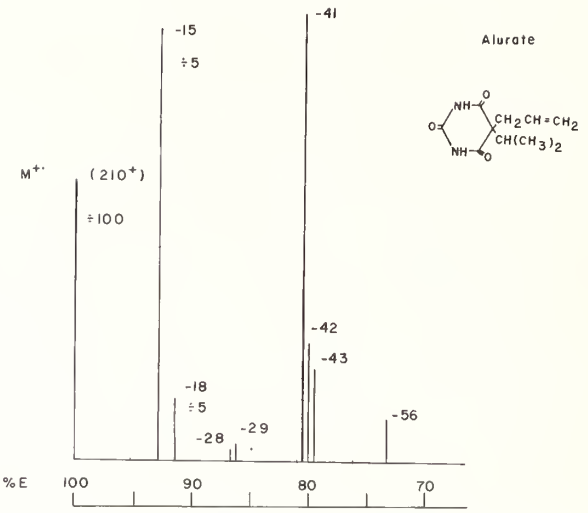

Figure 6. MIKE spectrum of the molecular ion of alurate (aprobarbital) illustrating structure-sensitive fragmentations in electron impact MIKES.

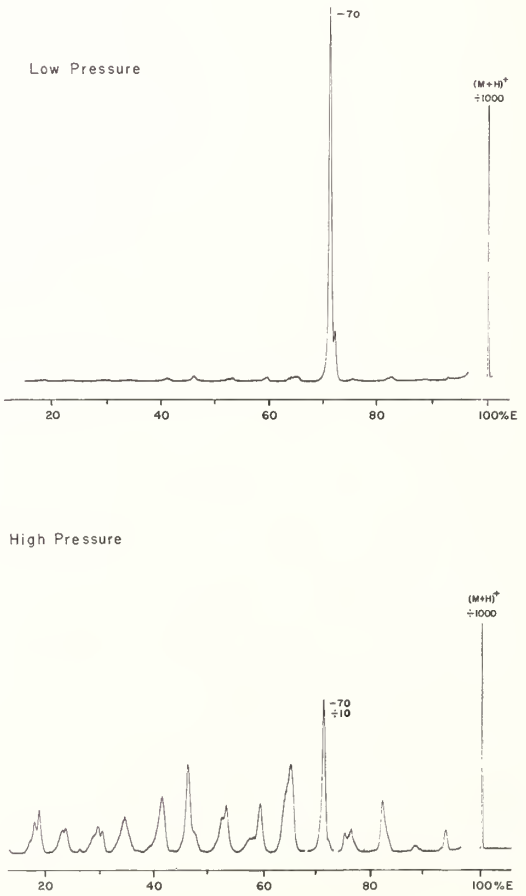

Figure 7. Simpler but less structurally informative MIKE spectra result when metastable ions are sampled (upper) rather than the usual collision-induced dissociation products (lower). Example given is protonated secobarbital. From ref. 16.

Implementation of the MIKES methodology requires that structure/spectral correlations be developed. Progress in gaining some of this knowledge has come from systematic investigations of the fragmentations of protonated ketones, ethers, amines and esters [18]. The prevalence of 1,3-rearrangements with elimination of alkane and alkene molecules, together with the breakdown of the even-electron fragmentation rule and the loss of alkyl radicals are highlights of this chemistry. These features are illustrated by the MIKE spectrum of protonated 3-hexanone (Fig. 8).

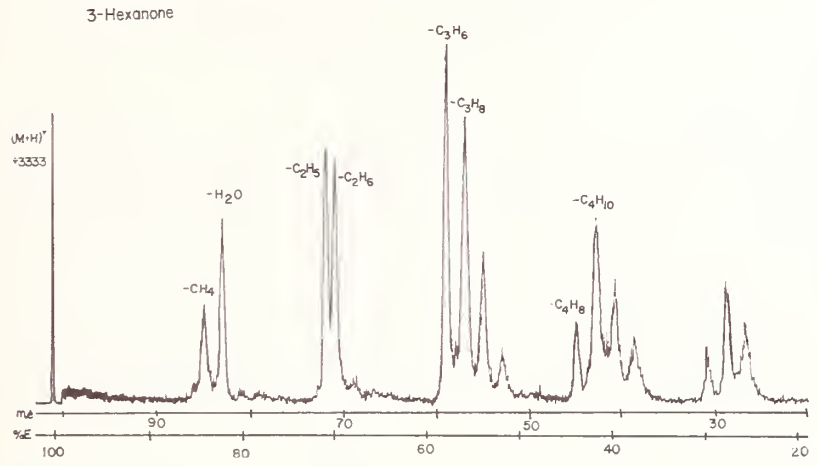

Figure 8. Characteristic losses of alkanes, alkenes, and alkyl radicals in MIKE spectra of protonated compounds. Ketones also show loss of water.

IV. Negative Ions

An important new development in mixture analysis by MIKES has been the use of negatively charged ions [19]. We now illustrate this development and use it as a vehicle for illustrating principles and procedures which apply to positive ions as well. We specifically give examples of applications to biological samples.

The transformation from a mixture of neutral molecules to a mixture of ions can be achieved in various ways. This is illustrated by the formation of molecular ions, $M^{+\cdot}$, by electron impact, vs. protonated molecules, $(M+H)^+$, and ethylated molecules $(M+C_2H_5)^+$, by chemical ionization. Within the realm of chemical ionization there exist numerous other ionization reactions, including the large class which yield anions derived from the sample. Two such reactions are those yielding $(M\text{-}H)^-$ by proton abstraction and $(M+Cl)^-$ by chloride attachment. The ability to select the ionization reaction represents a valuable additional filter in the mixture analysis scheme. In principle, this step could be used to make distinctions as subtle as that between optical isomers (using an asymmetric reagent ion). It also turns to advantage the fact that in MIKES ionization occurs in the presence of a mixture of compounds: by appropriate choice of ionizing reagent enhanced ionization of compounds with selected properties can be achieved. A simple example is Cl^- attachment, a reaction which is limited to certain substrates [3]. The attachment/detachment reaction sequence

$$M+Cl^- \rightarrow (M+Cl)^- \rightarrow (M\text{-}H)^- + HCl$$

can be used as a sensitive, but not particularly structurally diagnostic, means of analysis. Figure 9 shows the results of this series of reactions starting with 50 pg of glucose and monitoring the $(M\text{-}H)^-$ product from $(M+Cl)^-$ as a function of time.

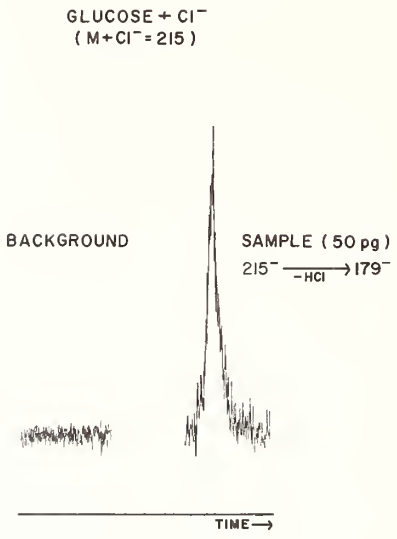

Figure 9. Detection of 50 pg glucose by single reaction monitoring. From ref. 19.

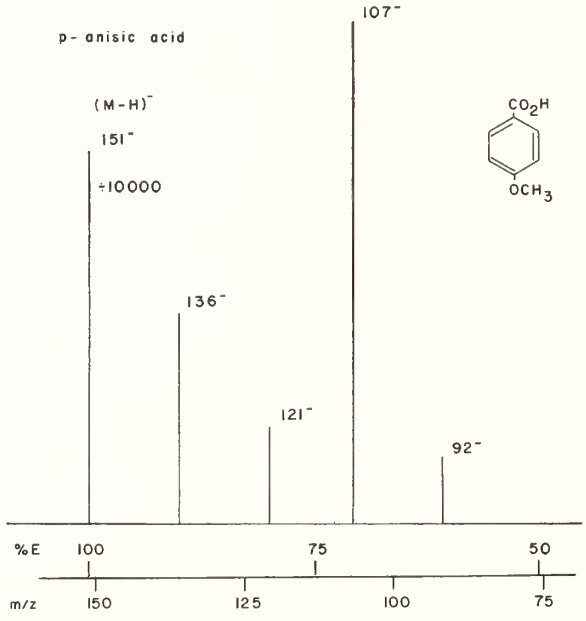

Figure 10. Negative ion MIKE spectrum. Negatively charged fragments arising by collision-induced dissociation of the selected anion $(M-H)^-$ are recorded.

The remaining experiments described below deal with primary $(M-H)^-$ ions. These ions fragment to provide structural information on the molecules from which they are derived, just as in positive ion MIKES. Thus, both molecular weight and molecular structure (or a characteristic molecular fingerprint), are obtained by mass-selecting $(M-H)^-$, inducing dissociation and recording the spectrum of the dissociation products. This is illustrated by the MIKE spectrum of the $(M-H)^-$ ion of p-methoxybenzoic acid (Fig. 10). The anions produced on dissociation include a prominent $(M-H-44)^-$ ion. This feature, the loss of CO_2, is a characteristic of carboxylic acids in these spectra. Note, too, the losses of methyl (136^-) and formaldehyde (121^-) which are both due to the methoxy substituent.

Alternative methods of characterizing the mass-selected $(M-H)^-$ ion exist. Instead of monitoring negative fragment ions, positive fragments can be monitored by a simple polarity change of the electric field. Thus, the high energy collision may be of the type

$$m_1^- + N \rightarrow m_2^+ + m_3 + N + 2e^-$$

including, for the special case $m_3 = 0$, the charge inversion (stripping) reaction

$$m_1^- + N \rightarrow m_1^+ + N + 2e^-.$$

The positively-charged products arising from $(M-H)^-$ ions have comparable abundances to the anionic fragments and they also provide valuable structural information. The complementary nature of the information yielded by anions and cations is shown in Figure 11 for p-chlorobenzoic acid. (Note that ^{37}Cl the minor isotope was selected for analysis; such isotopic selection is a feature of MIKES). Here, as in many other acids, the presence of the functional group is indicated by the characteristic CO_2 elimination (anion fragment) while the cation spectrum is typically more complex although no less informative. The unimolecular ion chemistry underlying these fragmentations is readily rationalized on the premise that the fragment cations arise from a nascent $(M-H)^+$ species. This ion will often be thermodynamically unstable (cf. RCO_2^+ vs. RCO_2^-) as well as being

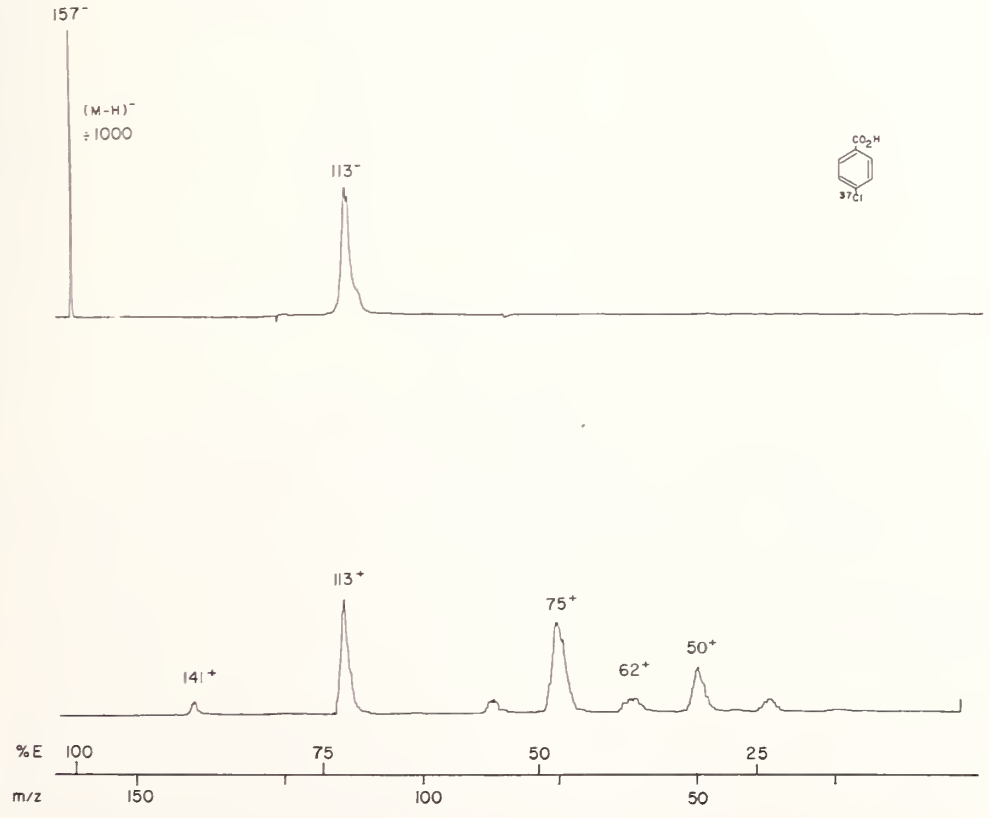

Figure 11. MIKE spectra showing positively and negatively charged products both arising from the $(M-H)^-$ ion of *p*-chlorobenzoic acid. The spectra have comparable sensitivities and are readily recorded in sequence. The anion spectrum characterizes the functional group while the cation spectrum provides further details on molecular structure.

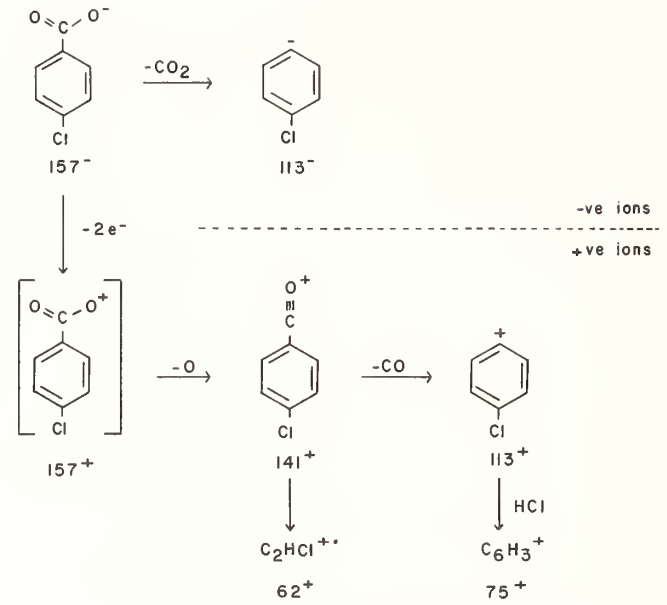

Figure 12. Proposed fragmentation pathways leading from $(M-H)^-$ to major anionic and cationic products for *p*-chlorobenzoic acid.

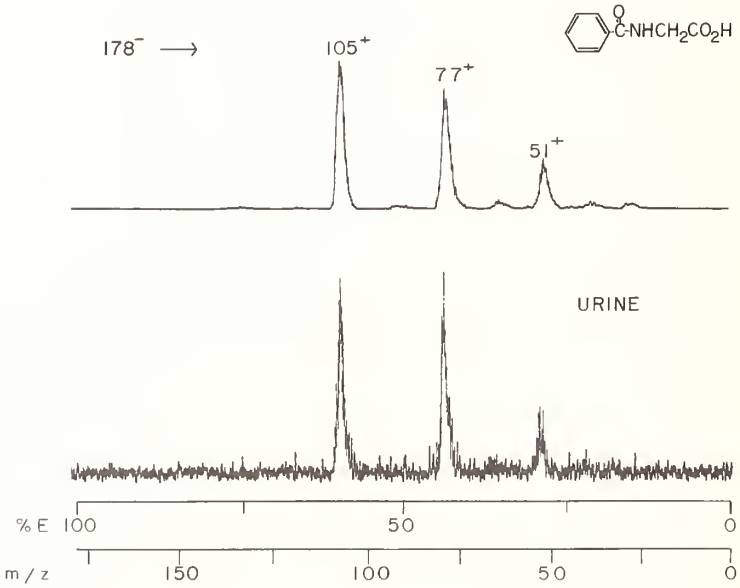

Figure 13. Identification of hippuric acid in urine by negative chemical ionization/MIKES. Untreated urine (total 2 μl) was used. From ref. 19.

generated with excess energy. The fragment cations arise from this species as illustrated in Figure 12. The anion reaction sequence is also shown.

Negative chemical ionization in conjunction with MIKES forms the basis for the identification of specific components in complex mixtures including those of biological origin [19,20]. This can be illustrated by the identification of hippuric acid in urine, the sample having undergone absolutely no pretreatment. The matching MIKE spectra of the authentic compound and the urine constituent are shown in Figure 13. As constituents present at still lower

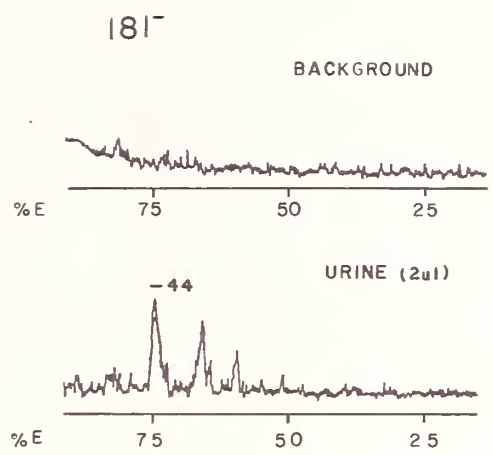

Figure 14. Identification of homovanillic acid (HVA) in urine by negative chemical ionization MIKES. Only the peak due to loss of CO_2 (44 amu) is due to HVA. Acidified urine (2 μl) was used. From ref. 20.

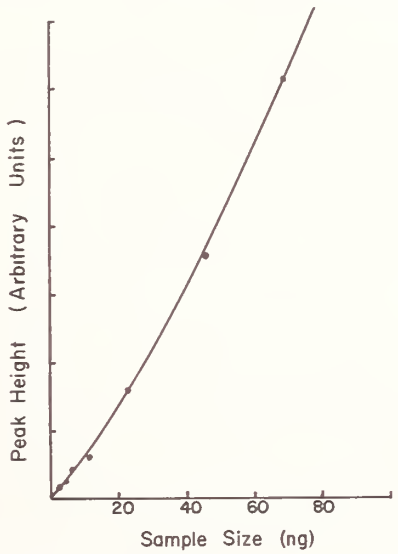

Figure 15. Quantitation of HVA using the single reaction $(M\text{-}H)^- \rightarrow (M\text{-}H\text{-}CO_2)^-$. From ref. 20.

concentrations are sought, MIKE spectra may show other peaks in addition to those for the component of interest. This simply means that mass separation does not yield complete molecular separations; the difficulty only arises in samples with components giving peaks of the same nominal mass. Even this does not preclude analysis as shown by the results for homovanillic acid (HVA) in urine given in Figure 14. In cases like this, single or multiple reaction monitoring would probably be chosen to maximize the time spent monitoring meaningful signal. In fact, HVA can be quantitated in this fashion as indicated by the calibration curve shown in Figure 15. The calibration curve is for the $(M\text{-}H)^- \rightarrow (M\text{-}H\text{-}CO_2)^-$ process and is specific for compounds which (i) have molecular weight 182 and (ii) form $(M\text{-}H)^-$ ions by NCI and (iii) fragment by loss of 44 amu. If, in any given situation, this set of criteria is not sufficiently specific for HVA then more criteria can be added (e.g., temperature profile, peak intensity, other fragment anions or cations, other primary ion formation and fragmentation reactions, etc.). Rapid switching between two sets of reactions makes more specificity available with minimum effort. Other illustrations of the use of NCI/MIKES in trace analysis of complex mixtures with no sample pretreatment include the

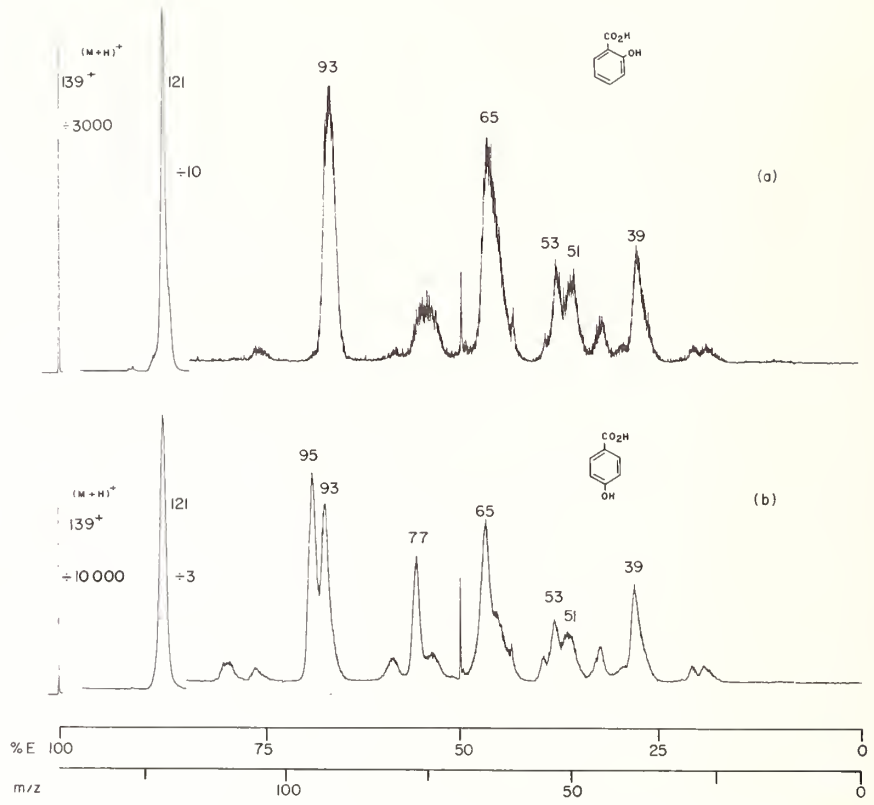

Figure 16. Isomers distinguished by the MIKE spectra of $(M+H)^+$ ions. o-Hydroxybenzoic acid (upper) and its para isomer. From ref. 19.

detection of salicyclic acid in aspirin, benzoic acid in foodstuffs and household products, butylhydroxyanisole (BHA) in yeast, ascorbic acid and glucose in urine and foods [19].

A final point, appropriately discussed under the negative ion heading, concerns the distinction between isomers. This may be possible using positive ion methodology, e.g., primary $(M+H)^+$ ions, Figure 16. It may also be possible from a consideration of the positive fragments from primary $(M\text{-}H)^-$ ions and an example of this is given in Figure 17. In other cases neither procedure is adequate. In principle, the charge stripping reaction (see above) ($m_1^- \rightarrow m_1^+$) is more sensitive to different isomers than is any type of fragmentation [21]. This is because stripping has an exceptionally high activation energy so that it minimizes selective sampling of ions of high internal energy. Reactions having lower activation energies selectively sample these ions which are just those most likely to have isomerized. The utility of charge stripping in this regard is illustrated by the fact that the $(M\text{-}H)^-$ ion of o-hydroxybenzoic acid undergoes stripping to 20 times the extent that the para isomer does, even though their MIKE spectra are very similar.

V. Prospects

The direct analysis of mixtures by mass spectrometry, and particularly by MIKES, is already established as a rapid, specific and extremely sensitive method which can be applied to samples of a variety of types [8a]. The near future will probably see a further broadening in the range of applications, specifically into more studies on biological systems, including examination of tissue for exogeneous and endogenous materials, surveys of plant material for components of pharmacological or chemotaxonomological interest and studies in drug metabolism and in biosynthesis. Instrumental refinements are also in prospect, particularly those which facilitate data acquisition and reduction and improve quantitative measurements. Further exploration of

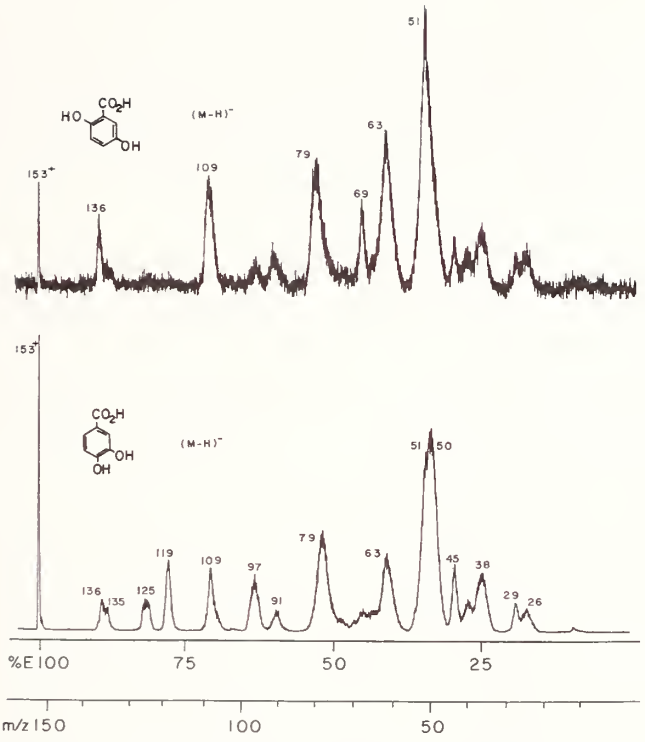

Figure 17. Isomers distinguished by the MIKE spectra of $(M-H)^-$ ions. From ref. 19.

ionization methodology is in hand, including choices of ionizing methods meant to promote more effective collision-induced dissociation. Ionization methods should also be varied in connection with a possible disadvantage of MIKES—the fact that ionization is effected in the presence of many constituents and so may be subject to matrix effects. The extent to which such effects occur should be one focus of future efforts.

We have not emphasized quantitative measurements in this paper because relatively little has been done. The results which have been taken have usually employed calibration curves [13a,20]. It is expected that future measurements will turn to the use of labeled standards. Any difficulties with matrix effects on ionization will be self-cancelling in such procedures. This methodology is well established in gc/ms and should be applicable intact to MIKES.

The least efficient aspect of present instrumentation is the dissociation process. New methods of achieving this are actively being sought. In this regard as well as others the instrumentation currently being used for mixture analysis is not optimized. It was designed for other purposes, specifically high resolution ion kinetic energy spectrometry. Considerable improvements in performance of MIKES instruments are therefore possible. In addition, some of the alternative methods of direct mixture analysis by mass spectrometry given in Table 3 are being or soon will be investigated. Hybrid magnet/quadrupole technology seems particularly attractive. Other important developments are also expected. For example, this methodology is uniquely compatible with pyrolysis techniques. Pyrolysis delivers complex mixtures of gaseous organic compounds in small amounts directly into the ion source; this is precisely the form in which samples are required for MIKES. The power of this combination has been demonstrated in studies on intact DNA [22]. Even the minor modified bases can be securely identified as illustrated in Figure 18. Isomeric bases can be distinguished and larger fragment ions containing several bases are now being obtained and analyzed [23]. These results, together with those on alkaloid identification in intact plant material [10,11] highlight the possible use of MIKES to investigate animal cell constituents.

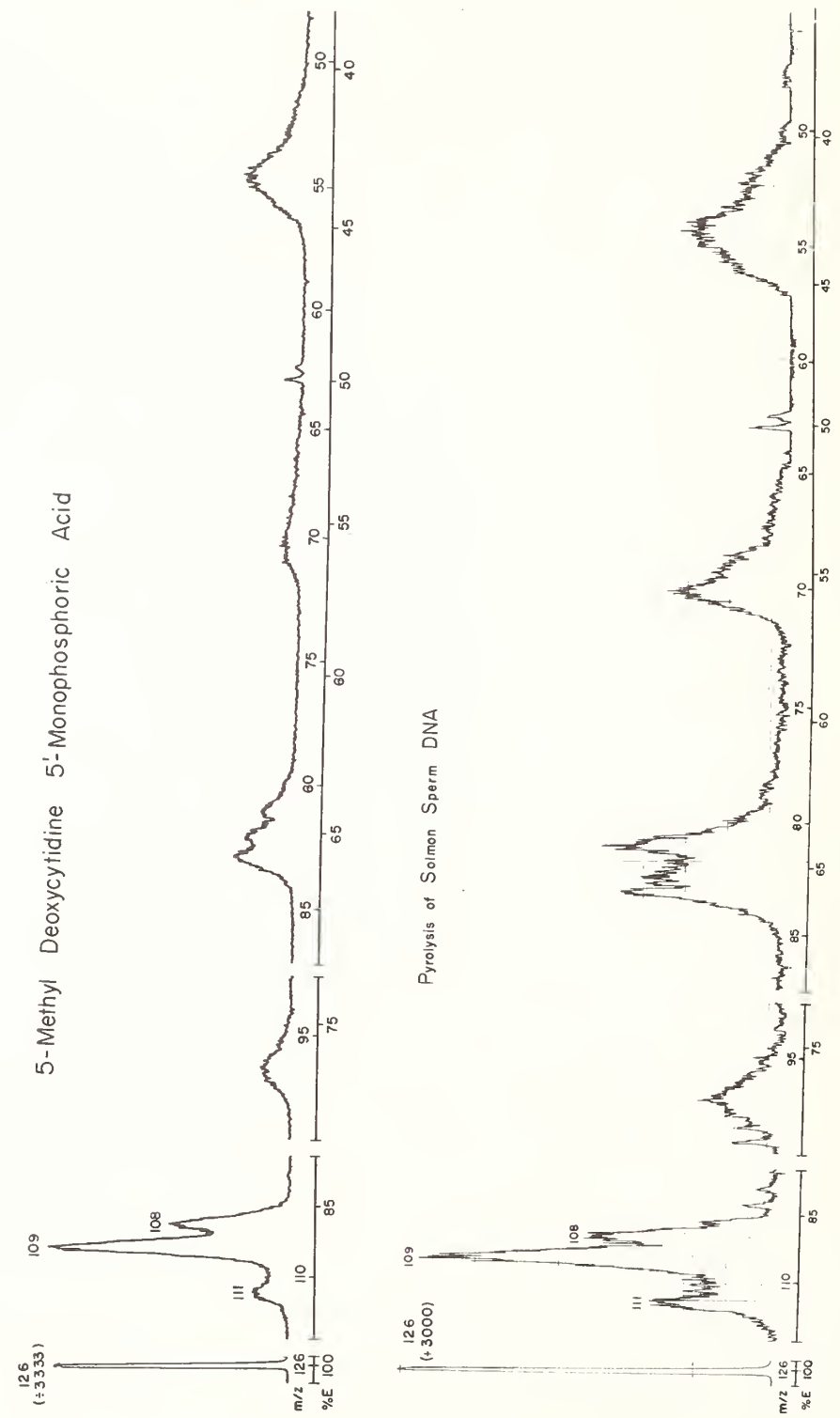

Figure 18. Application of MIKES to biopolymers. Detection of the rare base 5-methyl C in salmon sperm DNA. From ref. 8.

VI. Acknowledgment

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ORGANIC SECONDARY ION MASS SPECTROMETRY

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Secondary ion mass spectrometry (SIMS) provides information on the chemical composition of a solid surface by mass analysis of sputtered ions. Applying extremely small primary ion current densities, the analysis of a single monolayer becomes available (static SIMS). This technique has been applied to metal supported organic compounds such as amino acids, peptides, drugs, pharmaceuticals, vitamins, etc. From virtually all these compounds "parent-like" molecular ions of the general composition $(M+H)^+$ and/or $(M-H)^-$ are emitted with high secondary ion yields in the range of 0.1.

Experimental detection limits below 10^{-9} g were found. The technique offers promising applications in the field of trace detection and analysis, structural investigation, surface reaction studies etc. of high molecular weight organic compounds. It is applicable, without any restriction, to nonvolatile material.

Key words: Amino acids; drugs; nonvolatile organic compounds; organic trace detection; peptides; pharmaceuticals; secondary ion mass spectrometry; sputtering; structural information; vitamins.

I. Introduction

Application of mass spectrometry to nonvolatile samples of organic material presents a difficult problem [1]. Mainly two techniques are applied: field desorption [2] and californium-252 plasma desorption [3]. A new approach is secondary ion mass spectrometry (SIMS) [4–8].

New surface analytical techniques present high absolute sensitivities, in the range of 10^{-3} of one monolayer for Auger electron spectroscopy, 10^{-2} for photo electron spectroscopy, and $<10^{-6}$ for SIMS. It is only SIMS however, which has a compound selectivity sufficient for an application to organic compounds.

During the last years we have demonstrated the capacity of SIMS for the detection of organic compounds [4–8]. This paper gives a short summary of SIMS facts and of the present state of organic SIMS.

II. Secondary Ion Mass Spectrometry

During the penetration of an ion into a solid, its energy is dissipated in a small volume surrounding its way in the lattice. This energy dissipation results in various changes of the surface composition and structure on one hand and in a number of emission processes on the other [5].

In SIMS (schematically represented in Fig. 1), a mass analysis of the emitted positively or negatively charged surface atoms or clusters provides information on the composition of the uppermost molecular layers of the bombarded solid. This uppermost layer will be changed during ion bombardment by sputtering, chemical reactions, etc. The surface area damaged by one single primary ion can be described by a damage cross section σ (Fig. 2). The secondary ion spectrum is given by the emitted ion species X_1, X_2, ...X_i ..., e.g., $Me_nO_n^{\pm}$ in the case of a bombarded metal oxide [4,5].

Figure 1 presents the main elements of a SIMS arrangement. A target area A is bombarded by a primary ion flux density γ. The emitted secondary ions X_i are extracted, mass analyzed and detected, in general by a single ion counting device. The secondary ion yield $S(X_i)$ is the number of emitted ions X_i divided by the number of impinging primary ions. The overall transmission f of

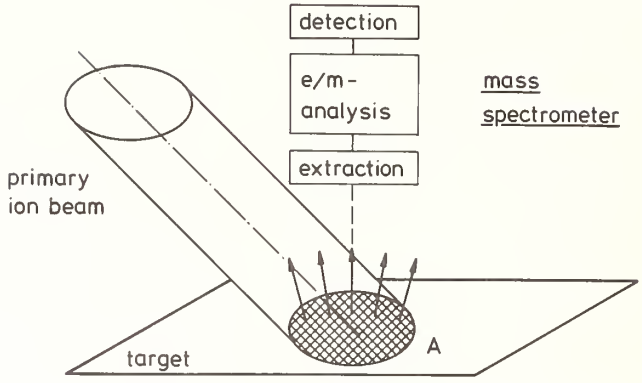

Figure 1. Principal arrangement of a secondary ion mass spectrometer. The sample material is deposited on the target surface.

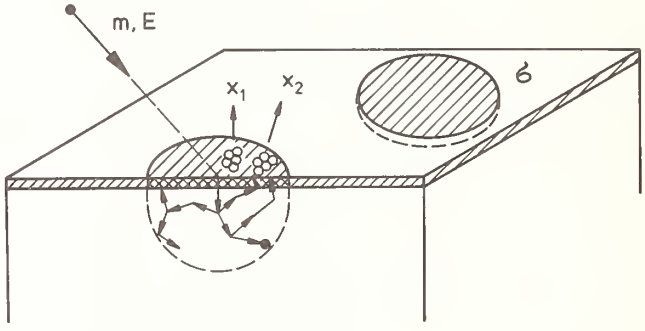

Figure 2. Ion surface interaction. A surface area σ is changed (sputtering, chemical changes etc.) by one impinging primary ion (m: mass; E: energy).

the spectrometer is given by the number of detected ions X_i, divided by the number of secondary ions X_i emitted from the target.

The counting rate of the detection unit for a certain ion species X_i is given by:

$$\delta = \gamma \cdot A \cdot S \cdot f \qquad (1)$$

This equation only holds for a target of the same composition on the surface and in the bulk. If a target is covered only by a single monolayer of the sample material, the damaging effect of the impinging primary ions has to be considered. This results in a time dependence of the relative surface coverage $\theta(t)$:

$$\theta(t) = \theta(o) \exp(-\sigma \cdot \gamma \cdot t) \qquad (2)$$

The average life of such a monolayer is given by $\Sigma = (1/\sigma \cdot \gamma)$. Now the counting rate δ for a secondary ion becomes proportional to $\theta(t)$:

$$\delta(t) = \theta(o) \exp(-\sigma \cdot \gamma \cdot t) \qquad (3)$$

This time dependence always is observed when a single monolayer on a surface is sputtered by a primary ion beam of uniform flux density γ. If the time t of ion bombardment is very small

compared against τ, the secondary ion emission originates from the virtually undisturbed upper most monolayer. This mode of SIMS operation with $t < \tau$, is called static SIMS [4,5].

III. Secondary Ion Emission of Organic Compounds

A. Historical Development

During the last decade we have studied systematically the emission of molecular secondary ions. As one of the general results we found a strong emission of negatively charged anion complexes (like SO_4^-, NO_3^- etc.) and their fragments from metal surfaces covered by the corresponding compound. More recently we found a strong emission of $(M+1)^+$, $(M-45)^+$ and $(M-1)^-$ from metal supported amino acids [6–8]. Continuing such investigations we studied the secondary ion emission of other organic compounds, e.g., peptides, derivatives of amino acids, various vitamins, pharmaceuticals, and drugs, and found strong quasimolecular ions for all these compounds [8,9].

B. Sample Preparation

Nearly all our investigations were carried out with silver supported samples. The routine preparation was as follows: The sample material was dissolved in water, typically 10 μg/μL. The silver target was cleaned with nitric acid, rinsed in distilled water and then dipped into the sample solution. After shaking off most of the solution the target was dried for some minutes and mounted in the mass spectrometer. No influence of dipping and drying time on the resulting spectra was observed.

A more defined amount of sample material could be deposited on a more defined target area with a micropipette: 1 μL of the solution mentioned above could be smeared out on a target area of 1 cm^2. By this way 1 μg of sample material could be deposited on the bombarded area of 0.1 cm^2.

C. Results for Different Groups of Organic Compounds

The SIMS behaviour of a metal supported organic sample can be described by its secondary ion spectrum X_1, X_2, ...X_i..., the yields $S(X_i)$ for these ions, and the damage cross section σ. Figures 3–6 give some typical examples of positive and negative spectra. In these, as in all spectra of organic compounds which we have recorded some ion groups can be distinguished:

- Quasimolecular ions such as $(M\text{-}OH)^+$ and $(M+H)^+$ for ephedrine, $(M-H)^-$ for nicotinic acid, or $(M+H)^+$ and $(M-H)^-$ for sulphanilamide.
- Secondary ions originating from the underlying silver (e.g., Ag^+, $AgCl_2^-$). The appearance of these ions indicates that the silver surface was not completely covered by the sample material.
- Characteristic large fragment ions in the positive as well as in the negative spectrum.
- Smaller fragment ions, mainly in the low mass range below m/e 100.

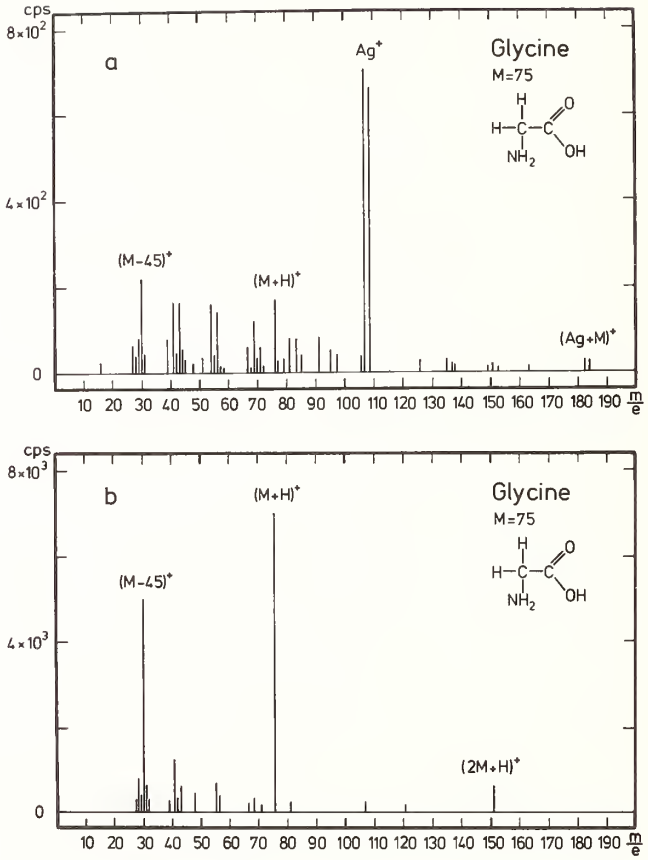

Figure 3. Positive secondary ion spectra of glycine on silver. a) routine preparation from a 10 μg/μL-solution; b) preparation from higher concentrated and acidified solution (50 μg/μL, pH=2. Primary ions: $4 \cdot 10^{-10}$ A, Ar^+, 2.25 keV. Bombarded target area: 0.1 cm^2. Scan time for one spectrum: 500's.

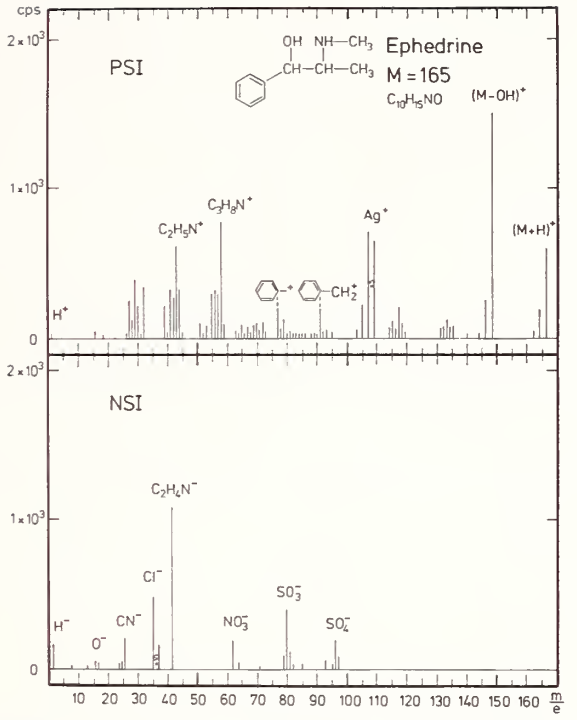

Figure 4. Positive and negative secondary ion spectra of ephedrine. Same experimental conditions as in figure 3a.

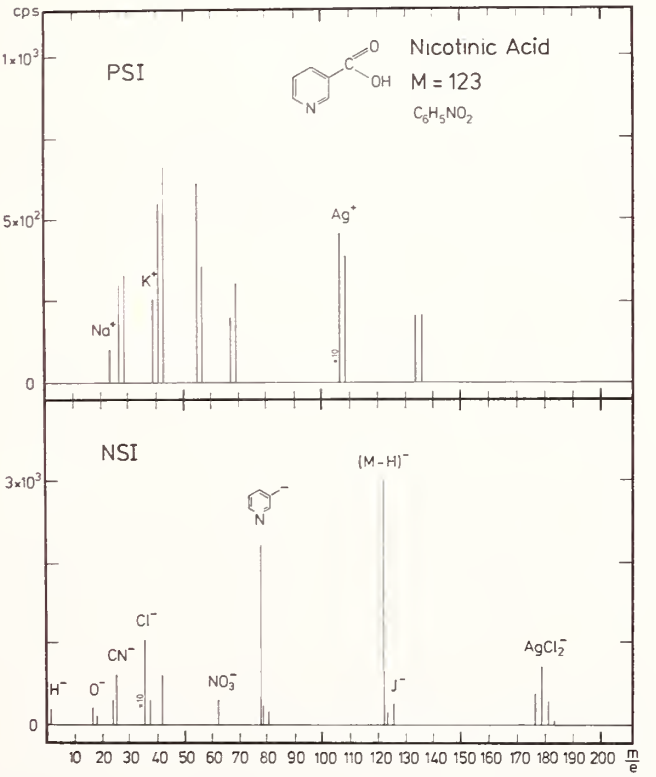

Figure 5. Positive and negative secondary ion spectra of nicotinic acid. Same experimental conditions as in figure 3a.

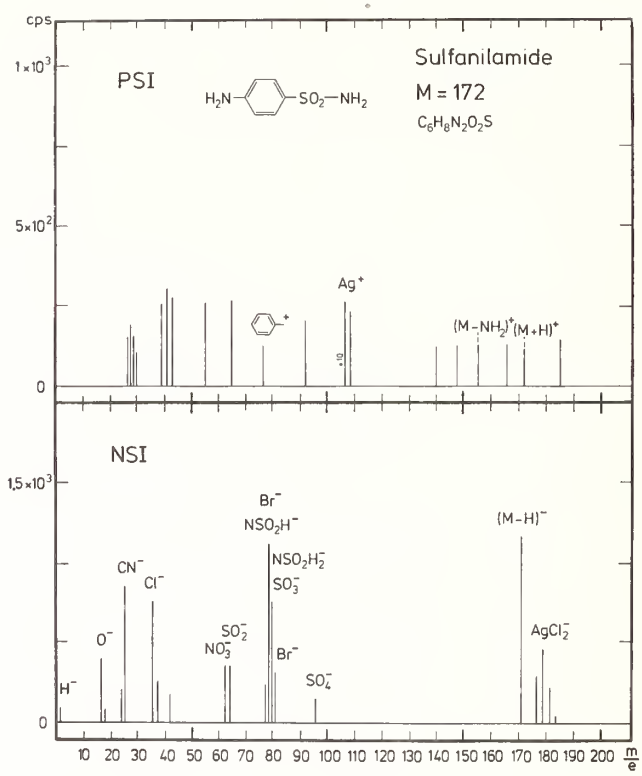

Figure 6. Positive and negative secondary ion spectra of sulfanilamide. Same experimental conditions as in figure 3a.

During bombardment of the samples with higher primary ion current densities, we always observed a decrease of the secondary ions which are characteristic for the sample material. This decrease follows roughly an exponential law (Fig. 7). $S(X_i)$ and σ can be derived directly from this time dependence. It is

$$\tau = \frac{1}{\sigma \cdot \gamma}; \quad \sigma = \frac{1}{\gamma \cdot \tau} \tag{4}$$

and

$$\delta(o) = \gamma \cdot A \cdot S \cdot \theta(o) \cdot f; \quad S = \frac{\delta(o)}{\gamma \cdot A \cdot \theta(o) \cdot f} \tag{5}$$

In Tables 1 and 2 the most important quasimolecular secondary ions emitted from 40 different organic compounds are listed. It should be mentioned here, that in equation 5 the surface coverage $\theta(o)$ has been supposed to be 1. This is not true for most of our targets as it can be seen from the appearance of Ag containing ions. Therefore the S values of the tables are probably too low.

The exponential time dependence observed for the characteristic secondary ion X_i may have two reasons: The sample material may be only in a monolayer on the metal surface or it is in a multilayer island structure on the surface, but during the removal of the first monolayer, the underlaying sample material is decomposed. In both cases an exponential decrease of δ results.

We have determined the damage cross section σ for glycine, cysteine and serine from equation (4). The resulting cross sections were in the range of 10^{-14} cm^2. Large errors due to surface roughness etc. could not be excluded.

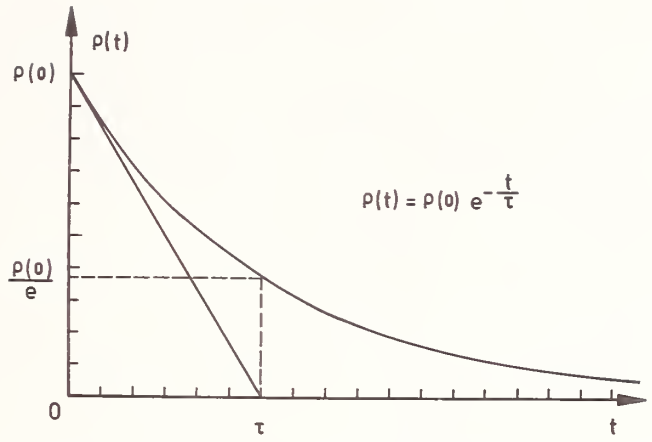

Figure 7. Decrease of the counting rate $\rho(t)$ for an ion species X_i, originating from a mono- or submonolayer sample on the target (see text, eqs. 1–5).

TABLE 1. *Absolute yields S(X) of "parent-like" secondary ions of 18 amino acids on silver. Primary ions: Ar^+, 2.25 keV, primary ion dose density $4 \cdot 10^{-6}$ A·s·cm^{-2} (static SIMS).*

Compound	Formula	Yield S(X)×100		
		$(M+H)^+$	$(M-H)^-$	$(M-COOH)^+$
Glycine	$C_2H_5NO_2$	120.0	-	52.0
A-Alanine	$C_3H_7NO_2$	21.0	40.0	53.0
B-Alanine	$C_3H_7NO_2$	88.0	19.5	7.2
Phenylalanine	$C_9H_{11}NO_2$	4.0	0.3	13.0
Serine	$C_3H_7NO_3$	61.0	18.0	61.0
Threonine	$C_4H_9NO_3$	8.3	1.6	13.8
Proline	$C_5H_9NO_2$	19.2	8.8	72.0
Valine	$C_5H_{11}NO_2$	8.0	8.3	32.0
Leucine	$C_6H_{13}NO_2$	0.8	26.4	40.0
Norleucine	$C_6H_{13}NO_2$	24.8	6.5	76.0
Arginine	$C_6H_{14}N_4O_2$	7.2	2.4	2.1
Tyrosine	$C_9H_{11}NO_3$	7.4	-	13.6
Tryptophan	$C_{11}H_{12}N_2O_2$	3.5	0.8	3.5
Cysteine	$C_3H_7NO_2S$	12.0	11.0	15.0
Cystine	$C_6H_{12}N_2O_4S_2$	4.0	1.6	1.8
Methionine	$C_5H_{11}NO_2S$	13.1	5.4	9.4
Ethionine	$C_6H_{13}NO_2S$	13.6	5.6	12.0
Glutamine	$C_5H_{10}N_2O_3$	7.2	8.3	4.3

TABLE 2. *Absolute yields S(X) of "parent-like" secondary ions of various organic compounds on silver. Same experimental conditions as in Table 1.*

Compound	Formula	Yield S(X)×100		
DERIVATIVES OF AMINO ACIDS		$(M-H)^-$	$(M'-H)^-$	$(M-Cl)^+$
Glycine ethylester HCL	$C_4H_{10}ClNO_2$	-	1.6	180.0
Alanine ethylester HCL	$C_5H_{12}ClNO_2$	-	-	48.0
Cysteinium HCL	$C_3H_8ClNO_2S$	-	4.0	19.7
Taurine	$C_2H_7NO_3S$	4.8	-	-
M′=mass of related amino acid, identical with $(M'+H)^+$				
PEPTIDES		$(M+H)^+$	$(M-H)^-$	$(M-COOH)^+$
Glycylglycine	$C_4H_8N_2O_3$	41.6	4.8	-
Glycylglycylglycine	$C_6H_{11}N_3O_4$	4.0	0.4	2.0
Glycylleucine	$C_8H_{16}N_2O_3$	1.6	4.2	3.0
Phenylalanylglycine	$C_{11}H_{14}N_2O_3$	3.8	1.6	-
DRUGS		$(M+H)^+$	$(M-H)^-$	$(N-OH)^+$
Barbital	$C_8H_{12}N_2O_3$	-	44.0	-
Ephedrine	$C_{10}H_{15}NO$	16.0	-	40.0
Atropine	$C_{17}H_{23}NO_3$	84.8*	-	-
Epinephrine	$C_9H_{13}NO_3$	-	6.4	-
*M^+				
VITAMINS		$(M+H)^+$	$(M-H)$	
Ascorbic Acid (C)	$C_6H_8O_6$	3.7	17.6	
Biotin (H)	$C_{10}H_{16}N_2O_3S$	0.3	4.2	
Nicotinic Acid (PP)	$C_6H_5NO_2$	-	46.4	
Nicotinamide	$C_6N_6N_2O$	2.1	15.2*	
*M^-				
SULFONAMIDES		$(M+H)^+$	$(N-H)^-$	
Sulfanilic Acid	$C_6H_7NO_3S$	-	16.3	
Sulfanilamide	$C_6H_8N_2O_2S$	0.6	17.6	
Sulfacetamide	$C_8H_{10}N_2O_3S$	-	20.8	
OTHER COMPOUNDS		$(M+H)^+$	$(M-H)^-$	
Thymidine	$C_{10}H_{14}N_2O_5$	1.9	1.3	
Acriflavine	$C_{14}H_{14}ClN_3$	-	-	96.0*
Creatine	$C_4H_9N_3O_2$	2.9	-	3.4**
Creatinine	$C_4H_7N_3O$	16.0	6.0	6.0***

*$(M-Cl)^+$
**$(M+H-H_2O)^+$
***$(M+H+H_2O)^+$

For practical application as well as for fundamental investigations an important question concerns the reproducibility of spectra, yields, and damage cross sections as well as its dependence of the sample preparation and excitation. Although not too many systematic investigations have been carried out up to now, some general features can be summarized.

A systematic investigation of the reproducibility of spectra, yield values $S(X_i)$ and damage cross sections σ for glycine, serine and cysteine on silver, excited by He, Ne, Ar, and Kr ions in an energy range between 0.75 and 2.25 keV have been carried out recently [10]. All experimental results came out to be reproducible within a factor 2, if the target preparation was carried out by the micropipette technique. No significant change in the secondary ion spectra was observed for the different primary ions in the investigated energy range. We found only a slight increase of S and σ with increasing primary ion energy, as is expected from the energy dependence of

sputtering yields in this energy range. A similar small increase of S and σ was observed for increasing mass of the bombarding ion.

We studied the influence of sample preparation in more detail for glycine and silver. A significant result was an increased of the ratio $(M+1)^+/(M\text{-}1)^-$ with increasing acidity of the sample solution [7–9]. This observation agrees with the general observation of increasing $(M+H)^+$ emission with increasing acidity of the solution for many compounds in field desorption.

D. Detection Limit

An important feature of an analytical technique is its detection limit or its sensitivity for a certain sample material. For organic SIMS this sensitivity will strongly depend on S and σ. The detection limit can be calculated in two different ways: We can start with the assumption of a metal, and the well known values of S and σ. From this results a more theoretical value for the detection limit. If we carry out this calculation for glycine on silver, taking $(M+1)$ with $S=1$ as the significant secondary ion, and assuming a damage cross section of about 10^{-14} cm^2, we result in a detection limit below 10^{-14} g. This is true only if we know the significant secondary ion and do not record the complete spectrum. Another more realistic estimation of the detection limit can be carried out in the following way: By the micropipette technique we are able to bring 1 μg glycine on the bombarded target area of 1 cm^2. This sample gives a secondary ion emission of $(M+1)^+$ in the 10^5 cps range for about 1000 s. If we don't know the characteristic ion, we have to record the complete spectrum. This results in a practical detection limit of $<10^{-9}$g.

E. Ion Formation

From the general mode of secondary ion emission, a strong fragmentation of compounds is expected, as is observed for metal oxides, e.g., [4,5]. The emission of unfragmented ions composed of about 30 atoms (see tables) should be a nearly impossible process. Therefore we have to consider new models for the emission of quasimolecular ions of high molecular weight organic compounds, as was observed in our investigation. Such emission processes may be the result of collective excitation of large surface areas by the impact cascades.

IV. Conclusion

Organic SIMS seems to be a powerful tool for the detection, structural investigation and analysis of high molecular weight organic compounds. High intensity emission of quasimolecular ions of the general composition $(M+H)^+$, $(M\text{-}H)^-$, $(M\text{-}45)^+$, etc. was observed for amino acids, peptides, pharmaceuticals, drugs, etc. Typical secondary ion yields were in the range of 0.1, for 2.25 keV Ar^+ as the primary ion. Typical damage cross sections are in the 10^{-14} cm^2 range. Therefore only static SIMS could be applied. It is difficult to understand the sputtering of nonvolatile organic compounds by the actual sputtering models. New models for energy transfer to surface molecules seems to be necessary.

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THE WIRE TRANSPORT LC/MS SYSTEM

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The two basic types of interface for LC/MS systems will be described and the characteristics of these interfaces that can affect column performance will be discussed in detail. The design of direct inlet systems developed by McLafferty will then be described and the results obtained from this device given. The wire transport interface system developed by Scott et al., will also be discussed, together with the results obtained using it in conjunction with high efficiency microparticulate columns. Finally, the modified form of wire transport system devised by McFadden et al. and utilizing a belt transport system will be described, and the results obtained from the Finnegan LC/MS system utilizing the belt transport interface given.

Key words: Liquid chromatography/mass spectrometry; pesticides; vitamin A; wire transport.

Over the past decade liquid chromatographic separations have become far more sophisticated as a result of the introduction of high efficiency microparticulate columns. The improved efficiencies have permitted more complex multi-component mixtures to be separated which in turn has introduced the problem of the structure elucidation of the individual components. Thus the concept of a LC/MS system has followed the development of liquid chromatographic separations in much the same way that GC/MS followed the development of gas chromatography. The association of the liquid chromatograph with the mass spectrometer, however, is far more complex than its GC/MS counterpart. In gas chromatography the mobile phase, normally helium or nitrogen, on entering the spectrometer does not interfere with the performance of the mass spectrometer, provided adequate pumping rates are employed. The LC/MS system, however, requires either the removal of the solvent prior to entering the mass spectrometer or its removal in the mass spectrometer. In the latter case the solvent can be used as a chemical ionization medium as in the interface developed by McLafferty [1–3].

In general, when two techniques are associated, in order to obtain the advantages of both, the interface system must be carefully designed so that the performance of neither system is compromised. In the case of the LC/MS system, from the point of view of the mass spectrometer the following conditions must be maintained.

1. Adequate low pressure must be maintained in the ion source to permit optimum performance of the mass spectrometer.

2. Any mobile phase entering the mass spectrometer must be consistent in properties or have no effect on the spectra produced. If the solvent composition changes, as with gradient elution development, and if the solvent is being employed also as the chemical ionization agent, the change in composition of the solvent must not affect the quality of the spectra produced.

3. Sufficient quantities of sample must be introduced by the interface to provide satisfactory spectra.

A further point needs to be considered, in that electron impact spectra generally provide more information for structural elucidation than chemical ionization spectra, particularly where a completely unknown substance is being identified. It follows that the interface should therefore permit both types of ionization methods to be used. Another important point to be considered in the interface system is that the majority of substances separated by liquid chromatography are relatively involatile. Otherwise it is likely that they would be more appropriately separated by a

gas chromatography procedure. Thus to obtain spectra from relatively involatile materials a type of probe sampling system would be preferred and an introduction system that depends on the volatility of the eluted samples at relatively low temperatures should be avoided if possible.

In a similar manner the liquid chromatograph will make demands on the interface system to insure that its performance is also not compromised. The following requirements are necessary for the interface to ensure optimum performance of the liquid chromatograph.

1. The versatility of the liquid chromatograph must be maintained. permitting free choice of solvent for the mobile phase, together with different development procedures such as gradient elution, flow programming or temperature programming.

2. The interface LC/MS combination must provide adequate sensitivity so that sufficiently small charges can be placed on the column to ensure that sample overload does not take place.

3. There must be zero or minimum dispersion in the interface to maintain chromatographic resolution.

The prevention of band dispersion in the interface is probably the major problem to be overcome in interfacing a liquid chromatograph with a mass spectrometer. If dispersion occurs the individual discrete bands eluted from the column can merge and the resolution obtained by the column is partly or completely destroyed. This dispersion problem is present even in liquid chromatography detectors, and this effect can be clearly seen in Figure 1. In Figure 1 the elution curves for a solute eluted from the same column under identical conditions but employing detectors having cell volumes of 25, 8 and 3 μL, respectively, is shown. Bearing in mind that a 5 cm length of capillary tubing 0.25 mm in diameter has a volume of 2.5 μL, it is obvious that in the LC/MS interface, tubular connections have to be kept to a minimum. It is also interesting to note that modern liquid chromatography detectors for the most part have such high cell volumes and connecting tubes that the full efficiency obtainable from microparticulate columns is never fully realized.

In order to meet all the requirements listed above, the wire transport system was considered the most appropriate interface [4,5]. The wire could take the sample as a thin film on its surface directly from the end of the column, thus eliminating all connecting tubing and band dispersion; the solvent could be evaporated from the wire, leaving the solvent on the surface prior to entering the mass spectrometer, in this way eliminating the contamination of the mass spectrometer with solvent vapor. Finally, the wire could act as a continuous probe sampling device and if designed correctly could be used to volatilize relatively involatile materials.

A diagram of the wire transport LC/MS system is shown in Figure 2. The wire drive mechanism was that used in the wire transport detector manufactured by Philips Chromatography. The wire, stainless steel, 0.1 mm in diameter, passed over an insulated pulley to a coating block

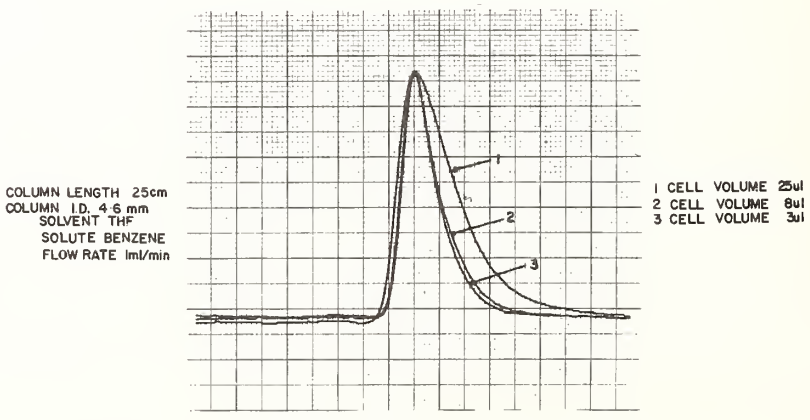

Figure 1. Peak profiles from detectors having different cell dimensions.

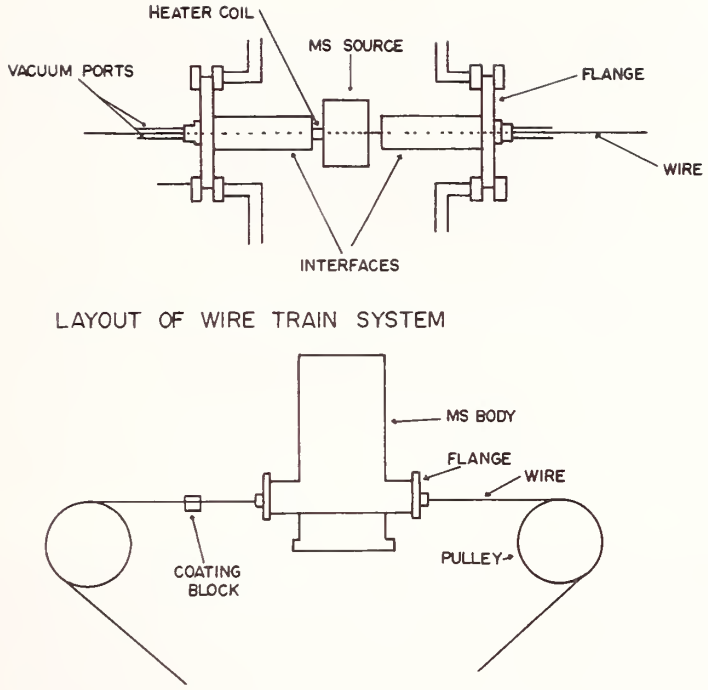

Figure 2. Diagram of interfaces located in MS source.

where the column eluent flowed directly over the wire and then through one interface into a Finnigan quadrupole mass spectrometer. The wire then passed directly through the source and out through a second interface over another insulated pulley, back to the winding spool. The quadrupole mass spectrometer was chosen because the ion source was operated at voltages close to earth potential, and thus the presence of an earthed wire close to the source would not affect the ion optics of the mass spectrometer. As seen in Figure 2 the interfaces were reentrant in design such that they terminated a few millimeters from the ion source. In order to volatilize the sample in the ion source a voltage was applied between the two pulleys. The current through the wire was about 200 mA, and while exposed to the air or in the interface the evolved heat was rapidly lost to the atmosphere by conduction and convection. When the wire passed through the ion source, however, the pressure maintained at this point was 10^{-6} mm of mercury and under these conditions heat could not be lost from the wire and thus the temperature of the wire rapidly rose and volatilized the sample directly in the ion source.

The interface is shown in Figure 3. Basically is consists of two small chambers separated and terminated by ruby jewels. Each jewel is 2.5 mm in diameter, about 0.5 mm thick and carries a hole in the center 0.25 mm in diameter to permit the passage of the wire. The jewels were set into the interface by high temperature thermal setting resin. The first chamber, between the first and second jewel, was connected to a rotary pump having a pumping capacity of about 150 L/min. The second chamber, between the second and third jewel, was connected to an oil diffusion pump which was also backed by a 150 L/min rotary pump. This system maintained a pressure of about 1 mm of mercury in the first chamber, about 5×10^{-3} mm of mercury in the second chamber and a pressure of 10^{-6} mm of mercury in the ion source. Two such interfaces were employed, one to permit the carrier to enter the source and the other to permit it to leave. There was little "scuffing" of the wire by the jewel aperture, as there was only point contact between the wire and the internal surface of the jewel aperture. Furthermore, any "scuffing" that did take place appeared to produce no "memory" effects that could be measured. As a result clean uncontaminated spectra could be obtained.

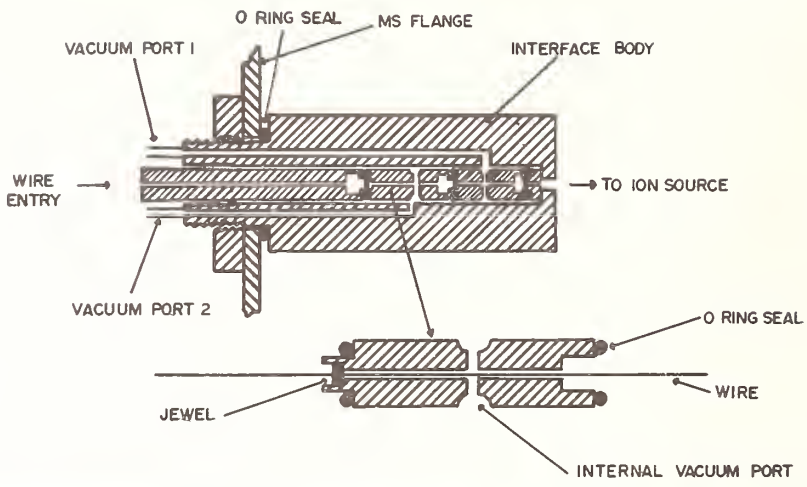

Figure 3. The LC/MS interface.

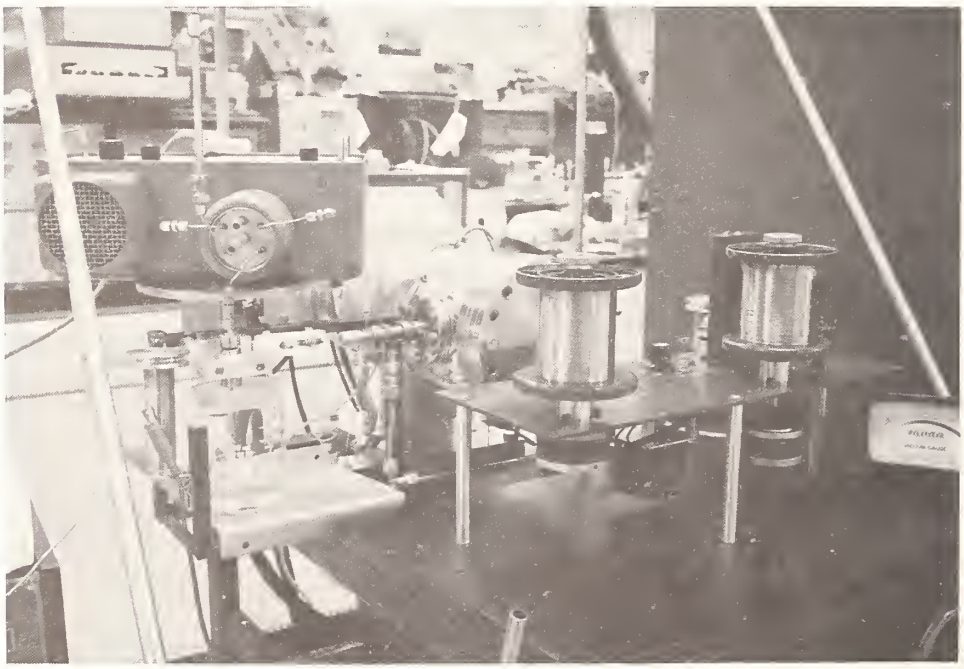

Figure 4. Photograph of the LC/MS apparatus.

The mass spectrometer was used together with the Finnigan data processing system. This consisted of a PDP8M computer fitted with a Diablo disc having a 1.7×10^6 word capacity, and a dual tape deck, operated by a Tektronix 4010-1 CRT terminal in conjunction with a Houston Complot Plotter and a Tektronix 4610 hard copier. Spectra could be obtained continuously during chromatographic development in much the same way as in GC/MS. Subsequent to the separation being completed, individual spectra could be obtained at any point during the elution of a solute, total ion current chromatograms could be obtained as required, and also chromatograms monitored on a particular ion mass.

A photograph of the original prototype instrument is shown in Figure 4. In Figure 4 the interfaces are not reentrant in design but are fabricated in exactly the same way as those shown in Figure 3 but are external to the ion source. On the left is shown the column connected to a LDC

refractometer which in turn is connected to the coating block through which the wire passes. The quadrupole ion source housing and the left hand interface are seen behind the wire train system. The connection between the interface and the respective pump are seen as vertical tubes joined to the horizontal interface. The two insulated pulleys are seen at the extreme left of the figure and behind the right hand wire spool.

The wire transport LC/MS system can be used very effectively as an automatic probe sampling device. In Figure 5 is shown the total ion current peaks for the manual spotting of individual samples onto the wire. The concentration of each solute was about 0.01% w/v. A sample was placed on the wire about every 25 s and thus, using a normal automatic gas chromatography sampling device, samples could be placed on the wire continuously and consecutively, which could provide a very useful automatic probe sampling system. In Figure 5 sample spectra are included together with examples of single ion monitoring, employing the major ion, of individual solutes and are shown on the left hand side of the figure. An example of an LC/MS analysis is shown in Figure 6. The chromatogram of the mixture obtained by total ion monitoring is shown at the top on the left hand side of the figure. The spectra of two of the individual components are shown on the right hand side, and the chromatograms obtained by single ion monitoring using the characteristic ion are shown again on the left hand side of the figure to permit comparison with the original chromatogram. Little or no band dispersion occurs in the interface, and this is clearly shown in Figure 7 by the total ion chromatogram obtained by gradient elution development of a sample of vitamin A acetate mother liquor. It is seen that excellent separation is maintained and the peaks remain sharp and well resolved. The wire carries about 10 μL/min of eluent from the column to the detector, and for normal liquid chromatography operation, column flow rates of about 1 mL/min are employed. It follows that the interface only takes 1% of the eluent into the mass spectrometer. This situation can be looked at in two ways. It either reduces the sensitivity by two orders when looking at trace materials or, conversely, allows most of the eluted solute to be recovered for other analytical procedures. As the sensitivity of the LC/MS system described above is only about 10^{-6} g/mL at a signal-to-noise ratio of two, adverse effects on sensitivity might be considered a serious limitation of the system. If microbore columns were employed normally 1 m long, 1 mm i.d., then flow rates of 10 to 40 μL/min would be employed and under such circumstances the whole of the eluent or 50% of the eluent could be passed directly to the mass spectrometer, and thus its sensitivity would be much improved. In absolute terms it was shown that 10 nanogram/s of solute entering the mass spectrometer was sufficient to provide good spectra.

To avoid the sensitivity problem when using normal wide bore columns, McFadden [6] developed the band interface system shown in Figure 8. In principle, the system is the same as the wire transport interface except that a continuous ribbon is employed as the transport device, which can sample a far greater quantity of column eluent and thus carry more of the solute into the mass spectrometer. McFadden used the same type of interface, in that he also employed two differential pumping chambers separated and terminated by ruby jewels. The solvent was evaporated from the ribbon by means of an infrared reflector before it entered the interface. The solute was vaporized from the ribbon and into the ion source by a separate heater situated close to the source filament. The ribbon then passed over a final heater for cleaning purposes before being recoated with more column eluent. In this way McFadden was able to take the complete eluent from a column operating at 0.8 or 1.0 mL/min solvent flow rate. The whole of the solutes contained in the eluent were then passed into the mass spectrometer. Examples of the spectra obtained from his device together with total ion current chromatograms are shown in Figure 9.

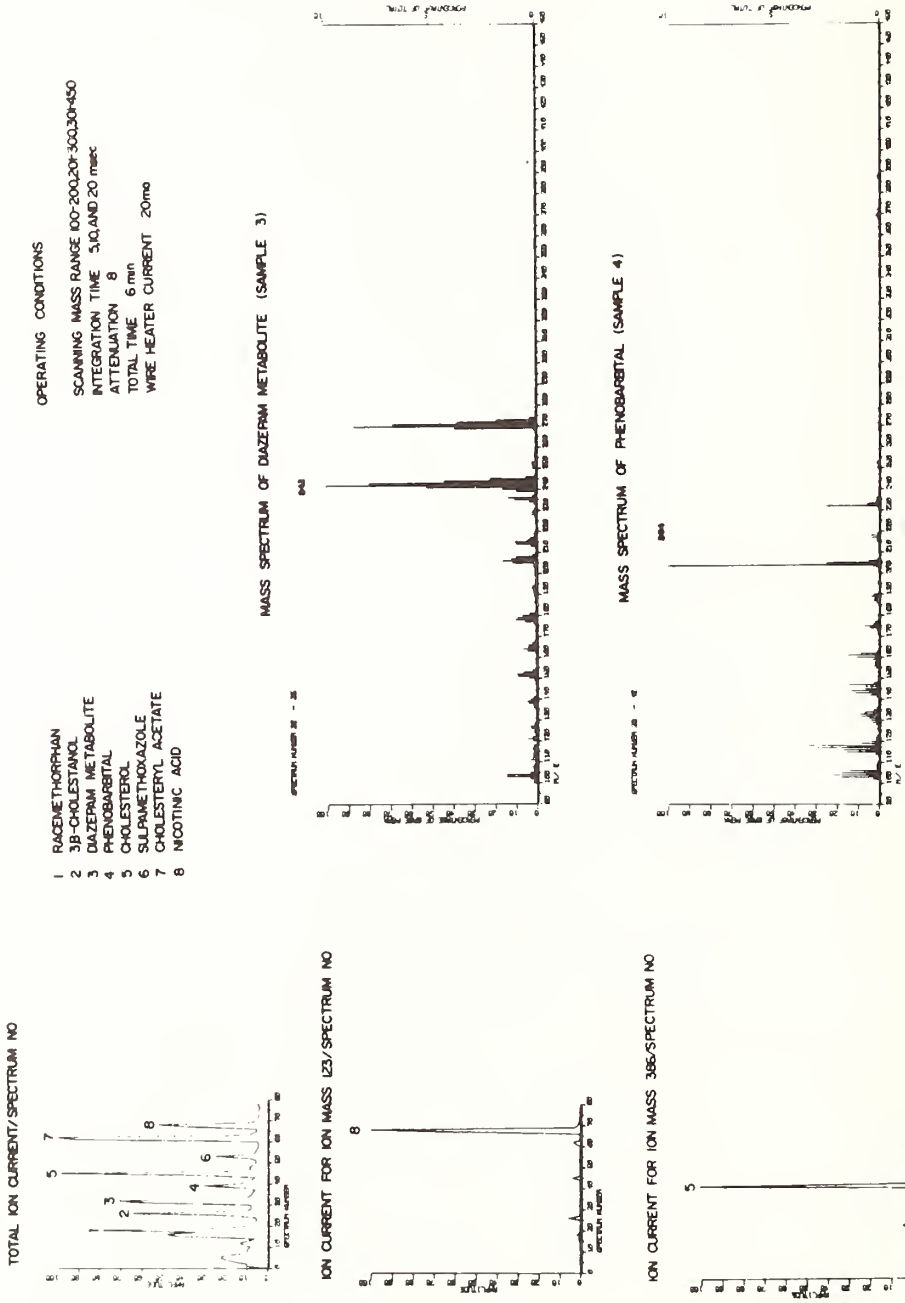

Figure 5. The application of the LC/MS system as an alternative to probe sampling.

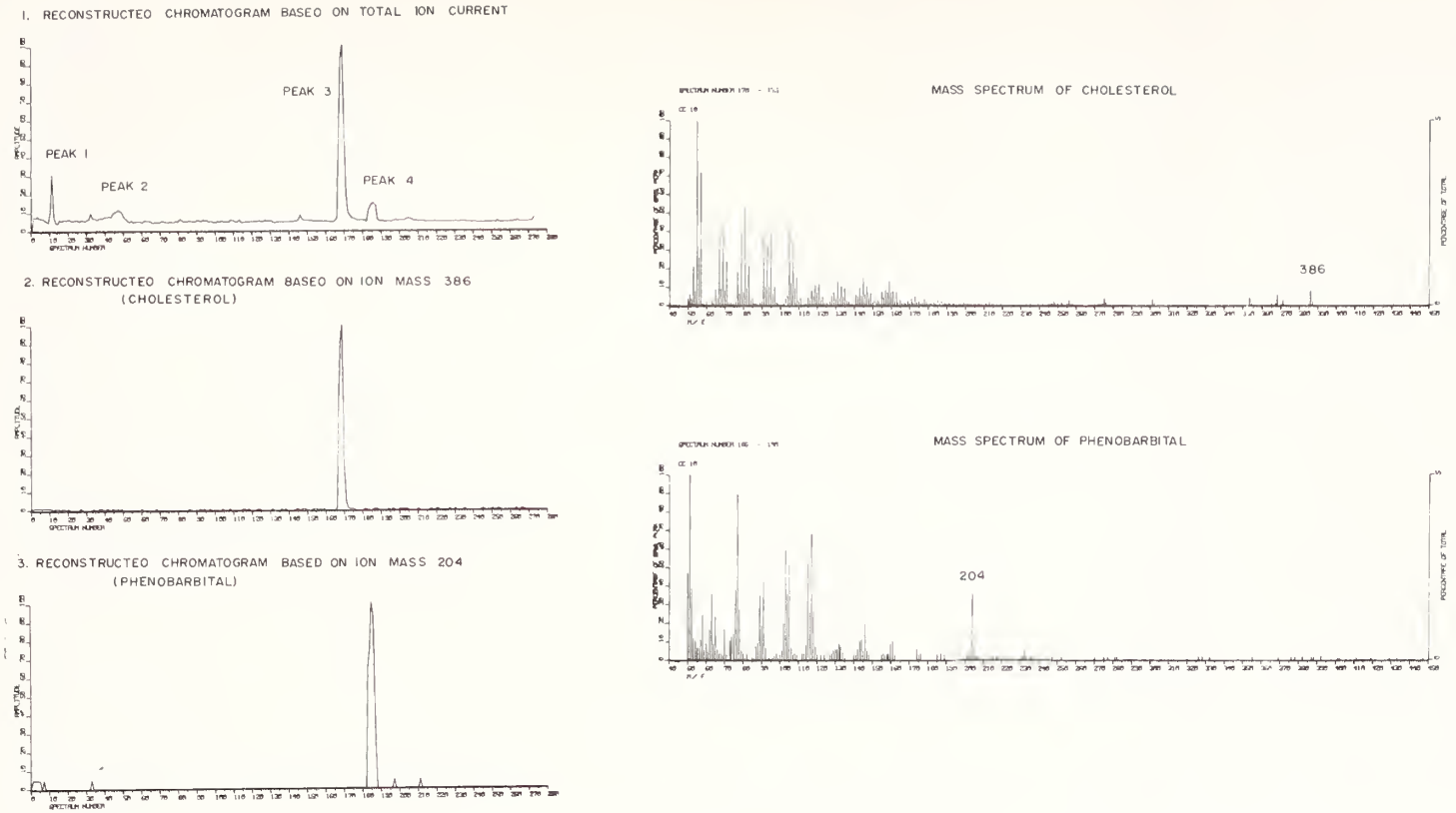

Figure 6. LC/MS analysis of a synthetic mixture.

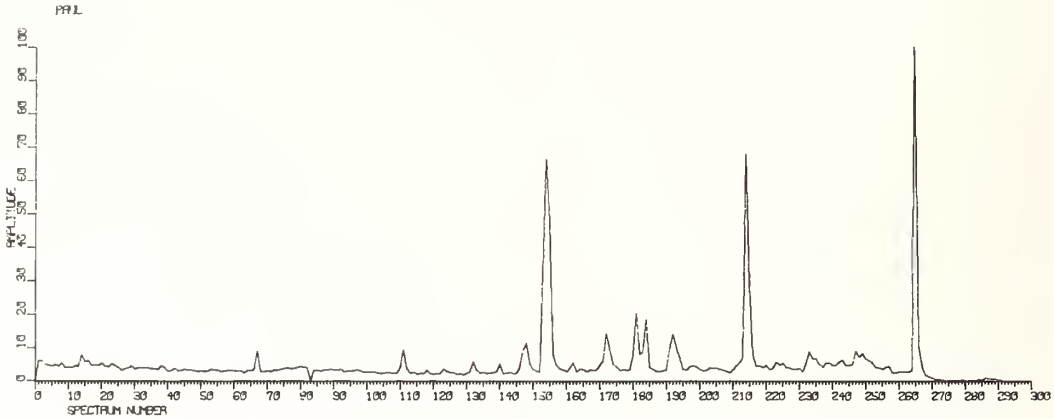

Figure 7. Total ion current chromatogram of vitamin A acetate mother liquor.

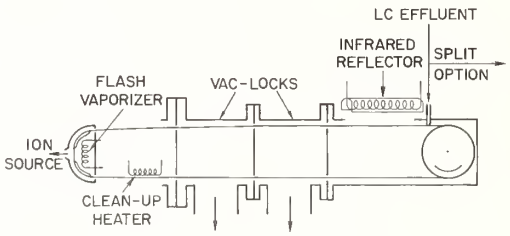

Figure 8. Finnigan belt transport interface system.

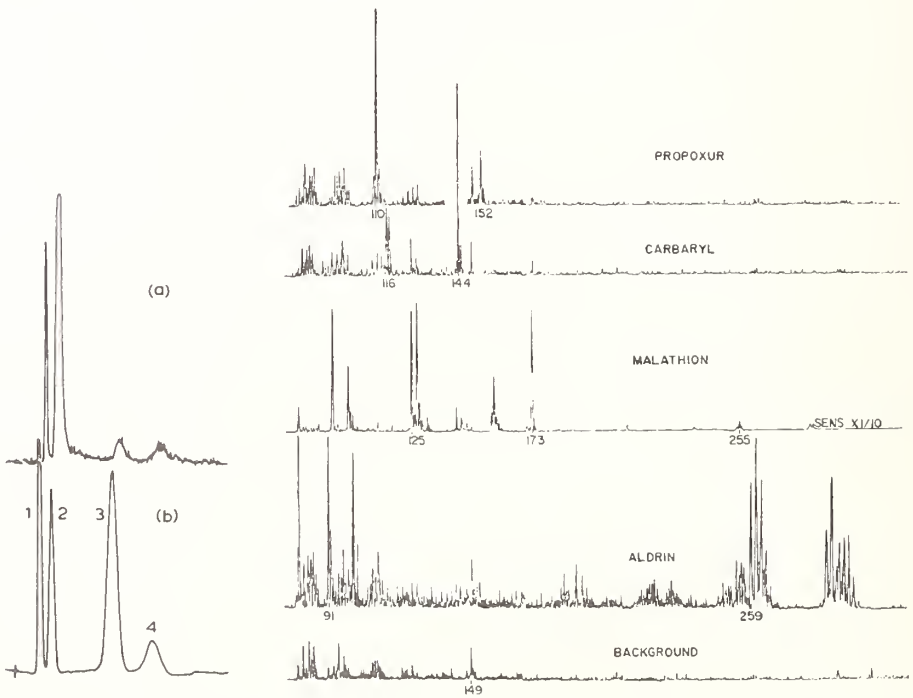

Figure 9. Example of the total ion current chromatograms and mass spectra for the Finnigan LC/MS systems.

The transport method for interfacing a liquid chromatograph with a mass spectrometer is still in the early stages of development, but so far it has shown great promise, and the device devised by McFadden is now commercially available. Summing up its advantages, it can be said that

1. It does not inhibit the conditions of operation of the liquid chromatograph in any way.
2. It can provide electron impact spectra or chemical ionization spectra as required.
3. Providing the potential of the source is not far from earth potential, it does not inhibit the performance of the mass spectrometer.
4. It will provide the mass spectrum of any sample that can be handled by a probe sampling device.
5. It can be used exclusively as an automatic probe sampling system.

The systems presently available do suffer from relatively low sensitivity, however. As stated in the original paper [1] these can probably be improved significantly by the use of an alternative vaporizing procedure by heating the wire or ribbon with infrared radiation or by laser light.

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TECHNIQUES FOR COMBINED LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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High pressure liquid chromatography (HPLC) has recently become established as a powerful separation technique with many environmental and biomedical applications. HPLC is especially valuable for polar, involatile, or thermally unstable compounds not amenable to gas chromatography. The most serious obstacles to further rapid development of HPLC appear to be problems associated with the versatility, selectivity, and sensitivity of available detectors. The conbination of mass spectrometry with HPLC is an obvious possible solution to these detector problems, but the technical problems involved in the LC-MS interface are substantially more difficult than those encountered in the analogous development of GC-MS.

This paper presents a review of the current status of combined LC-MS systems and focuses on techniques which have demonstrated capabilities for trace organic analysis not amenable to GC-MS. Several successful approaches to LC-MS interfacing are described and the demonstrated performances of the various techniques are compared. Selected examples are given of applications of combined LC-MS systems to trace organic analysis of environmental and biomedical samples.

Key words: Laser ionization; laser vaporization; liquid chromatography; liquid chromatography/mass spectrometry.

I. Introduction

Several approaches are currently being studied for LC-MS interfacing. These include a simple split (*ca.* 1/1000) of the LC effluent, [1–6] use of atmospheric pressure ionization (API) mass spectrometry, [7–9] enrichment of the LC effluent with a membrane separator, [10–13] use of mechanical transport of the sample with solvent evaporation in vacuum lock chambers, [14–17] and the approach involving laser vaporization combined with molecular beam technology developed in this work.

Our approach employs laser vaporization to rapidly vaporize both the solvent and the sample and molecular beam techniques to transport and ionize the sample with minimal contact with solid surfaces. The rationale for our approach is based in part on the work of Friedman and coworkers, [18,19] in which it was shown that quite involatile samples, e.g., underivatized peptides, can be vaporized intact by employing rapid heating and vaporizing the sample from weakly interacting surfaces such as Teflon.

A key feature of our approach to interfacing the liquid chromatograph with the mass spectrometer is that solute molecules eluting from the LC are vaporized, ionized and mass analyzed with minimal contact with solid surfaces. The defining characteristic of molecules which cannot be analyzed by gas chromatography appears to be their instability with respect to solid surfaces. On contacting a surface, involatile molecules may either adhere or be decomposed. By using the laser vaporization and crossed beam ionization techniques, contact with solid surfaces is avoided.

An obvious potential problem area for any LC-MS technique applied to involatile solids is the possibility of decomposition of solid contaminants on critical surfaces in the instrument. This may be particularly troublesome in the mass spectrometer if the deposited materials are electrical insulators; surfaces covered by insulating layers may be charged by ion or electron bombardment

causing erratic performance or complete loss of the ion beam. Disassembly and cleaning is often required before satisfactory performance can be restored. Contamination problems may be particularly severe in the combination of mass spectrometry with ion-exchange chromatography, where the mobile phase typically contains macroscopic quantities of dissolved salts as buffers. Unfortunately, many of the most promising biomedical applications of LC employ ion-exchange separation techniques.

The crossed beam LC-MS interface possesses the characteristics necessary to cope with potential contamination problems. Since the sample molecules, which are ionized and eventually detected, are transmitted through the apparatus in a molecular beam with little contact with surfaces, the critical surfaces may be heated quite hot to prevent deposition of the solids, without causing pyrolysis of the sample. Thus the critical beam defining apertures, e.g., the sampling orifice, skimmer, and collimator are heated electrically. While the contamination problems cannot be avoided entirely, these techniques are sufficient to extend the mean operating time between cleanings to the point that down time is not a serious impediment to the application of the instrument.

II. The Crossed-Beam LC-MS Interface

The crossed-beam LC-MS interface initially developed in this work, shown schematically in Figure 1, is described in detail elsewhere [20]. Briefly, the LC-MS interface consists of three essential elements: (1) the laser beam vaporizer; (2) the differentially pumped, free-jet, molecular beam forming apparatus; and (3) the crossed beam CI-EI ionizer. The effluent from the liquid chromatograph enters the vaporization region through a stainless steel capillary tube, and the liquid jet emerging from the capillary is intersected by the focused beam from a 50 watt CO_2 laser (Coherent Radiation Model 42). By using a 12.5 cm focal length zinc selenide lens, the beam of 10.6 micron radiation is focused to a diameter of 0.015 cm at the intersection with the jet, corresponding to a power density of 10^5 W/cm^2 at the intersection. This radiation intensity is sufficient, if entirely absorbed, to vaporize up to 1 mL/min of water in the approximately 10^{-4} s required for the liquid jet to pass through the laser beam.

The rapid vaporization converts the liquid jet into a vapor jet which is sampled by a small orifice in a thin molybdenum plate located a short distance downstream. The sampling orifice serves as the nozzle for the supersonic molecular beam system employed in the crossed-beam ionizer. In the region between the nozzle and skimmer the vapor undergoes adiabatic expansion. By proper choice of the nozzle to skimmer distance and the skimmer diameter, the transition from

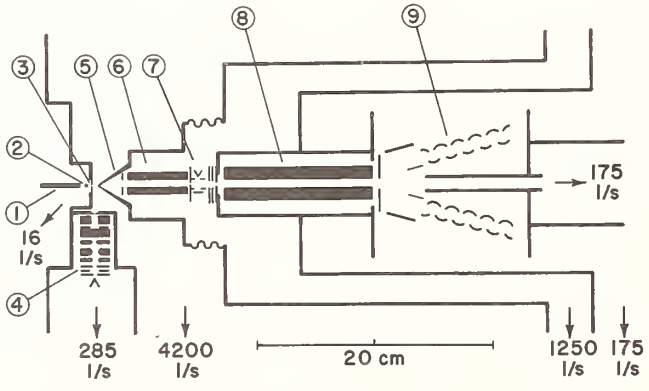

Figure 1. Schematic diagram of the crossed-beam LC-MS interface. (1)–Liquid nozzle; (2)–laser beam; (3)–sampling aperture; (4)–CI electron gun; (5)–skimmer; (6)–quadrupole with RF only; (7)–EI ion source; (8)–quadrupole mass analyzer; and (9)–multipliers.

viscous flow to free molecule flow can be made to occur near the skimmer orifice, i.e., the mean free path at the skimmer is approximately equal to the skimmer orifice diameter.

In the region between the nozzle and the skimmer, the molecular beam is intersected by a focused electron beam, which produces ionization, mainly of the solvent molecules. As a result of frequent collisions occurring in this region, the ionization is preferentially transferred to the solute molecules by ion-molecule reaction. In this way, chemical ionization (CI) of the solute is produced with the solvent as the reagent. During the adiabatic expansion, the temperature of the vapor is substantially reduced and some condensation may occur.

A second electron beam intersects the molecular beam in the region between the skimmer and collimator. In this region, the beam is characterized by free molecule flow, i.e., molecular collisions in the beam are negligible. Thus, ionization of the beam in this region produces the normal electron impact spectra of both the solvent and the solute. By operating one or the other electron gun, or both, we may produce either CI spectra or EI spectra, or the combination of the two.

The mass analyzer is a high-performance ELFS™ quadrupole (Extranuclear Model 4-162-5 with Model 011-1 RF power supply) with a mass range of 1–1000 amu. After exiting the quadrupole, ions may be detected by either of two bakeable electron multipliers. These multipliers are displaced laterally from the quadrupole exist to minimize noise due to photons or excited neutrals emerging from the analyzer and to minimize contamination by involatile substances in the molecular beam. Deflectors are provided to direct the ions into the active surface of one or the other multiplier. The anode of each multiplier is connected to the amplifier outside the vacuum envelope through a high voltage triaxial feedthrough allowing either one to be wired for detecting either positive or negative ions. In normal operation the deflectors and multiplier bias voltages are set up so that one multiplier detects positive and the other negative ions. The ion intensities may be measured either by ion counting or by current amplification.

A high performance liquid chromatograph (Perkin-Elmer Model 601) is equipped with a variable wavelength detector. This system provides pulse-free reproducible flows from 0.05 mL/min to 6 mL/min at head pressures to 3000 psi. The system is operated with the UV detector prior to, but in series with the mass spectrometer to allow direct comparisons of the two detectors.

Two alternate systems are provided for measuring the ion intensities transmitted by the quadrupole mass analyzer. One of these consists of a 100 Mhz ion counting system followed by a D-A convertor; the other is a 10 khz current amplifier with a dynamic range of 10^4. The ion counting system may be used for the measurement of the intensities of either positive or negative ions, but is routinely used on the negative ion channel.

The output of either data channel may be connected to the oscillographic recorder for recording spectra; alternatively, both channels may be connected to the computer through A-D convertors and pseudo-simultaneous measurement of both positive and negative ion spectra may be accomplished using the modulation technique described previously by Hunt and coworkers [21]. The computer system is the Finnigan/Incos Model 2300.

III. Results

An example of the performance of the crossed-beam LC-MS run in the EI mode on a mixture of aromatic hydrocarbons is shown in Figure 2, where the output of the UV detector (254 nm) is compared to mass chromatograms on the parent masses and the reconstructed liquid chromatogram obtained by summing all of the ions detected. This data was obtained using 5 second repetitive scans from 60 to 350 amu, and the chromatographic conditions are given in the caption of Figure 2. Under these conditions the widths of the peaks at half-height are approximately 30 seconds and no difference is detectable between the widths of the peaks on the UV trace and those in the mass chromatograms. Thus, at least at the LC resolution displayed in this example, there is no apparent degradation of effective LC efficiency as the result of using the

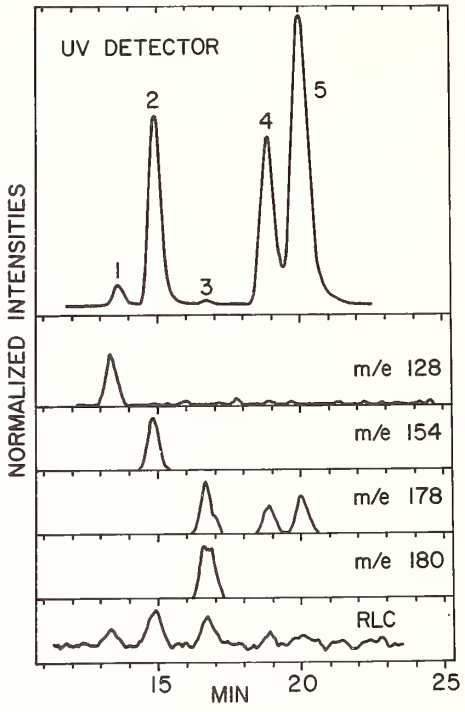

Figure 2. UV (254 nm) detector trace and reconstructed mass and liquid chromatograms for an aromatic mixture. (1)–2.4 μg naphthalene; (2)–5.4 μg biphenyl; (3)–4.8 μg 9,10-dihydroanthracene; (4)–2.0 μg phenanthrene; and (5)–2.4 μg anthracene. The mixture was separated using a 25 cm×4.6 mm Partisil-10 ODS-2 column with a 20% water in acetonitrile isocratic mobile phase at 0.5 mL/min.

mass spectrometer as a detector. For the *ca.* 2 μg samples shown in Figure 2 the peak sample elution rate is about 70 ng/s, and except for mass 128 (naphthalene) no background signal was detected at the parent mass with the detector sensitivity used in these measurements. The minimum detectable signal per amu for these detector conditions is about 0.4% of the maximum signal observed for biphenyl at mass 154. We conclude from this result, and a series of similar measurements that the minimum detectable effluent rate using the mass chromatogram is about 1 ng/s corresponding to a total sample injection of about 30 ng for these admittedly favorable compounds. The detection limit using the reconstructed liquid chromatogram corresponds to about 1 μg of sample as the result of base line noise caused by flow fluctuations and variations in the molecular beam intensity. An advantage of the mass spectrometer over the UV detector is illustrated by 9,10-dihydroanthracene, which, due to its weak UV adsorption at 254 nm, is barely detectable in the UV trace, even though it is a major component in the mixture. The mass chromatograms show nearly the same sensitivity for all five components.

Examples of the mass spectra obtained for some of these aromatic hydrocarbon samples are shown in Figure 3. Except for differentiating between anthracene and phenanthrene which have very similar EI spectra, all of these components were easily identified by automatically searching the computer library file of some 23,000 spectra.

Evaluation of the performance of the instrument has primarily employed samples of biological significance with special emphasis on the components of nucleic acids—bases, nucleosides, and nucleotides—which provide an interesting series of biologically important compounds of increasing volatility and corresponding increasing difficulty for mass spectrometry. Most of our work has employed reverse phase LC separation with water, acetonitrile, and water/acetonitrile mixtures as solvents; however, we have also done some work using ion exchange chromatography using both phosphate and acetate buffers.

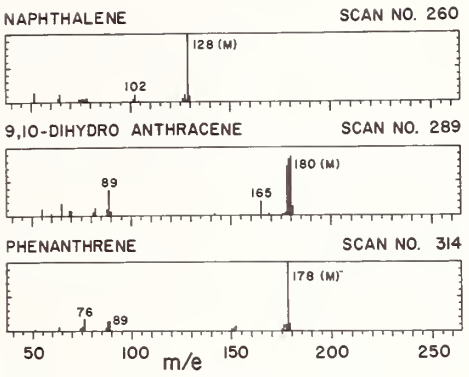

Figure 3. EI mass spectra obtained for some of the aromatics shown in Figure 2.

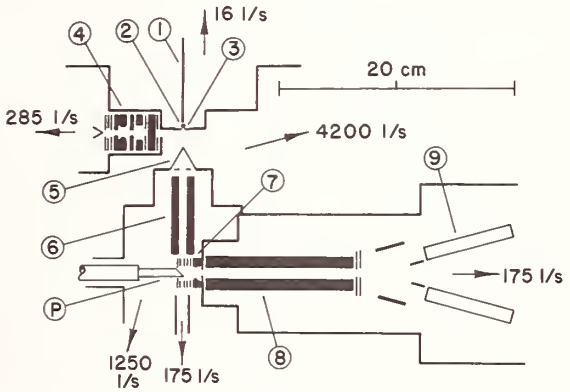

Figure 4. Schematic of LC-MS with transversely mounted quadrupole. (1)–liquid nozzle; (2)–laser beam; (3)–sampling aperture; (4)–CI electron gun; (5)–skimmer; (6)–quadrupole with RF only; (7)–EI ion source and transverse extraction region; (8)–quadrupole mass analyzer; and (9)–multiplers, (p)–heated direct insertion probe.

The evaluation work employing the instrumental configuration shown in Figure 1 is described in detail elsewhere [20]. Although some useful results were obtained in this work, a number of problems were identified which need to be solved before fully satisfactory operation of the crossed-beam LC-MS can be achieved. These include the following:

(1) The pressure in the mass analyzer was often too high, sometimes reaching 10^{-4} torr at high solvent flow rates.

(2) The sensitivity for relatively involatile molecules was about two orders of magnitude lower than expected.

(3) The spectra of relatively involatile, thermally labile molecules showed evidence of pyrolysis prior to ionization.

(4) Large oscillation of the ion beam were observed when formed under CI conditions.

(5) A mist was sometimes formed in the molecular beam due to incomplete vaporization or partial condensation.

Many of these problems are related and now it appears that a common solution may be possible. We have recently modified the instrument to incorporate a transverse mounting of the quadrupole as shown schematically in Figure 4. This modification solves the problem of high pressure in the mass analyzer and leads to improved sensitivity due to reduction in ion losses by small angle scattering. However, the major loss of sensitivity as well as the other problems noted

above appear to be related to mist formation. In recent experiments we have established that the onset of the mist depends on laser power, flow rate, and very critically on the diameter of the liquid jet orifice. By reducing the diameter of this orifice from 0.25 mm the flow rate of heptane at which the onset of mist formation occurs can be increased dramatically from *ca.* 0.3 mL/min to above 1 mL/min.

The oscillations observed in the CI ion beam have been definitely related to the formation of fine droplets or mist in the molecular beam, and it now appears that this effect is also responsible for the low sensitivity observed for involatile samples and may be implicated in some of the apparent pyrolysis that has been observed. It appears that involatile sample molecules, which serve as nuclei for condensation, are preferentially contained in the droplets even though most of the solvent is vaporized. These sample-containing droplets may be carried along by the molecular beam and pass through the ion source and mass analyzer without being detected. However, these droplets which strike heated surfaces in the vicinity of the ion source may be vaporized on the surface and sample molecules vaporized, ionized and detected. Since the EI source was operated at *ca.* 350 °C during the measurements of spectra of nucleosides and nucleotides, this effect probably accounts for the observed pyrolysis rather than excessive heating by the laser beam.

The present configuration of the LC-MS system is equipped with a heated direct insertion probe as shown schematically in Figure 4. The tip of this probe is made of nickel-plated copper; the end is a flat surface approximately 5 mm high by 10 long inclined at 45 degrees relative to the direction of travel of the molecular beam. The probe tip is heated by a 100 watt cartridge heater and the temperature is monitored by a chromel-alumel thermocouple embedded in the copper tip. This probe design allows solid samples deposited on the flat surface of the probe tip to be vaporized directly into the ion source and makes it possible to evaluate the sensitivity of the mass spectrometer independent of the efficiency of the LC interface. Also, the probe can be inserted into the ion source in the path of the molecular beam to study the mass spectra obtained by impact vaporization of droplets or mist which may be carried by the molecular beam.

The system shown schematically in Figure 4 has been in operation only a very short time, but already some very interesting results have been obtained. With the heated probe removed from the source, mass spectra of aromatic hydrocarbons evaporated in the LC interface from methanol solution were measured with sensitivities comparable to those shown in Figure 2; however, the sensitivity for less volatile compounds, such as adenosine and other nucleosides was much lower. However, the spectra of the nucleosides showed no evidence of pyrolysis as was observed in our earlier work using the instrumental configuration shown in Figure 1. As an example, a spectrum of adenosine obtained under these conditions is compared to a spectrum retrieved from the computer library in Figure 5. When the probe was inserted into the source in the path of the molecular beam, the sensitivity for adenosine in the LC effluent was significantly improved, but no changes in the spectrum were observed which would indicate that pyrolysis was occurring. Also, a virtually identical spectrum of adenosine was obtained by thermal vaporization from the direct insertion probe. Similar results have been obtained for all of the commonly occurring nucleosides from *t*-RNA including inosine and guanosine which were previously thought to be insufficiently volatile for thermal vaporization and electron ionization.

Mass thermograms of the principal ions obtained for guanosine vaporized from the direct insertion probe are shown in Figure 6. These data were obtained by heating the probe at about 80 °C per minute and scanning the mass range from 20 to 320 amu in 5 second repetitive scans. The parent ion, mass 283, is first detected at 180 °C, reaches a maximum intensity near 210 °C, and vanishes above 240 °C. All of the structurally significant ions with masses greater than that of the guanine base (151 amu) show a dependence on probe temperature similar to that of the parent ion while the lower mass ions behave differently. The low temperature evolution of mass 151 is probably due to a guanine impurity in the sample while the higher temperature evolution of mass

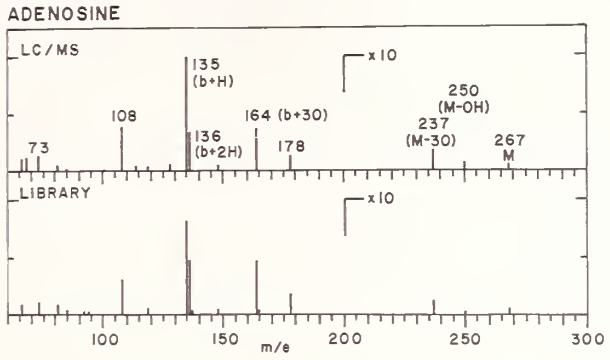

Figure 5. Comparison of EI mass spectrum of adenosine obtained on the LC-MS with that on file in the computer library. This spectrum was obtained using the transverse geometry shown in Figure 4 under conditions in which the molecular beam does not contact heated surfaces in the vicinity of the EI source.

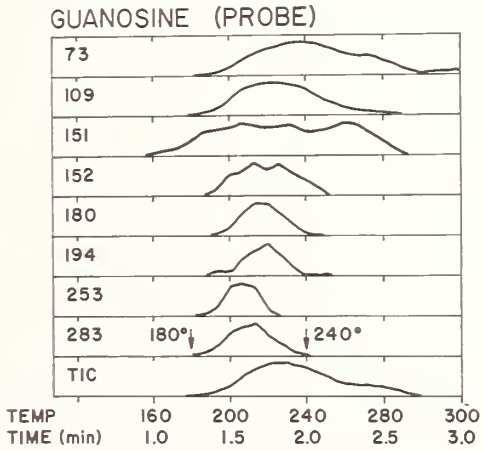

Figure 6. Mass thermograms of major ions in EI spectrum of guanosine. The mass corresponding to each trace is indicated on the left. The bottom trace (TIC) is the total ion current.

151 and the guanine and sugar fragments at masses 109 and 73, respectively, are probably due to pyrolysis of the guanosine.

The EI spectrum of guanosine corresponding to a probe temperature of 210 °C is shown in Figure 7. The major ions observed are satisfactorily explained by the fragmentation mechanisms for purine nucleosides established earlier by labeling and high resolution studies, principally on adenosine [22]. Our EI spectra of guanosine are essentially independent of whether the sample is vaporized from the direct insertion probe or by combined laser and impact vaporization from the LC effluent. Earlier work under conventional EI, FI, and CI conditions failed to yield ions characteristic of the molecular weight; however, the molecular ion was previously observed under FD conditions and MH^+ has been observed by using an FD emitter as a solids probe under CI conditions [21].

In summary, we have successfully used laser heating to rapidly vaporize both the sample and the solvent eluting from the liquid chromatograph and have developed molecular beam techniques to transport and ionize the sample with minimal contact with solid surfaces; however, we seem not to have achieved complete vaporization of the less volatile compounds particularly when dissolved in highly polar solvents such as water and methanol. Our recent results on guanosine and other relatively involatile nucleosides and nucleotides seem to indicate that thermal vaporization without pyrolysis is possible for a wider range of compounds than has previously been demonstrated.

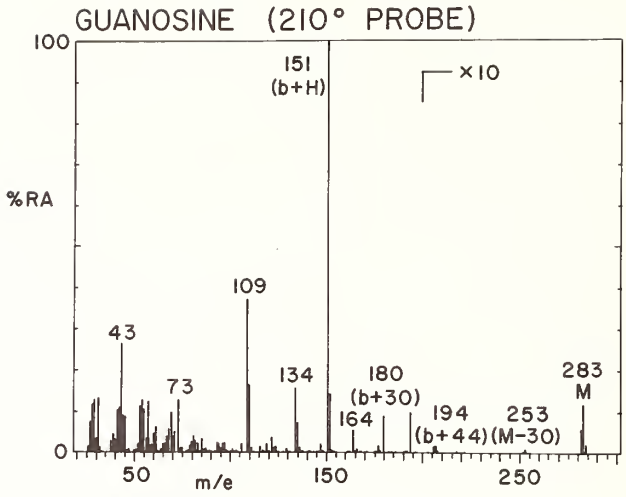

Figure 7. EI mass spectrum of guanosine by vaporization from direct insertion probe at 210 °C.

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ATMOSPHERIC PRESSURE IONIZATION MASS SPECTROMETRY

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Atmospheric pressure ionization mass spectrometry differs in several significant ways from conventional mass spectrometry. The ionization of samples occurs in a small reaction chamber at atmospheric pressure, negative as well as positive ions are detected, and the source does not contain a heated filament. Samples can be introduced in organic solvents by direct injection or in the effluent stream from a liquid chromatographic column, as well as in the usual ways. Very short-lived, as well as very long-lived, ions have been detected, and subpicogram sensitivity of detection has been achieved for both positive and negative ions. Ionization occurs through a sequence of ion molecule reactions, or through electron attachment. Current studies are concerned with negative ion mass spectrometry and with conditions for attaining high sensitivity of detection in bioanalytical studies.

Key words: Atmospheric pressure ionization; bioanalytical systems; mass spectrometry; negative ions; positive ions.

During the initial studies of atmospheric pressure ionization (API) mass spectrometry, there were several major differences between the prototype instrument and conventional mass spectrometers. The ionization process was carried out with a non-filament source that was external to the mass analyzer region of a quadrupole mass spectrometer, and the reaction chamber was at atmospheric pressure. The instrument was capable of detecting negative as well as positive ions, and samples were injected in solution after the fashion of gas chromatography as well as by conventional techniques. At that time it was widely believed that negative ion studies were not of theoretical or practical interest, that filament sources would fill all forseeable needs in bioanalytical mass spectrometry, and that ionization reactions conducted at atmospheric pressure could not be adequately controlled or used for analytical purposes. It is now recognized that negative ion mass spectrometry may prove to be highly useful in analytical applications, that non-filament sources will be needed for some purposes, and that very high sensitivity of detection can be achieved in analytical applications of API mass spectrometry. The outstanding difference at this time between API techniques and the most recently employed negative ion and positive ion CI techniques lies in the gas pressure in the source. Source pressure, however, is essentially a technical detail rather than a matter of fundamental significance; the significant difference between API mass spectrometry and conventional mass spectrometry is that the product ions are in thermal equilibrium within the source when the usual API conditions are used, while this is not true for conventional procedures. It is our belief that thermal equilibrium conditions are advantageous when selective ionization circumstances are employed in quantitative work, but non-equilibrated conditions are required for some types of analyses, and for typical fragment ion studies.

A corona discharge source is shown in Figure 1. When the electrode point is 4 mm from the aperture, the ions which are observed are the same as those seen when a ^{63}Ni foil is used as a primary source of electrons, and they are in thermal equilibrium within the source. If the electrode point is 0.5 mm from the aperture, ions which are not in thermal equilibrium within the source are observed. This effect is shown in Figures 2 and 3. Figure 2 shows the ions observed for 0.1% argon in helium; the He^+ and He_2^+ ions would not be observed in the presence of 0.1% argon if

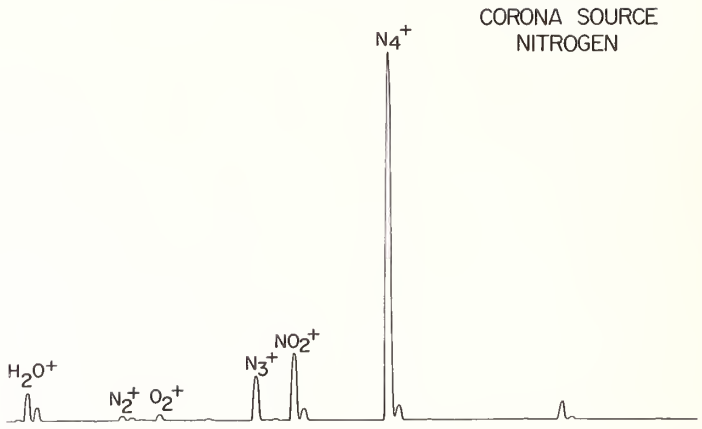


Figure 1. Schematic diagram of corona discharge API source, with provision for varying the distance of the point of the electrode to the aperture from 0.5 mm to 4 mm.

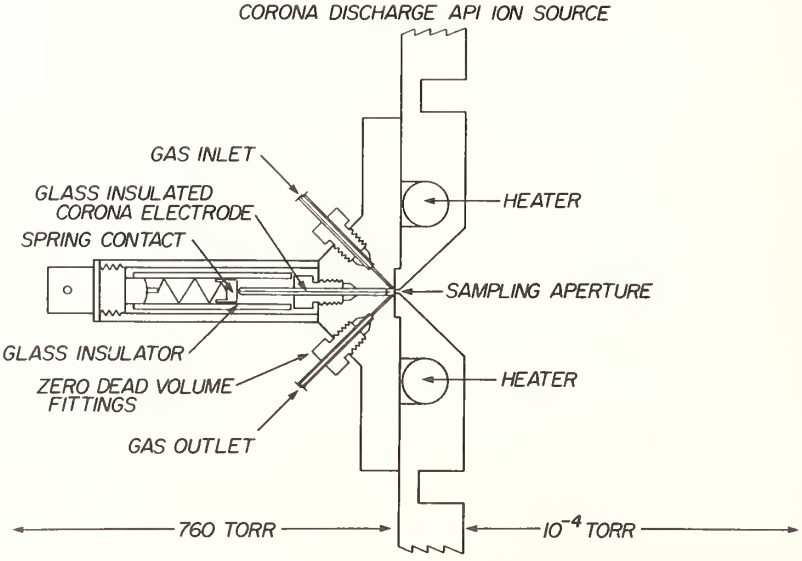


Figure 2. Ions observed for 0.1% argon in helium, with 0.5 mm corona electrode distance. These ions have a very short residence time and are not in thermal equilibrium within the source.

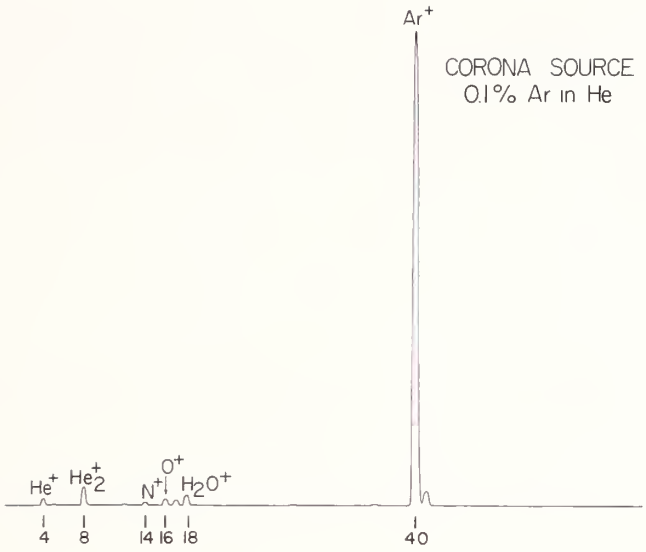


Figure 3. Ions observed for nitrogen carrier gas, with 0.5 mm corona electrode distance. The nitrogen ions are not in thermal equilibrium within the source; the distribution corresponds to a temperature of 540 °C.

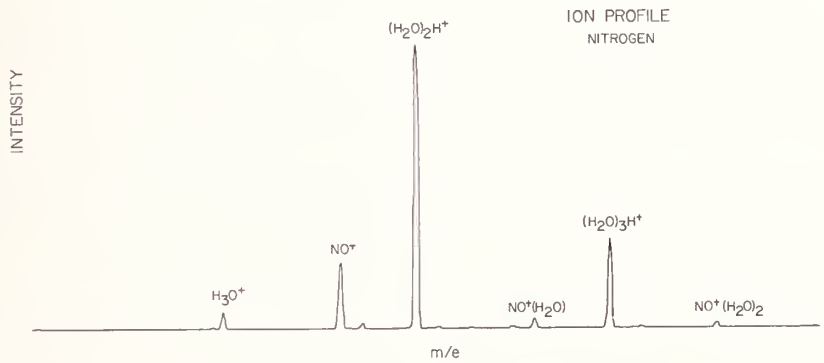


Figure 4. Reagent ions observed for nitrogen carrier gas with a corona electrode distance of 4 mm, and with a ^{63}Ni foil source. The ions are thermally equilibrated. Nitrogen ions are no longer observed; the ion current is due almost entirely to hydrated protons arising from a trace of water in the carrier gas. Nitric oxide ions (NO^+ and NO^+ hydrates) are also present.

equilibrium conditions prevailed. A trace of water, which is always present, is evident as H_2O^+; these water ions left the source before encountering a water molecule. Similar effects are illustrated in Figure 3. The principal ions are N_4^+ and N_3^+ when nitrogen is the carrier gas and when the distance is 0.5 mm. When the distance is 4 mm, all ions have equilibrated and the major ion is $(H_2O)_2H^+$ as shown in Figure 4. Calculations indicate that the effective ion temperature is 540 °C for the circumstance shown in Figure 3; the wall temperature was 200 °C.

The addition of an appropriate reagent leads to the formation of positive reagent ions, and positive sample ions are formed by ion molecule reactions that are well understood. The usual API condition involves 0.1% or less of reagent in a carrier gas. The common reagents are isobutane, ammonia and water for protonation reactions, and nitric oxide for charge transfer reactions. The typical reaction sequence:

primary electrons
↘
carrier gas ions
↘
reagent gas ions
↘
sample ions

was discussed in the initial API report [1]; the series of ion molecule reactions occurring in nitrogen carrier gas without added reagent were postulated as involving N_4^+ as the principal carrier gas ion, hydrated protons as the principal reagent ions, and protonated sample ions as the terminal ions expected for organic compounds containing carbon-carbon unsaturation or oxygen, nitrogen or sulfur atoms. All of the expected ions have been observed experimentally [2]. A similar sequence occurs when nitric oxide is used as the reagent gas, but the sample ions are formed by charge exchange or other reactions, rather than by protonation. For example, Figure 5 shows the M^+ products from two amides (acetylcodeine and diacetylmorphine) when 0.1% nitric oxide in

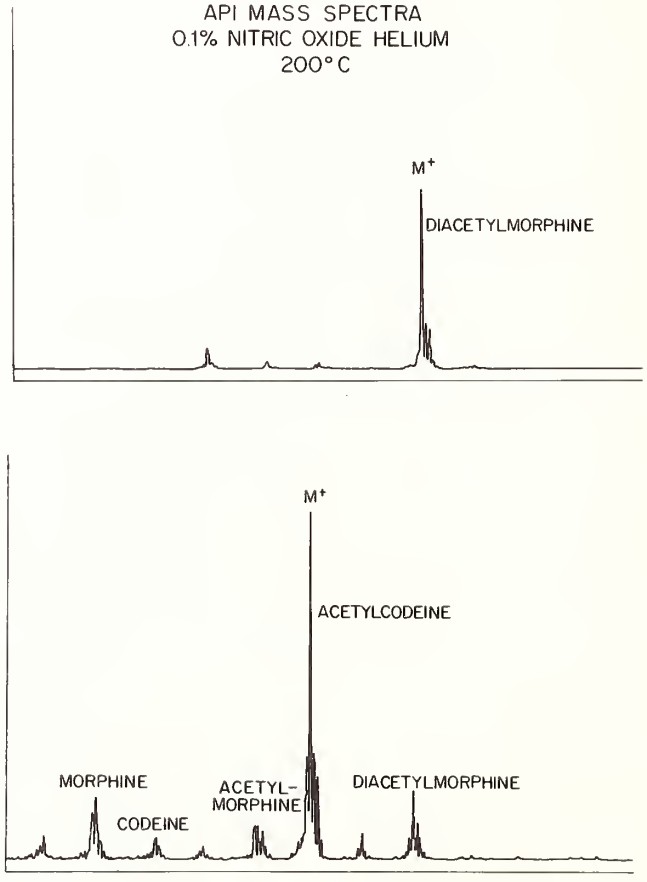


Figure 5. These two API analyses of diacetylmorphine and acetylcodeine were carried out with a 4 mm corona electrode distance and with 0.1% nitric oxide in helium. The amides were converted to molecular radical ions by reaction with NO^+. This experiment was carried out by J-P. Thenot.

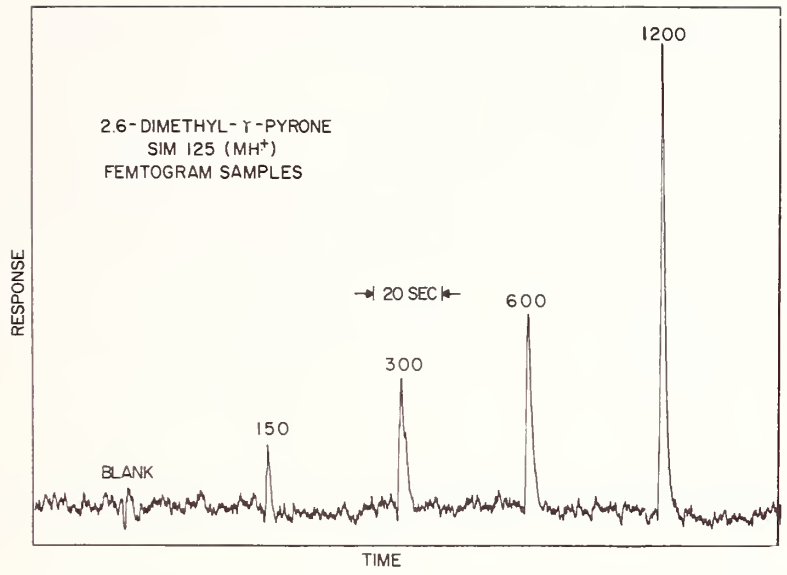


Figure 6. Subpicogram samples of 2,6-dimethyl-γ-pyrone were detected using a ^{63}Ni foil source and nitrogen carrier gas. Samples were injected in solution, in increasing amounts, with a series of syringes that were never exposed to high sample concentrations. Selected ion detection of MH^+ ions at 125 amu was employed.

helium is employed. It should be noted that positive sample ions generated in this way under API conditions should be the same as those observed under 0.5–1 torr pressure (conventional GI conditions) for all highly stable ions. Higher energy ions which are observed under CI conditions should not be observed under API (4 mm) conditions, but these should be seen under API non-equilibrated conditions. Theoretically, it should be possible to duplicate any CI mass spectrum by varying the distance of the API corona electrode from the aperture.

Subpicogram sensitivity of detection has been demonstrated for positive ions [3]. This is illustrated in Figure 6. Samples of 30–50 fg have been detected; it may be possible to lower this limit.

Samples may be injected in solvents after the fashion of gas chromatography, and the effluent stream from a liquid chromatograph may be directed through the source with or without splitting. The source design must be modified to provide a vaporization zone, but the general design of a HPLC-MS-COM system, shown in schematic form in Figure 7, is analogeous to current GC-MS-COM systems. The reagent ions are derived from the solvents; for example, isooctane forms the familiar $C_4H_9^+$ ion. Figures 8 and 9 illustrate the results obtained for a HPLC-MS-COM separation of a mixture of three amino acids as methylthiohydantoin derivatives. The technique of selected ion detection, which is universally used in GC-MS-COM quantitative analyses, gave the result shown in Figure 8. The spectra in Figure 9 show that each compound was ionized to MH^+ and to a solvated ion. The reagent ions were ethanol-solvated protons. While there were obvious limitations in applications of HPLC-MS-COM procedures, the necessary instrumental capability for quantitative analysis has been achieved. A limitation which is not always mentioned is illustrated in Figure 10. Compounds are eluted relatively slowly from HPLC columns, and the limiting sensitivity of detection of a HPLC-MS-COM bioanalytical system is about 0.5 ng. This is not very different from the sensitivity of detection expected for an ultraviolet absorption detector of the usual type. The detection limit on a concentration basis for an API mass spectrometer is about the same for sample injection in solution (Fig. 6 was obtained in this way) and for sample introduction in a HPLC effluent stream (Fig. 10); the relative slowness of sample elution shown in Figure 10 is

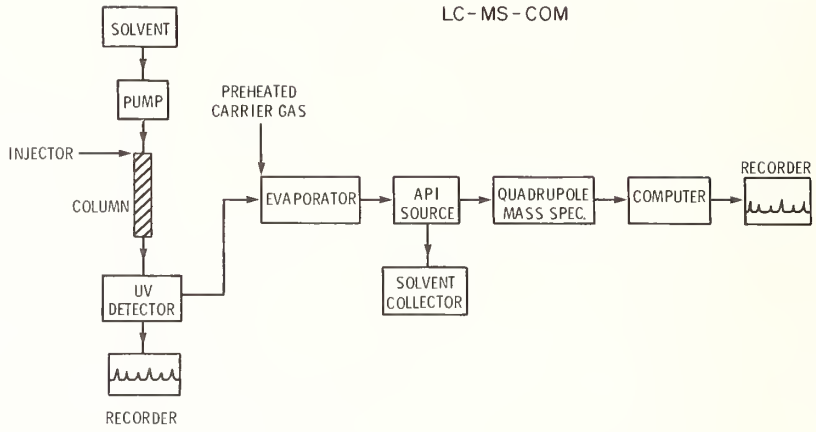


Figure 7. Schematic outline of a HPLC-MS(API)-COM bioanalytical system. The HPLC UV detector was retained in order to provide a comparison record of separations. Reagent ions are generated from the LC solvents and the usual product ion is MH^+.

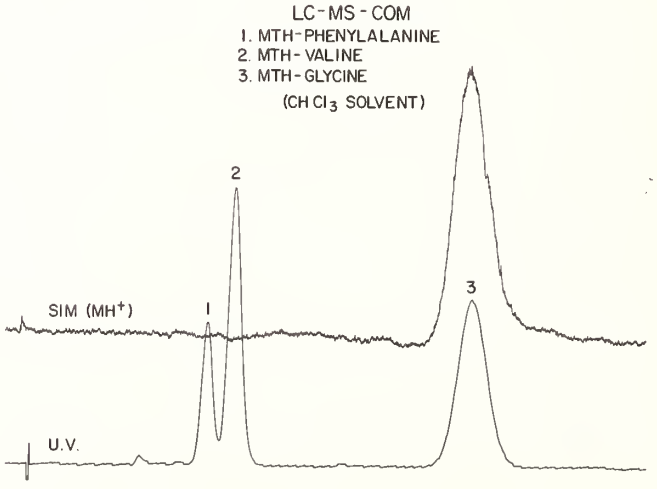


Figure 8. Chart showing UV and API selected ion monitoring responses for a separation of methylthiohydantoin derivatives of three amino acids. The bioanalytical system was that shown in Figure 7. The eluting solvent was chloroform containing ethanol (silicic acid column) and the mass spectrometric response was for MH^+ ions generated from ethanol-solvated protons. In this instance, the glycine derivative was detected.

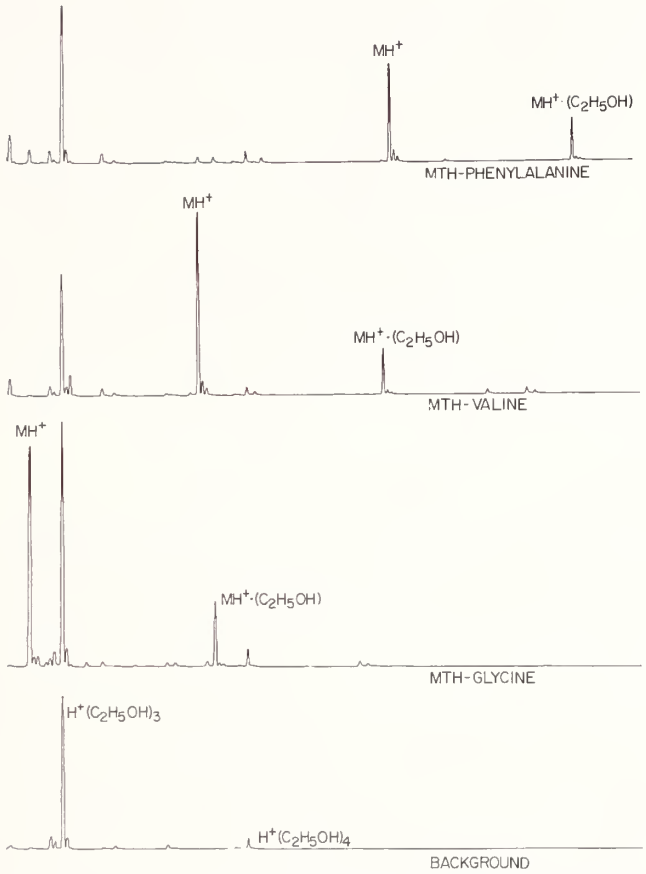


Figure 9. Mass spectra of methylthiohydantoin derivatives of three amino acids. These API spectra were obtained by scanning the peaks shown in Figure 8. The product ions correspond to MH^+ and ethanol-solvated MH^+ ions. This experiment was carried out by K. D. Haegele.

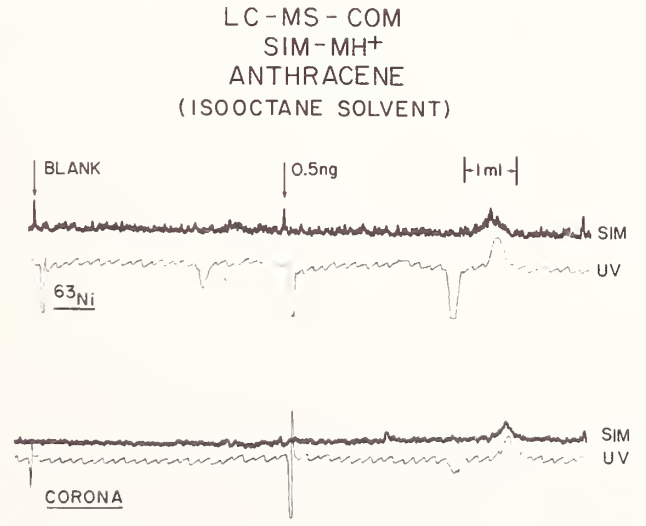


Figure 10. These charts show the responses found for anthracene in a HPLC-MS(API)-COM bioanalytical system, for a conventional UV detector, and for selected ion detection of the MH^+ ion of anthracene (isooctane was the eluting solvent). The responses for a 0.5 ng sample were approximately the same. No difference in sensitivity of detection was observed for a ^{63}Ni foil source and a corona discharge (4 mm) source.

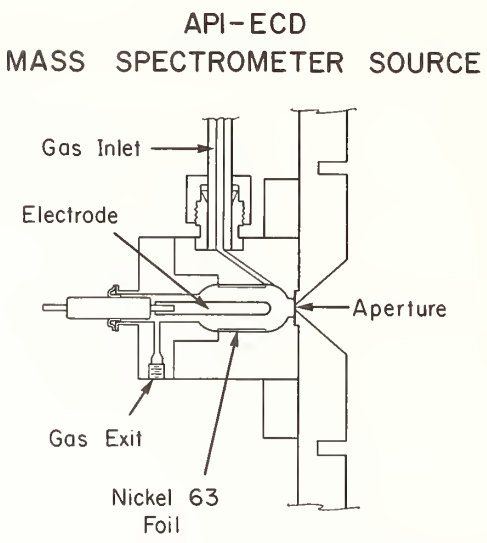


Figure 11. Schematic diagram of an API source corresponding in physical dimensions to a conventional electron capture (EC) detector. The ions present in the chamber are detected by scanning during the period of an EC response.

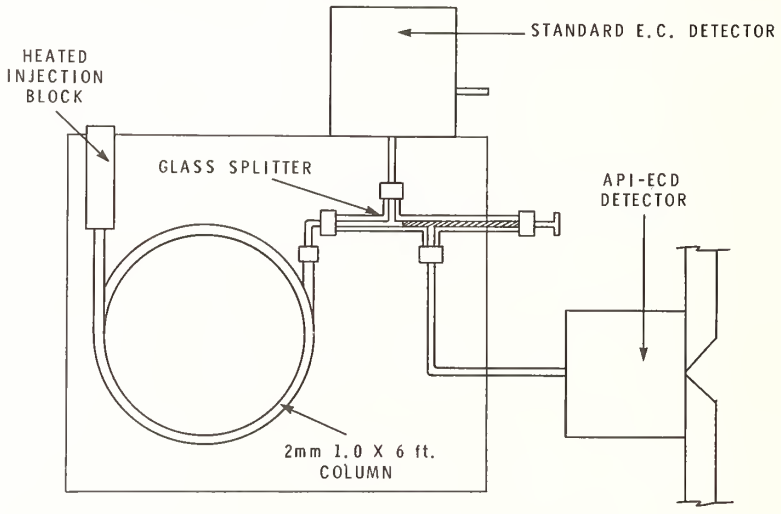


Figure 12. Schematic diagram of the GC unit of a GC-MS(API)-COM bioanalytical system with the source illustrated in Figure 11. The effluent stream was split in order to obtain EC responses from a conventional EC detector. Three responses were compared. The response of the conventional EC detector of the GC unit; the response of the EC detector which was also the API ion source; and the API selected ion detection response. Scans for positive and negative ions were obtained while compounds under study were present in the source chamber.

responsible for the apparent loss in sensitivity of detection as far as sample size is concerned. This work is described in greater detail in other papers [4–6].

Negative ion formation was studied recently [7] with the source design shown in Figure 11. The API source was constructed to correspond to the physical dimensions of a commercially available electron capture (EC) detector, and the EC response was determined for this source at the same time that mass spectra were obtained. A second EC detector was employed in the usual way; the GC unit is shown in schematic form in Figure 12. Three responses were recorded: an EC response from the conventional detector, an EC response from the API source, and an API negative ion response at a selected mass. API spectra were recorded for both positive and negative ions when compounds under study were present in the source.

Negative sample ions are generated by reactions with electrons or with negative reagent ions. In nitrogen alone (oxygen must be excluded) and in argon-methane (95:5; this mixture is commonly used with electron capture detectors), the reactant species consists of thermalized electrons. The reaction sequence is:

primary electrons
↘
secondary electrons
↘
thermal electrons
↘
negative sample ions

The formation of negative ions under these conditions involves resonance electron capture to form a stable M^- ion, or dissociative electron capture to form a stable anion of a gas phase acid and a neutral radical which is not detected. These events occur as indicated:

$$M + e \rightarrow M^-$$

$$AB + e \rightarrow [AB]^- \rightarrow A^- + B^\cdot$$

Stable negative radical molecular ions are readily recognizable intermediates in one-electron reduction/oxidation reactions; for example, quinones and related compounds (benzil) are reduced in the gas phase to stable semiquinone negative radical ions. The protonated radical is a neutral semiquinone radical. Similar reactions occur for aromatic nitro compounds of appropriate structure. Dissociation occurs in instances where an anion of a strong gas phase acid can be formed by elimination of a neutral radical; the parent negative radical molecular ion is never observed, but one or more stable anions are seen as reaction products. An example is shown in Figures 13 and 14. Methyl parathion is ionized in nitrogen and in argon/methane to four negative ions. These are the thiophosphate ion corresponding to loss of a methyl radical, the thiophosphate ion and the nitrothiophenoxide ion. All of these are anions corresponding to strong gas phase acids. The compound may be detected and quantified as shown in Figure 13; the negative ion that was monitored was $(M\text{-}15)^-$, and the two electron capture responses are also shown. The ion products are shown in Figure 14.

Anions of gas phase acids may also be formed directly from acids by reaction with a basic ion. The direct gas phase ionization of acids with O_2^- has been studied under API conditions [8]; the reagent ion is usually generated with 0.1–1% oxygen in nitrogen, but concentrations up to 20% have been used.

The superoxide ion, O_2^-, is a useful reagent for the detection of certain types of halogenated compounds. Aromatic halides are converted into phenoxide ions through a substitution reaction that apparently occurs through an intermediate radical ion with the structure $[MO_2]^-$; this transition state ion is not observed, since it is converted into a stable phenoxide ion [9]. The reactions are:

$$\begin{array}{ccccc} & O_2 & & & M \\ e & \downarrow\ (1) & & (4) & \downarrow\ e \\ & & (2)\quad (3) & & \\ O_2^- & + & M \rightarrow [MO_2]^- \rightleftharpoons & O_2 & + \ M^- \\ & & (6)\downarrow\quad (5) & & \\ & & (M{+}O{-}Cl)^- & + & ClO^\cdot \end{array}$$

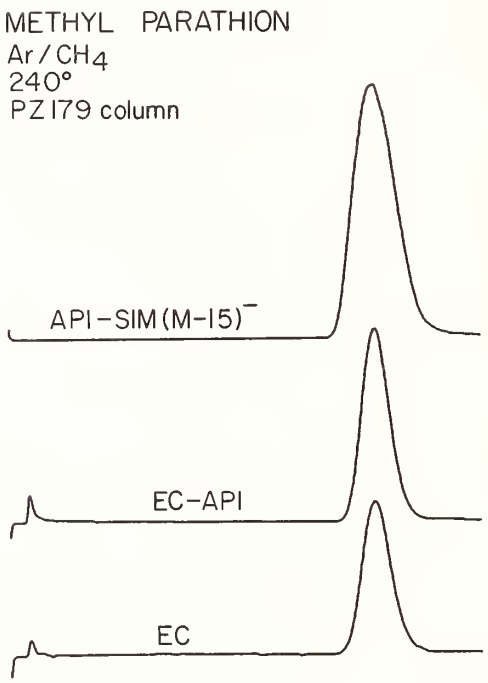


Figure 13. Comparison of three responses for methyl parathion: the response observed for a conventional EC detector (EC); the response observed for the EC detector/API ion source (EC-API); and the selected ion monitoring response (API-SIM) for the (M-15) negative ion corresponding to loss of a methyl radical from methyl parathion. The carrier gas was argon-methane (95:5).

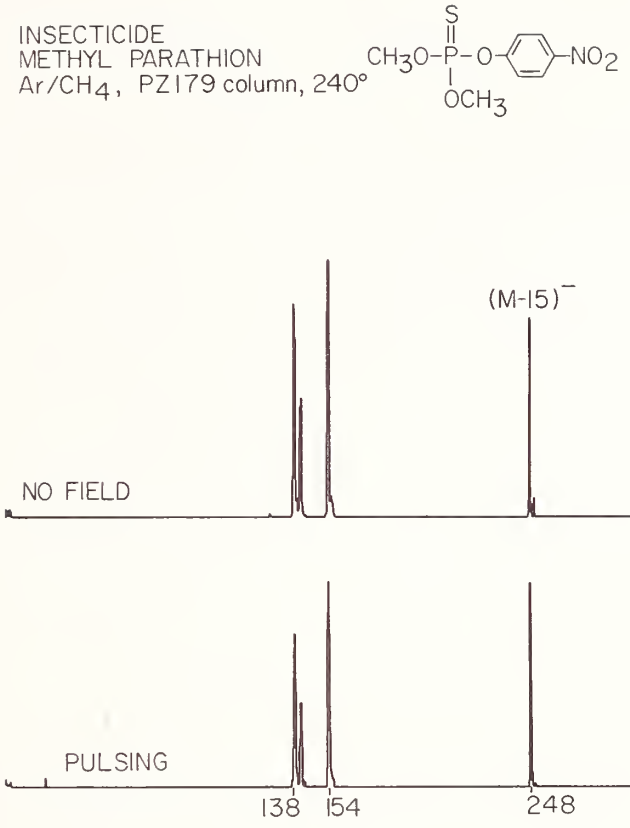
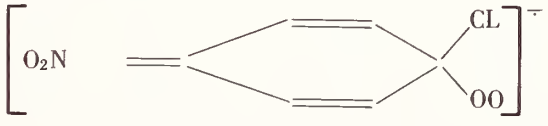


Figure 14. Negative ions observed for methyl parathion with the source described in Figure 11. The ions are stable anions of strong gas phase acids. They are the thiophosphate ion resulting from loss of a methyl radical, the thiophosphate ion resulting from loss of a nitrophenyl radical, the nitrophenoxide ion, and the nitrothiophenoxide ion. The parent M- ion was not observed; the reaction products result from dissociative electron capture without and with rearrangement. In this instance the ion products were the same when the EC/APl detector was in operation, and when a field was not present in the ion source.

When a relatively high concentration of oxygen is present, reaction (4) is suppressed and the reaction sequence is $1 \rightarrow 2 \rightarrow 6$, with $(M+O-Cl)^-$ as the only observable product. As the amount of oxygen is decreased, reaction (4) can be detected and the sequence $4 \rightarrow 3 \rightarrow 6$ occurs. Reaction (5) apparently does not occur because reaction (6) occurs instead. The nature of the intermediate radical ion $[MO_2]^-$ is not known, but since these reactions have been observed for *o*- and *p*-nitrochlorobenzene, while *m*-nitrochlorobenzene forms an M^- ion in the presence of oxygen, it is assumed that the transition ion is probably of the type:



for *p*-nitrochlorobenzene, with an analogous structure for the *o*-isomer. This reaction also occurs for polyhalogenated aromatic compounds with chloro, bromo and iodo substituent groups. Hexachlorobenzene, for example, forms the pentachlorophenoxide ion, and polychlorobiphenyls form the corresponding phenoxides. Direct evidence for the radical $XO^{\cdot}$ has not been found, but this is believed to be the additional product. The overall reaction for a polyhalide in the presence of oxygen is electron capture with formation of an anion of a gas phase acid and a neutral radical.

The competing reaction of dissociative electron capture for halogen compounds:

$$M \xrightarrow{e} [M]^- \rightarrow (M\text{-}X)\cdot + X^-$$

occurs in the absence of oxygen. This can be demonstrated for many halides, with the usual reaction rate order of I>Br>Cl. Trace concentrations of oxygen, however, are sufficient to divert the course of the ionization process for aromatic compounds to phenoxide formation. Reactions of the superoxide ion with aliphatic halides have not been studied under API conditions, and it is not known if a substitution reaction involving O_2^- will occur in competition with the usual dissociative electron capture reaction; an alkoxide ion, however, would not be an expected reaction product. A neutral alkylhydroperoxide radical might be formed along with a halide ion.

Subpicogram sensitivity of detection has been observed for negative ions. Figure 15 shows the detection of the phenoxide ion from 2,3,4,5,6-pentachlorobiphenyl with O_2^- as the reagent ion, with subpicogram samples. In other studies, it has been found that the limiting sensitivity of detection of positive and negative ions is about the same. There is a very great difference, however, in selectivity in the ionization process when negative ion mass spectrometry is compared with positive ion mass spectrometry. Most organic compounds of biomedical interest contain oxygen, nitrogen or sulfur atoms in addition to the carbon skeleton, and it is difficult to form MH^+ ions in selective fashion except for strongly basic compounds. An analogous situation is present for M^+ ion formation, although it should be possible to establish selective conditions for some types of structures. Negative ion formation, however, occurs for relatively few compound types when thermal electrons or O_2^- ions are used as reagents.

API mass spectrometry has been used in a variety of studies where a relatively high limiting sensitivity of detection was required. An early study of nicotine in non-smokers was carried out to determine if the positive values observed in other studies for non-smoking individuals were due to nicotine [10]. Urinary analyses by API techniques are shown in Figure 16. Nicotine can be detected in the urine of smokers by numerous analytical procedures, but the analysis for a non-smoker shows clearly that the ions at M^+ and MH^+ for nicotine are also present, although in this instance the subject was not exposed to tobacco smoke in any unusual way. In this study, nicotine was found to be present in the circulating air, and it is believed that in an urban environment virtually all adults excrete nicotine because of air transfer and the prevalence of smoking. A study of the presence of nicotine in skin surface lipids was carried out recently. The index and adjacent finger on each hand were washed with hexane (2 mL), for smokers and non-smokers in the same

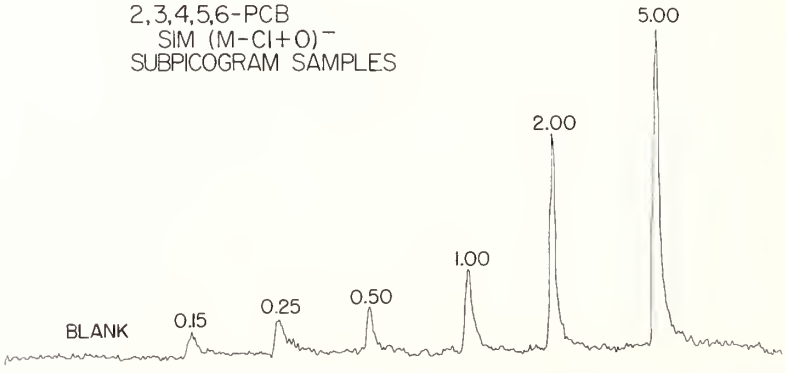


Figure 15. Negative ion responses observed for subpicogram samples of 2,3,4,5,6-pentachlorobiphenyl. The nitrogen carrier gas contained oxygen as a reagent, and the product ion was $(M\text{-}Cl+O)^-$. Selected ion monitoring was employed.

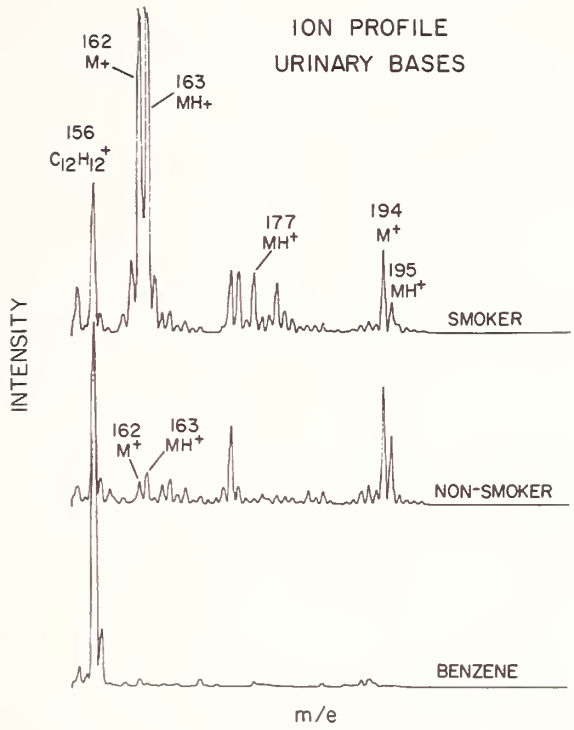


Figure 16. These API mass spectral scans are for benzene, and for benzene extracts of urine from a non-smoker and a smoker. Nicotine is ionized to M^+ and MH^+ ions at 162 and 163 amu under these conditions. Non-smokers in an urban environment excrete nicotine, which is acquired by air transfer. The ions at 194 and 195 amu are M^+ and MH^+ ions from caffeine.

environment. The hexane washes were analyzed by API mass spectrometry; a typical result is shown in Figure 17. Non-smokers showed traces of nicotine in finger skin surface lipids, with an approximately equal amount on each hand. Smokers showed a greater amount of nicotine on both hands, with a much higher concentration for the hand used in smoking (the smoker in Fig. 17 was right-handed). In this work it was necessary to take special precautions to avoid contamination from nicotine in the air, but when this was done satisfactory control analyses were achieved. The instrumental samples which gave these analytical data ranged up from a few picograms.

API mass spectrometry was used in a recent study of the absorption of *tris*(2,3-dibromo-1-propyl) phosphate through the skin of children wearing *tris*-treated sleepware [11]. A metabolite, 2,3-dibromo-1-propanol, was found in the urine in greatly increased amount when a child, who had been wearing laundered sleepware, used new *tris*-treated sleepware. Negative ion mass spectrometry was used (nitrogen was the carrier gas) with the GC-MS(API)-COM system illustrated in Figure 12. Bromide ions at 79 and 81 amu were monitored for the metabolite and for an internal standard (1,4-dibromo-2-butanol). The metabolite was not found in adult and child samples where no exposure had occurred, but it was found in low concentration in urine samples from children wearing well-washed sleepware. Examples of analyses are in Figures 18 and 19.

During the initial developmental period of bioanalytical mass spectrometry, almost all analyses were carried out by EI techniques, and the usual objective was to identify components of biologic samples. While this is still an important function, it is now usually assumed that quantitative analyses will be needed for most substances of interest, and the emphasis in much current work is upon the development of quantitative procedures at or near the limiting sensitivity of detection of bioanalytical systems. It is our belief that most future quantitative work will be based upon API or CI techniques, rather than upon EI techniques, and that selective ionization conditions leading to highly stable ions will be used whenever possible. Source conditions that

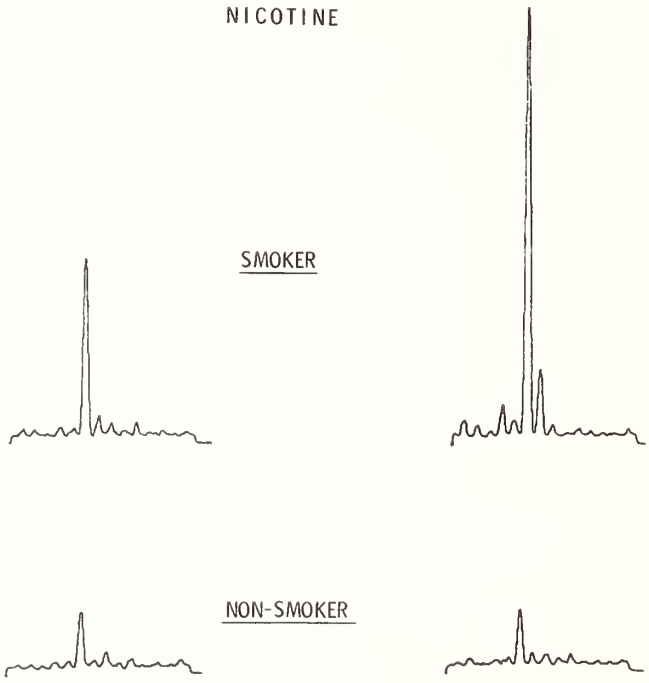


Figure 17. API selected ion detection responses for nicotine (163 amu) with hexane solutions of finger skin surface lipids. Under these conditions MH^+ ions were formed. The solvent alone gave no response, but it was necessary to use special handling procedures when the sample was introduced into the API mass spectrometer. Washes from the left and right hands are in the left and right chart positions. Non-smokers always had approximately the same amounts of nicotine on the skin on both hands (absorbed from the air), while smokers showed unequal and higher concentrations. The nicotine is not removed by ordinary washing with soap; it is in the skin surface lipid film. The responses for non-smokers are for API samples of a few picograms.

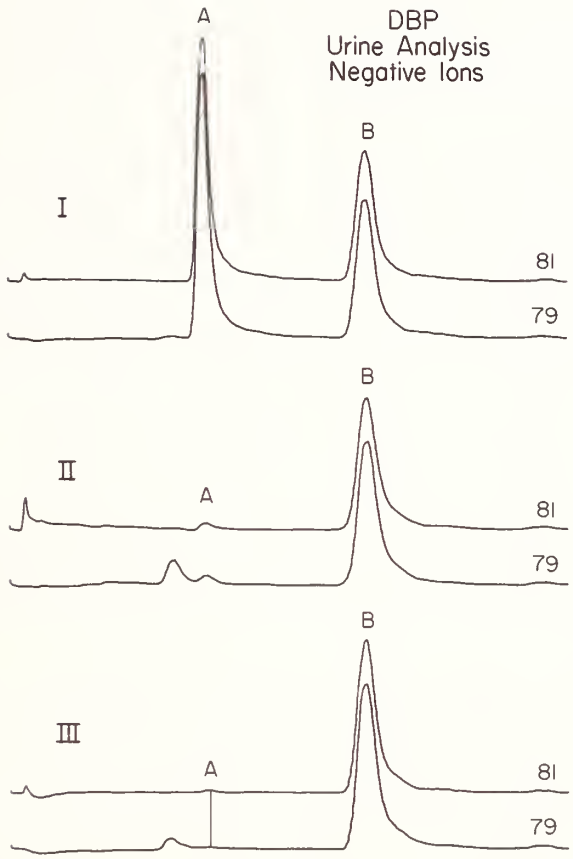


Figure 18. Negative ion API mass spectrometric analyses of human urine samples for 2,3-dibromo-1-propanol by selected ion detection of bromide ions at 79 and 81 amu. A: 2,3-dibromo-1-propanol; B:1,4-dibromo-2-propanol (internal standard). Chart I: urine sample collected first day after wearing new *tris*-treated sleepware. Chart II: urine sample collected day before wearing new sleepwear; child had been wearing well laundered *tris*-treated sleepwear. Chart III: urine sample from child who had never worn *tris*-treated sleepwear. The analyses were carried out with the system and source described in Figures 11 and 12.

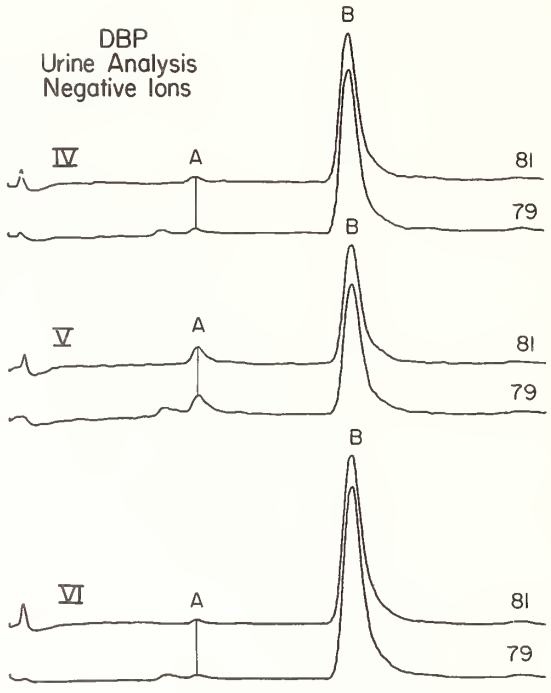


Figure 19. Negative ion API mass spectrometric analyses of human urine samples for 2,3-dibromo-1-propanol, conducted in the same way as for Figure 18. These three charts are for samples from three different children in two families, wearing well laundered *tris*-treated sleepwear. Concentrations of 0.5-5 ng/mL of 2,3-dibromo-1-propanol have been found under these circumstances.

result in thermally equilibrated product ions are likely to be preferred, but in some instances it may be necessary to employ non-equilibrium conditions in order to obtain suitable sample ion products. This can be done by decreasing the ion residence time under API corona discharge conditions, or by employing CI conditions that lead to ions of much higher effective temperature than the source.

Although source pressure is a significant factor governing certain design aspects of bioanalytical systems based on mass spectrometry, it is not known if the limiting sensitivity of detection for an API system will be greatly different from that found for well-designed CI systems, and it is not known if design changes can be made that will result in subfemtogram sensitivity of detection for either API or CI systems. These are important issues that require further study.

Acknowledgment

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

^{252}CF-PLASMA DESORPTION MASS SPECTROMETRY (PDMS) OF ANTIBIOTIC MOLECULES

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The use of mass spectrometry for the analysis of antibiotic molecules is mainly to confirm the molecular weight of a structure deduced by NMR or x-ray analysis. The chemistry of Cf-plasma desorption mass spectrometry (PDMS) makes it possible to produce molecular ions of the molecule by initiating fast chemical reactions in solid films. The fission track formed by the passage of a high energy heavy ion affords a means of exciting an ensemble of molecules at solid-state density in times on the order of 10^{-16} s. Reactions that occur depend on the nature of the inter-molecular interactions and can include electron transfer (hedamycin), proton transfer (erythromycin) and ion-dipole attachment (β-endorphin). Reaction products can be desorbed from the solid surface by the shock wave that follows the initial excitation. Although the application is in mass spectrometry of biomolecules, PDMS may be another approach to the study of solid state organic photochemistry.

Key words: β-endorphin; charge transfer; chlorophyll; erythromycin; fast reactions; fission track; ion-dipole; ^{252}Cf.

I. Introduction

Natural products continue to be a source of molecules with unusual structures that have biological activity. Some of these molecules like bleomycin [1] and hedamycin [2] have potent anti-tumor activity. An understanding of the relationship between molecular structure and anti-tumor activity may lead to the development of more effective chemotherapy agents. The current approach to the elucidation of an unknown structure involves a combination of NMR and x-ray analysis of the parent molecule and degradation products. A molecular weight determination by mass spectrometry is part of the data required to confirm a proposed structure. This is done by producing gas phase molecular ions by a combination of physical and chemical processes. If the molecule is involatile, the solid → gas phase transition is difficult and many analyses are not possible because the phase transition cannot be detected. Field desorption [3] solves the problem in many cases by imposing a high electric field gradient at the solid-gas interface which perturbs the dipole interactions. The rapid heating technique introduced by Friedman et al. [4] is based on the premise that the rate of sublimation is faster at higher temperatures than is thermal decomposition. The fastest heating techniques using high energy heavy ions (nuclear fission fragments) [5] or pulsed lasers [6] form shock waves as the medium for rapid heating, and these techniques represent the limit for the speed of heating a sample.

II. ^{252}Cf-Plasma Desorption Mass Spectrometry (PDMS)

This technique, introduced by Macfarlane and Torgerson in 1974 [5], employs the fission fragments emitted from the radioactive decay of ^{252}Cf to rapidly excite an ensemble of molecules in a solid matrix. A typical fission fragment (^{105}Tc-100 MeV) passing through a solid film will deposit $\sim 10^4$ eV/nm in its path. The initial interaction produces electron-hole pairs ($\sim$3 eV/pair) by energy transfer via the intense electric field pulse of the moving fission fragment and the electric

dipole moments of the molecules in the medium. The interaction can extend to ~20 nm from the trajectory. The electron-hole recombination results in a collection of excited molecules at high density. The nature of the excited states depends not only on individual molecular properties but also on the intermolecular interactions and can in the case of ordered arrays give rise to exciton bands [7]. Exciton-mediated processes can transfer electronic energy to neighboring molecules, deposit energy in traps generated by impurities or transform electronic excitation into lattice vibrations (photons). Thus, the ensemble of excited molecules in the fission track is sensitive to properties of the aggregate state such as relative orientation and impurities. Both field desorption and plasma desorption depend upon interactions of aggregate molecules and both have shown sensitivity to the presence of certain impurities.

For PDMS, chemical reactions occur in molecular excited states. (Proton transfer occurring readily between amino acid molecules in PDMS is endothermic by ~6 eV). The molecular ions formed are desorbed by the intense shock wave that arrives after many of the fastest reactions and fragmentations have taken place. The shock wave is so intense that it displaces atoms of crystals from their equilibrium position producing a radiation-damaged area of ~20 nm diameter that can be directly observed by electron microscopy. A schematic diagram of the sequence of events that takes place in PDMS is shown in Figure 1. The initial excitation is to the intrinsic conduction band to form the electron-hold pair followed by de-excitation to lower-lying states where the fast chemical reactions take place. For a molecule like anthracene, which has a strongly fluorescing S_1-state, emission from this state can be observed when excited by fission fragments.

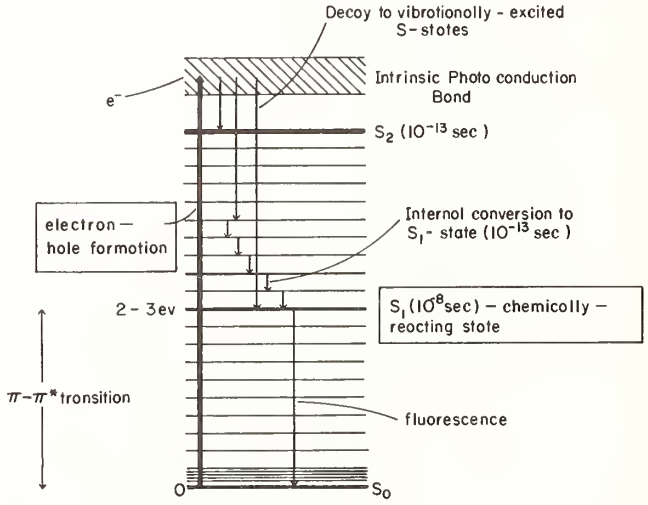

Figure 1. Schematic Jablonski diagram of the excitation of a molecule by fission fragments. Initial process produces an electron-hole pair which decays to lower-lying excited states. Fast chemical reactions may occur during the cascade de-excitation.

III. Electron Transfer

For molecules that exhibit significant π-π intermolecular interactions, electron transfer can take place producing an $M^{\pm}$ geminate transient species. This can be detected directly if the molecular ions are desorbed by the shock wave pulse. Chlorophyll was first observed to exhibit this reaction. The antibiotic, hedamycin having an extensive conjugated π system, also undergoes this reaction (Fig. 2).

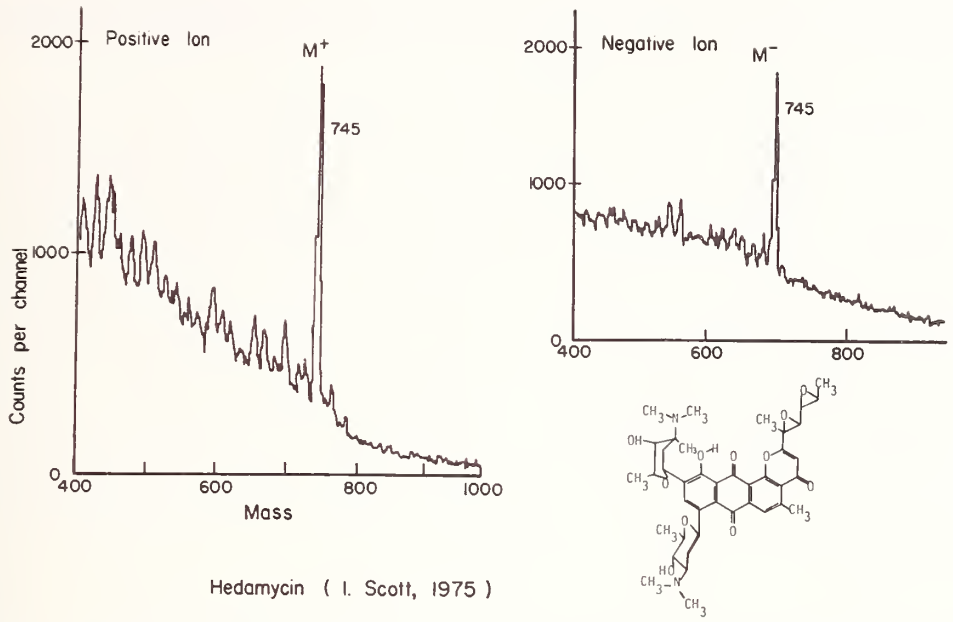

Figure 2. ^{252}Cf-PDMS spectra of hedamycin in the region of the molecular ion: an example of e^--transfer to produce an $M^{\pm\cdot}$ geminate.

IV. Proton Transfer

If the excited states of the aggregate system are mediated by H-bonding as in the case of amino acids, and mononucleotides, H^+-transfer can result in the formation of an $(M \pm H)^{\pm}$ geminate. We have shown this reaction mechanism to be valid using deuterium-labelled valine as a model compound and have also demonstrated the equivalence of positive and negative molecular ion yields. Erythromycin also undergoes this reaction.

V. Ion-Dipole Attachment

There is evidence that the charge transfer reactions are among the fastest. If this reaction is retarded because the coupling between molecules does not contain a significant charge transfer component, then slower reactions can occur. The most common of these is alkali metal ion attachment. Ions like Na^+ are present at some level in all materials and solvents. In the formation of a solid film of a compound from a solution, the alkali metal ions will settle into polar sites around a large biological molecule. When the molecular aggregate is excited the alkali metal ion can form a chemical bond at the polar site. The reaction probability is dependent on the concentration of alkali metal ions. We have studied this reaction using α-cyclodextrin, a cyclic hexose, as a model compound. It forms $(M+Na)^+$ ions using the Na-impurities in the sample. If we add Li or Cs-salts to the sample we form $(M+Li)^+$ or $(M+Cs)^+$ ions. At higher metal ion concentrations, we observe $(M+3Li\text{-}2H)^+$ ions, indicating that H-Li exchange reactions can also occur in the excited molecular aggregate. We have on occasion observed $(M\text{-}H)^-$ ions of this molecule, an indication that H^+ transfer is possible under certain conditions. That the yield is not reproducible suggests that the degree of intermolecular ordering for a particular sample can effect the probability for a particular reaction to occur.

Large peptides, like β-endorphin, undergo cation attachment to form $(M+Na)^+$ ions. Figure 3 shows the PDMS spectrum of this molecule. This result also demonstrates that the desorption of large molecules is possible by this technique.

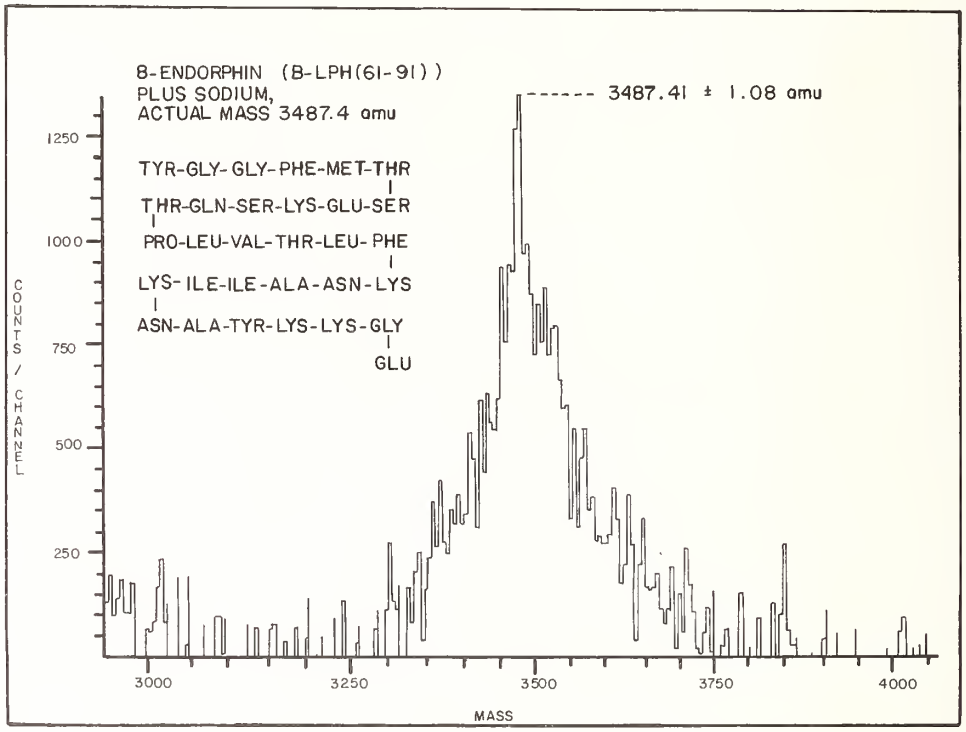

Figure 3. ^{252}Cf-PDMS of β-endorphin; an example of the desorption of a large molecule after alkali metal ion attachment.

VI. PDMS and Molecular Weight Determination

The central theme of this paper is the molecular weight determination of unknown antibiotics by PDMS. There are some important technical aspects of the PDMS measurement that relate to this problem. The method, as utilized by us, involves time-of-flight techniques. It is also a measurement that can be made over a relatively long period of time (many hours), if the molecular ion yield is weak. We can monitor a wide mass range (e.g., 500–3000) in a dynamic mode for as long as is necessary without detectable sample loss or degradation. Utilizing these principles, we have high sensitivity for detecting molecular ions of an unknown molecule if they are formed.

We consider the problem of molecular ion formation in PDMS as one of identifying a particular solid state organic photochemical reaction that produces molecular ions as products. We have listed in this paper some of the reactions we believe occur in PDMS. The signatures of these reactions, e.g., geminate ion pair formation and cationization, give ions whose mass can be used to deduce the molecular weight of the parent molecule. The problems arise when, for example, an antibiotic, capable of producing molecular ions by one of these reactions does not do so because the solid matrix contains impurity species that inhibit the particular reaction by a variety of solid state processes that might include charge and energy, trapping and quenching. Perhaps this is the reason why some antibiotic molecules only give molecular ions after a certain level of purity has been attained.

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RECENT ADVANCES IN SYNCHRONOUS LUMINESCENCE SPECTROSCOPY FOR TRACE ORGANIC ANALYSIS

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The use of the synchronous excitation technique and second-derivative luminescence spectroscopy for monitoring organic pollutants is reported. The combination of these two methods can significantly improve the selectivity of luminescence spectroscopy.

Key words: Cresol; luminescence spectrometry; organic pollutants monitoring; synchronous excitation spectroscopy.

I. Introduction

In recent years, the public concern about the occurrence of many organic pollutants, such as the polynuclear aromatic compounds (PNA), in the environment has created an urgent need to develop instrumentation and methods that can quickly analyze environmental samples. Most characterizations of PNA's in environmental samples have required elaborate and time-consuming separation techniques. The need for relatively simple, on-the-spot techniques is particularly strong if the analyses are to be performed routinely on a large scale. One technique, described in this work, that offers rapidity and simplicity while improving compound selectivity is the synchronous excitation luminescence method.

With its inherent sensitivity, luminescence spectroscopy has provided an important tool for monitoring trace organic pollutants. The use of conventional luminescence methods with fixed excitation wavelength or fixed emission wavelength, however, has practical limitations because of the frequent overlap of the various emissions from the numerous components in complex mixtures. In order to combat this difficulty, a methodology was developed [1] that is based on the idea of synchronous excitation [2]. The synchronous excitation technique can be applied to obtain not only spectroscopic fingerprints from complex samples, such as oil spills [3], but also specific information of analytical interest. The second-derivative (d^2) technique has also been used to further enhance the selectivity of the synchronous method.

II. Synchronous Excitation Spectroscopy

Conventional luminescence spectroscopy uses either a fixed wavelength excitation (λ_{ex}) to produce an emission spectrum or a fixed emission wavelength (λ_{em}) to record an excitation spectrum. With synchronous spectroscopy, the luminescence signal is recorded while both λ_{em} and λ_{ex} are simultaneously scanned. A constant wavelength interval $\Delta\lambda$ is maintained between the excitation and the emission monochromators throughout the spectrum. As a result, the intensity of the synchronous signal I_s can be written as a product of two functions (1):

$$I_s(\lambda_{ex},\ \lambda_{em}) = k \cdot c \cdot E_x(\lambda_{ex}) \cdot E_M(\lambda_{em}), \qquad (1)$$

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where

k = a constant,
c = concentration of the analyte,
E_X = excitation function,
E_M = emission function.

For a single molecular species the observed intensity I_s is simplified (often to a single peak), and the band width is narrower than for the conventional emission spectrum. Figure 1(a) shows the fluorescence excitation and emission spectra of phenol in ethanol. In Figure 1(b), the synchronous signal of the same sample is given; in this example a 3-nm interval ($\Delta\lambda$) between λ_{em} and λ_{ex} was used. Note the broad and featureless structure of both conventional spectra and the narrow peak of the synchronous signal. This feature can significantly reduce spectral overlap in multicomponent mixtures. Correlation of the signal wavelength position with the structure of the compounds becomes easier. For example, the spectrum of a higher ring-number cyclic compound occurs generally at a longer wavelength than the spectrum of a lower ring-number compound. With conventional spectroscopy, this basic rule cannot often be utilized advantageously due to severe spectral overlap. By confining each individual spectrum to a narrow and definite spectral band, the synchronous method offers the possiblity of identifying specific compounds or a group of compounds in a mixture [1].

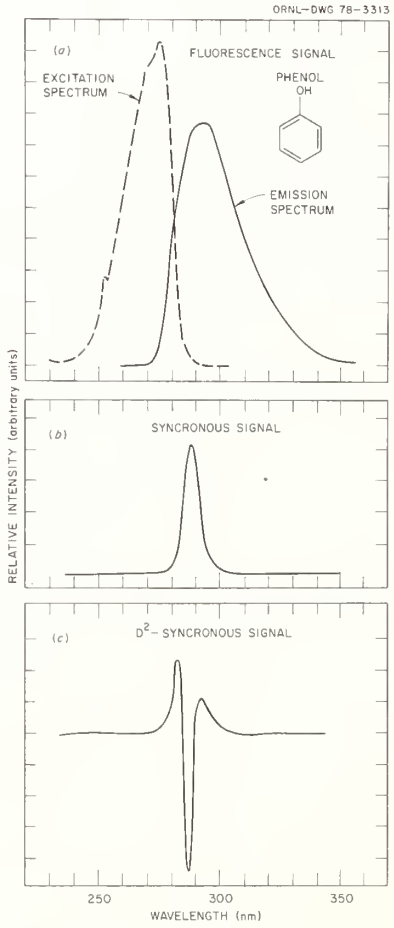

Figure 1(a) Fluorescence emission (λ_{ex}=265 nm) and excitation (λ_{em}=300 nm) spectra of phenol in ethanol (42 μg/mL). (b) Synchronous signal of phenol. (c) Second-derivative synchronous signal of phenol.

III. Second-Derivative Technique

The second-derivative technique can be used to enhance the selectivity of spectroscopic techniques. In this mode of data representation, a signal proportional to the second-derivative of the spectrum with respect to wavelength is produced. Second-derivative signals can be obtained by numerical differentiation [4], modulation technique [5–7], and by direct electronic differentiation [8]. For absorption or flame emission spectroscopy the modulation technique is usually employed because direct analog differentiation of the detector output may substantially degrade the signal-to-noise (S/N) values, whereas it was found for luminescence spectrometry that there is no significant difference between the S/N values in the d^2 spectra obtained by these two techniques. The present availability of inexpensive commercial devices that can be added to an existing spectrometer makes the choice of electronic differentiation most suitable for our application.

In this study the output of the spectrofluorimeter is converted by a commercial electronic differentiator (Perkin Elmer, Model 200-0507) into the first (d^1) and second (d^2) derivative with respect to time. Since the monochromator is scanned at a constant rate, the signal obtained is also equivalent to a wavelength derivative spectrum.

In the d^2 mode one measures the rate of change of curvature of a peak. Broad peaks are eliminated in the recording while sharp spectral features are intensified. Hence, one has a means for improving compound selectivity. Figure 1(c) shows the d^2 response of the synchronous signal of phenol. In this case, the second derivative and synchronous methods of signal generation and recording are coupled to produce an improvement in selectivity.

IV. Applications

The synchronous and second-derivative techniques have useful applications in many cases of environmental sample analysis. They are most valuable in situations where the variations in the composition of complex samples (containing a large number of organic compounds, e.g., oil spills, exhaust soot, by-product water) fail to provide significant changes in the conventional fluorescence spectrum.

The simple method of analysis using synchronous excitation opens up a host of possibilities for monitoring organic pollutants by luminescence spectroscopy. Some recent practical applications include the characterization of PNA's in by-product water and the multicomponent analysis by room temperature phosphorimetry of organic compounds absorbed on filter paper [9].

An example showing two d^2 synchronous signals is given in Figure 2. This figure shows the signal of phenol and p-cresol in ethanol. The complete separation of the two peaks illustrates the spectral specificity of the technique. The wavelength difference in the peak positions between phenol and its methyl derivative is sufficient to allow its use for qualitative analysis. Comparison with the d^2 synchronous spectrum of a solution of synthane coal gasifier by-product water in ethanol (1 mL/L) reveals the presence of both compounds (Fig. 3). For comparison, the conventional fluorescence spectra of the same by-product water sample are given in Figure 4. The sharp peaks in the d^2 synchronous signals, contrasted with the broad and featureless structure of the conventional spectra, illustrate the significant improvement in selectivity.

The simplicity of the technique makes it particularly attractive as a "rapid screening" method for monitoring organic pollutants. It can be easily applied to spectrofluorimetry as well as to spectophosphorimetry. No additional equipment is required and synchronous measurement can be performed directly using any commercial spectrofluorimeter in which excitation and emission monochromators can be interlocked.

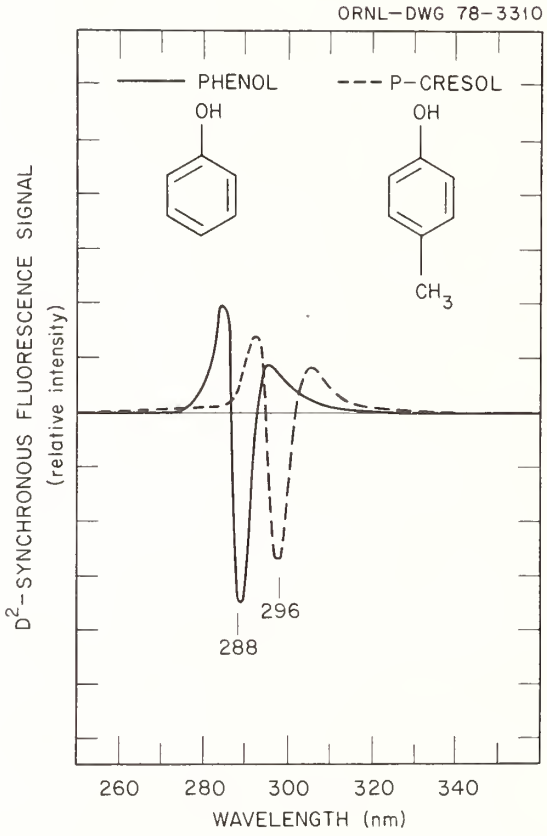

Figure 2. Second-derivative synchronous signal ($\Delta\lambda = 3$ nm) of phenol (30 μg/mL) and p-cresol (41 μg/mL) in ethanol.

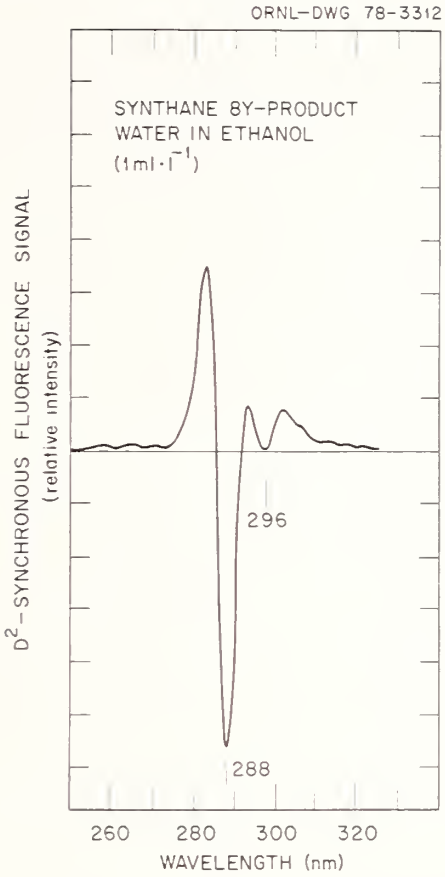

Figure 3. Second-derivative synchronous signal ($\Delta\lambda = 3$ nm) of synthane by-product water in ethanol (1 mL/L).

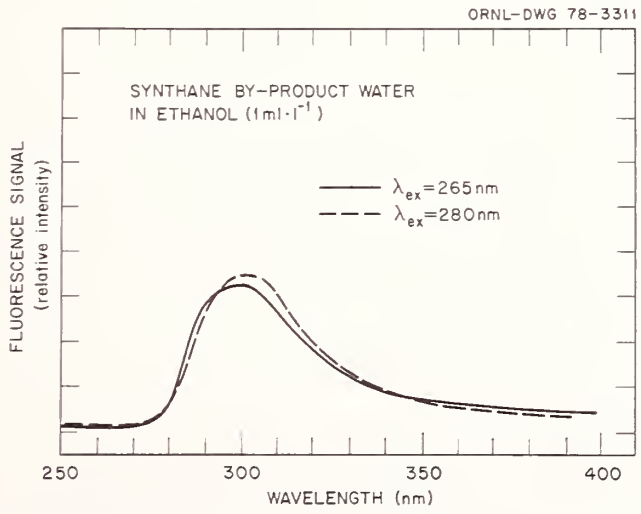

Figure 4. Conventional fluorescence spectra of by-product water from the synthane coal gasifier in ethanol (1 mL/L).

V. Acknowledgment

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

ANALYSIS OF INDIVIDUAL POLYCHLORINATED BIPHENYLS (PCBs) AND THEIR HEPATIC MICROSOMAL METABOLITES BY HPLC-MS

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The new technique of liquid chromatography interfaced mass spectrometry (LCMS) was applied to reactant and product identification in the in vitro metabolism of 2-, 3-, and 4-chlorobiphenyl by hepatic microsomal cytochrome P-450. Individual monochlorinated biphenyl (MCB) isomers were purified and quantitated by HPLC with a reverse-phase C_{18} column (67.5% water/acetonitrile solvent mixture). This system proved to be incompatible with LCMS and therefore a normal phase Porasil column method (0.5% methanol/methylene chloride solvent mixture) was developed. The direct introduction of LC effluent into the mass spectrometer (via interface) supported the purity of the reactant materials. Analysis of the metabolized mixtures identified, in addition to several nonchlorinated interferents, a monohydroxychlorobiphenyl and, at trace levels, a dihydroxychlorobiphenyl for each of the three MCBs. A second monohydroxychlorobiphenyl product was also identified in the metabolism of 3-chlorobiphenyl with a significantly different retention time from the other products. Kinetic analysis of the metabolism of the three MCBs showed that, for the production of the monohydroxylated product, 4-chlorobiphenyl has a V_{max} significantly greater than those of the other two MCBs, while 3-chlorobiphenyl has a significantly lower K_m.

Key words: Cytochrome P-450; liquid chromatography-mass spectrometry; PCB metabolism; polychlorinated biphenyls.

I. Introduction

The widespread introduction of mixtures of PCBs into the environment [1] and the subsequent persistence of the more highly chlorinated constituents in animals [2] and man [3] has potentiated numerous investigations into their metabolism.

The study of PCB metabolism has been hindered by the complexity of the metabolic and excretory pathways in experimental animals and the indirect methods of analysis [4–7]. In the present study the investigation of the in vitro metabolism of individual monochlorobiphenyls (MCBs) was greatly facilitated by the application of the new technique of high performance liquid chromatography directly interfaced to a mass spectrometer (LCMS) for metabolite identification.

The hepatic microsomal mixed function oxidase catalyzed metabolism of a substrate (S) can be described as,

$$NADPH + O_2 + S + H^+ \rightarrow NADP^+ + SOH + H_2O \quad (1)$$

where S, in the present case, is a monochlorinated biphenyl and the available proton is supplied by the NADPH. The hydroxylated product is monohydroxymonochlorobiphenyl (MCB-OH). Note that if SOH is substituted for S, the product (SOH)OH or dihydroxymonochlorobiphenyl(MCB-$(OH)_2$) would be the expected product.

II. Materials and Methods

A. MCB Metabolism

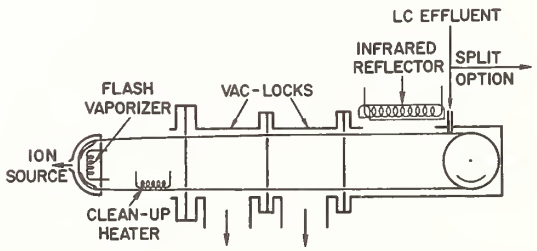
Individual MCB isomers (Analabs, North Haven, CT) were purified by HPLC. A Waters Model 244 HPLC with a reversed-phase microparticle silica column (μBondapak/C_{18}) and a mixture of 67.5% acetonitrile/water was used for purifying and analyzing the MCBs and the metabolites. Individual PCBs and commercially available MCB-OHs (RFR Corp., Hope, RI) were chromatographed and used for identification and quantitation.

The in vitro metabolism of individual PCBs by hepatic microsomal cytochrome P-450 was studied using the above system. 2-, 3-, and 4-chlorobiphenyl were used in this study. Male Wistar rats (250$\pm$10 g) were utilized to provide the hepatic microsomes. Each MCB (0.1 to 2.0 mmol/L) was incubated at 37 °C with a microsomal preparation (2 mg protein/mL, 1 nmole cytochrome P-450/mL) from rat liver together with NADPH (2.25 mmol/L) for 5 minutes. The MCB and its metabolites were extracted with ether and the residue taken up in tetrahydrofuran solution and analyzed. The identification of various metabolites was supported by both retention time data and mass spectrometry analysis.

B. Mass Spectrometry

A Finnigan 4000 mass spectrometer was equipped with the first commercially available liquid chromatograph interface (Finnigan) comprised of a mechanical moving belt which transfers samples and evaporates volatile solvents (from ambient pressure) through two differentially pumped chambers to the high vacuum of the mass spectrometer (Fig. 1). This process is efficient for volatile solvents with sample transfer rates of 30% and EI mass spectral conditions [8]. The limiting solvent is water (high polarity and surface tension) which beads up and freezes on the transfer belt, thus reducing the evaporation rate and causing "bumping" of the MS pressure as frozen droplets reach the high vacuum. This limits the water flow rate from the LC to 0.4 mL/min onto the belt. The present reversed phase system, where desired separation is lost with less than 20% water/acetonitrile, requires split ratios of 5:1 to meet the above criteria, severely limiting "trace" analysis by LCMS.

An alternative system was developed using a normal phase Porasil column with a 0.5% methanol/methylene chloride solvent system at 0.6 mL/min. All solvents were HPLC grade (Burdick and Jackson, Muskegon, MI). This system was used with the LCMS interface.



Courtesy of W. H. McFadden, Finnigan Corporation, Sunnyvale. California.

Figure 1. Liquid chromatograph-mass spectrometer interface.

III. Results and Discussion

The mass spectra of a MCB, MCB-OH and MCB-$(OH)_2$ are shown in Figure 2. Under the best conditions, the mass spectral scan is limited to greater than m/e 100 due to background from

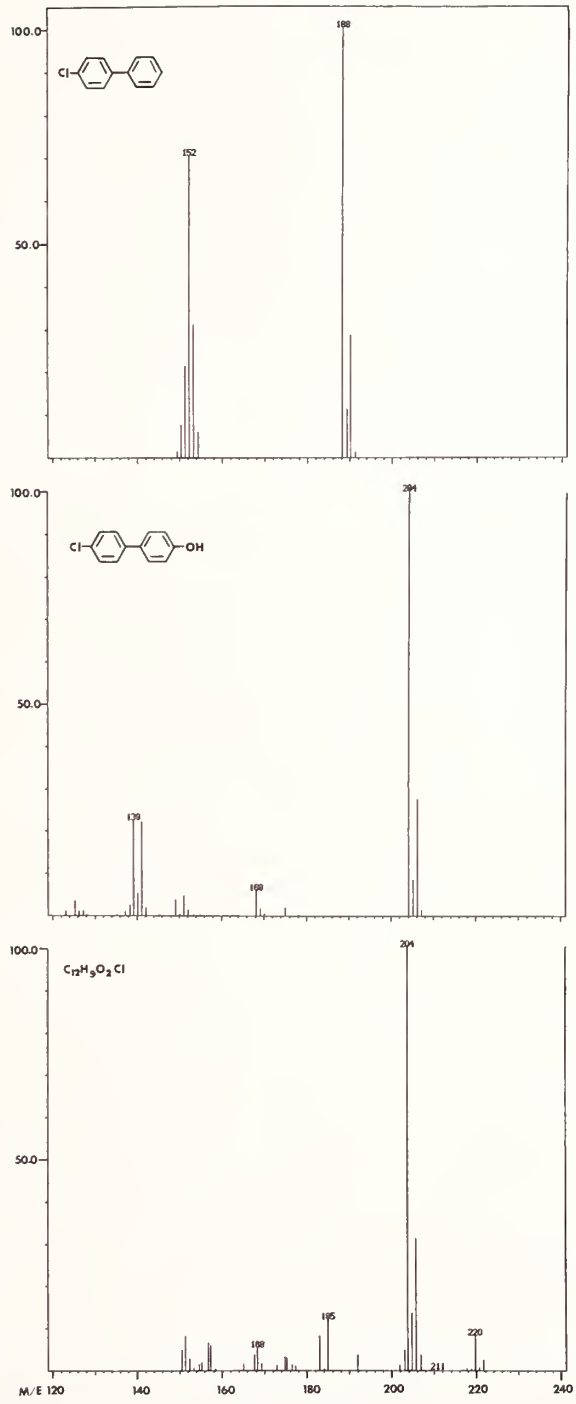


Figure 2. The mass spectra of monochlorobiphenyl (MCB), monohydroxychlorobiphenyl (MCB-OH), and dihydroxychlorobiphenyl (MCB-$(OH)_2$) show characteristically different mass spectral patterns.

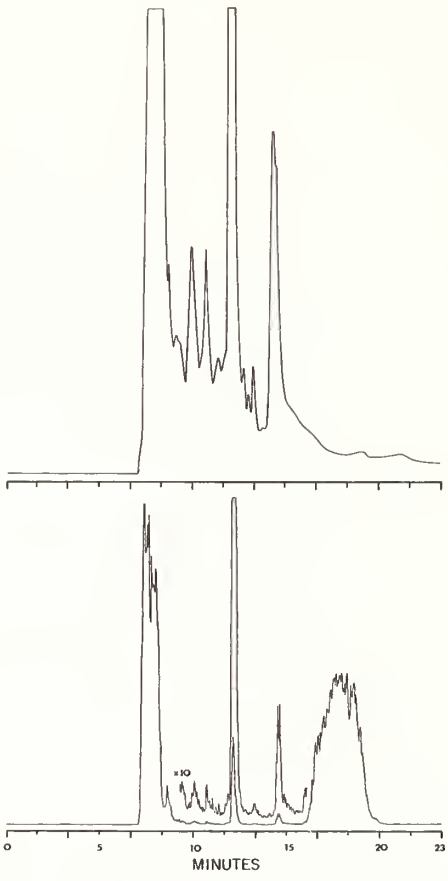


Figure 3. LC (254 nm) and LCMS-RIC (m/e 100-600) traces of the cytochrome P-450 catalyzed metabolite mixture from 4-chlorobiphenyl. Identified elutants are: 4-chlorobiphenyl plus nonchlorinated interferents (7.7 min.), monohydroxychlorobiphenyl (12.1 min.), and dihydroxychlorobiphenyl (21.2 min., not visible on chromatogram, but centered around scan 650 in Fig. 4).

the solvent impurities. Comparison of a HPLC (detected at 254 nm) and a LCMS-RIC trace (m/e 100–600) shows the differing responses of the two detection methods (Fig. 3).

Selected ion monitoring of the chlorine cluster for each expected compound (i.e., MCB m/e 188, 189, 190) discriminated against the background and permitted quantitation of the individual compounds. On column injections of standards produced linear calibration curves to 1 ng for MCB (m/e 188) and MCB-OH (m/e 204). Conditions deteriorated rapidly, however, with a 5-fold loss in sensitivity in 40–50 hours running time. Due to the limited number of SIM mass peaks for these light compounds (which gave unambiguous identification but produced spurious RIC peaks), an additional criterion was imposed on the SIM-RIC plot in Figure 4. A peak was plotted only when the chlorine isotope ratio was correct for M^+:$(M+2)^+$, namely 3:1. The resulting plot enhances the chlorinated reactants and products and, in addition, defines the second monohydroxylated MCB for the metabolism of 3-chlorobiphenyl not previously identified in LC studies (Fig. 4).

Thus, based on retention times and mass spectral analysis, the metabolism of 4-chlorobiphenyl only gives rise to 4′-chloro-4-hydroxybiphenyl and traces of 4′-chloro-3,4-biphenyldiol; 3-chlorobiphenyl gives rise to 2 monohydroxylated biphenyls and a dihydroxylated biphenyl; and 2-chlorobiphenyl gives rise to a mono and a dihydroxylated compound.

The rates of formation of the monohydroxylated products of 2-, 3-, and 4-chlorobiphenyl were determined as a function of substrate concentration using the HPLC. The results are

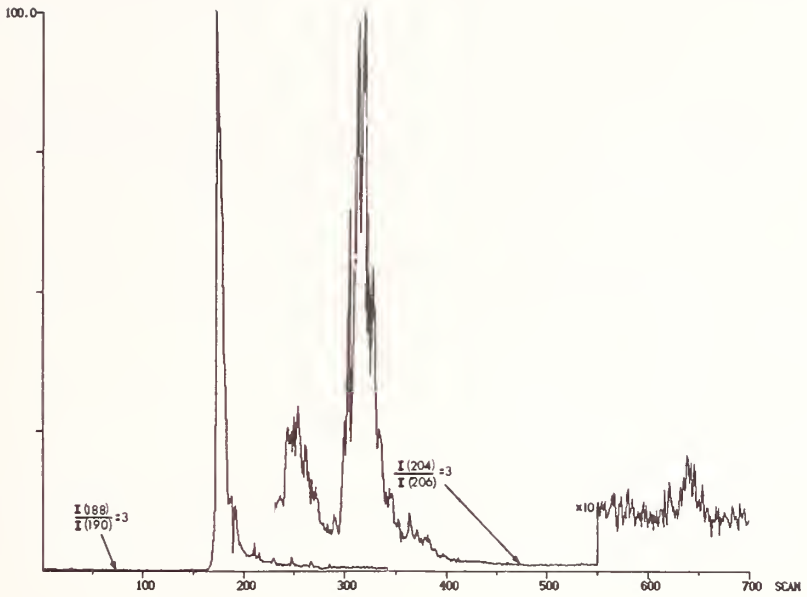


Figure 4. The mass spectral selected ion trace of the metabolized mixture from 3-chlorobiphenyl enhances the chlorinated products when only peaks meeting the criterion of $M^+/(M+2)^+=3$ are plotted. The peak at scan 250 is a second monohydroxychlorobiphenyl.

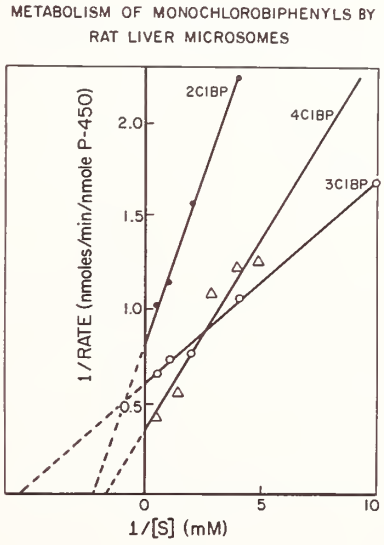
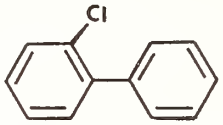
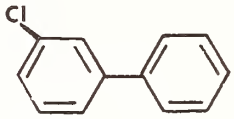
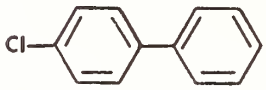


Figure 5. Lineweaver-Burk plot of the hepatic microsomal metabolism of 2-, 3-, and 4-chlorobiphenyl. Reactions were performed at 37 °C in the presence of air. Reactions were initiated by the addition of NADPH.

presented in the form of a Lineweaver Burk plot in Figure 5. Apparent V_{max} and K_m values were calculated from these results and are shown in Table 1. The position of the chlorine on the biphenyl nucleus affects the affinity for the cytochrome P-450, with a 3-chloro substituent producing the highest affinity. This does not, however, permit the fastest rate of metabolism which is conferred by the 4-chloro substituent.

TABLE 1. *Metabolism of monochlorobiphenyls by rat liver microsomes*

	K_m (mM) (n=4)	V_{max} (nmoles/min/ nmole P-450)
	0.59 ± 0.10	1.02 ± 0.14
	0.27 ± 0.09	1.77 ± 0.16
	0.63 ± 0.10	3.07 ± 0.21

IV. Acknowledgments

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SUB-PART-PER-TRILLION DETECTION OF ORGANICS IN AQUEOUS SOLUTION BY LASER INDUCED MOLECULAR FLUORESCENCE

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Pulsed laser irradiation has been demonstrated to be a superior excitation source for fluorescence analysis. Three different configurations have been compared experimentally, and the superior system used as the excitation source in the trace analysis of several organics in aqueous solutions. In favorable cases sub-part-per-trillion detection limits can be achieved.

Key words: Fluorescence; organics in water; pulsed laser.

I. Introduction

Fluorescence techniques have long seen extensive application in chemical analysis. The analytical potential of fluorescence techniques has been developed to the point where fluorescence spectrometers routinely provide sensitive and selective analysis of trace quantitites in complex mixtures. The power of the technique is directly attributable to the numerous parameters which can be varied: excitation wavelength, emission (fluorescence) wavelength and fluorescence lifetime. In addition, non-fluorescent materials can be analyzed by covalently attaching fluorescent labels. Finally, fluorescence properties have been shown to be a sensitive function of the local environment and can, for example, be used to distinguish free dye molecules from those bound to DNA.

The development of laser techniques has greatly augmented the analytical applications of fluorescence spectroscopy. The most notable change has been to extend the limits of detection, usually by several orders of magnitude [1,2]. Some of this improvement can be traced to the use of time resolved fluorescence techniques with pulsed laser sources.

This paper describes an experimental comparison of three laser fluorimetry systems. Two of the systems used analog signal processing while one system used digital photon counting. One system used a high peak power, low repetition rate laser while the other two systems used a low peak power, high repetition rate laser. For many applications a nitrogen pumped dye laser with boxcar integrator signal processing is currently the most desirable fluorimetry system. The limits of detection for a number of polycyclic aromatic hydrocarbons and biochemicals in aqueous solution were determined with this system.

II. Experimental

Figure 1 is a schematic of the experimental apparatus used to evaluate the merits of a high peak power, low repetition rate laser as the excitation source for laser fluorimetry. A Molectron UV1000 nitrogen laser is used to pump a dye laser operated in the DL200 configuration. A small portion of the beam is picked off and used as a trigger and reference signal. Typical peak powers

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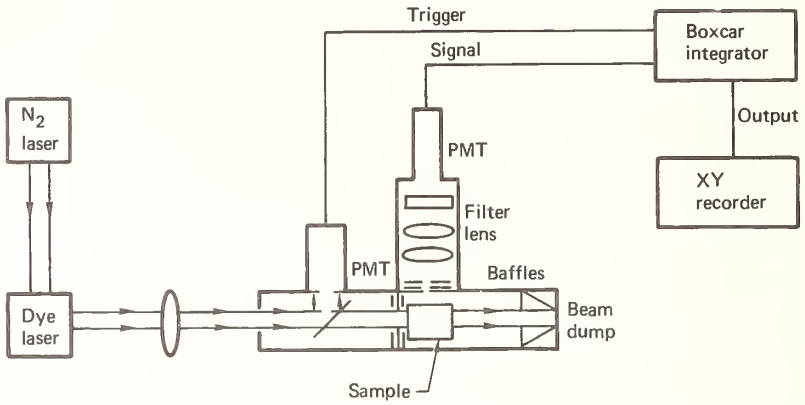

Figure 1. Schematic of the experimental apparatus used to evaluate the high peak power, low repetition rate laser excitation source with analog detection.

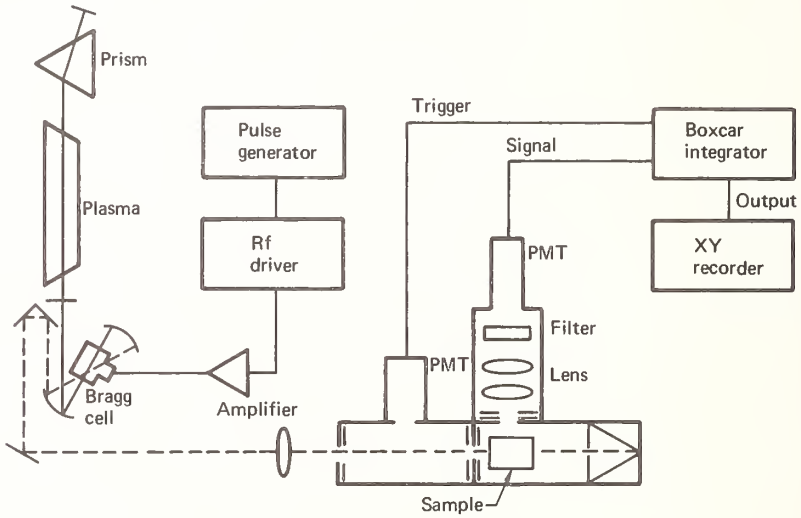

Figure 2. Schematic of the experimental apparatus used to evaluate the low peak power, high repetition rate laser excitation source with analog detection.

actually delivered to the cell were 2 KW at 30 Hz. The fluorescence is viewed at right angles through a cut-off filter, detected by an RCA 8850 photomultiplier tube, and subsequently processed by a dual channel PAR 162/163/164 boxcar integrator.

Figure 2 is a schematic of the experimental apparatus used to evaluate the merits of a low peak power, high repetition rate laser as the excitation source. A Spectra-Physics cavity dumped 170–09 argon ion laser, operating at 514.5 nm, was used. Typical peak powers actually delivered to the cell were 5 W at ~1 MHz. The sample chamber, detection and signal processing were identical to those used with the high power laser.

Figure 3 is a schematic of the experimental apparatus used to evaluate the low peak power, high repetition rate laser using digital signal processing. A Lexel 96 argon ion laser operating at 514.5 nm was mode locked, typical peak powers being 1 W at 120 MHz. External to the laser cavity, an acoustically modulated cell was used to select a single pulse. For this experiment single pulses were deflected into the sample chamber used in the previous experiments at a rate of 1 MHz. An EMI 9789 photomultiplier tube was used to detect the fluorescence pulses. Ortec pulse counting modules were used to digitize the analog signal.

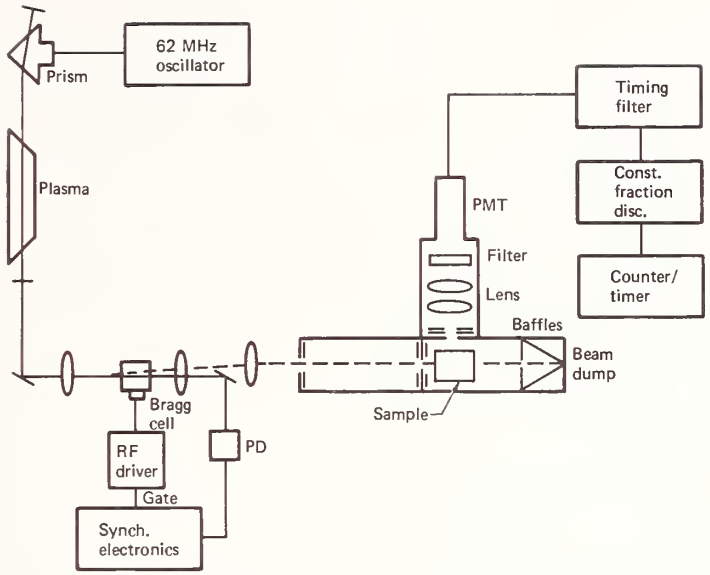

Figure 3. Schematic of the experimental apparatus used to evaluate the low peak power, high repetition rate laser excitation source with digital detection.

III. Results

Rhodamine B was chosen as the fluorophor to compare the three experimental configurations [3]. The nitrogen pumped dye laser was tuned to 514.5 nm. The results are summarized in Table 1. The configuration shown in Figure 1 had the least scatter in the data. This was determined by a linear least squares fit to the fluorescence intensity versus concentration analytical working curves. The detectability power product is a useful figure of merit in determining the absolute sensitivity in terms of parts-per-trillion (ppt) detected per unit energy delivered per second.

The nitrogen pumped dye laser and cavity dumped argon ion laser were shown to perform equally well under identical conditions for trace detection. However, other factors should be considered in choosing a laser system for analytical applications. Rhodamine B was specifically chosen as the test fluorophor so as not to unduly handicap the ion laser systems. The cavity dumped ion laser offers only a few select lines; the output can be used to pump a dye laser but at a considerable power loss. The smaller signal size associated with smaller peak powers leads to a loss in flexibility. For example, including a monochromator decreases the signal intensity dramatically. This can be mostly compensated for by increasing the photomultiplier gain with the nitrogen pumped dye laser, but with the lower power laser system all the gain is needed with just the filter.

TABLE 1. *Comparison of different experimental configurations for rhodamine B detection*

Apparatus	Limit of Detection (ppt)	Slope	Detectability power product (ppt-mW)
Figure 1	1.0	0.916±0.016	0.5
Figure 2	0.5	0.84 ±0.03	0.5
Figure 3	15	0.96 ±0.06	2.5

Tunability is much larger with the nitrogen pumped dye laser through a wider selection of both dyes and doubling techniques. Furthermore, the higher peak powers permit the application of nonlinear optical techniques to analytical applications. Consequently, the nitrogen pumped dye laser offers greater flexibility in potential analytical applications. These results indicate that the synchronously pumped dye laser might prove to be another reasonable alternative although it is more laser-technology oriented.

Table 2 summarizes the limits of detection achieved for polycyclic aromatic hydrocarbons (PAH) using laser induced molecular fluorescence [2]. The temporal resolution inherent in a nitrogen pumped dye laser was instrumental in achieving the particularly large improvement in detection limits for fluoranthene and pyrene. The detection limits for representative PAH have been extended sufficiently to consider PAH detection in ground water [4].

TABLE 2. *Limits of detection of PAH in water*

PAH	Transition of interest	Excitation wavelength (λ_{ex}) nm	Absorptivity at λ_{ex} $L \cdot mol^{-1} \cdot cm^{-1}$	Fluorescence wavelength (λ_{ex}) nm	Limits of detection (μg/L) LIMF	Conventional[c]
Benzene	$1_A \rightarrow 1_{L_b}$	259.95	9×10^{1}[a]	302,273	19	--
Naphthalene	$1_A \rightarrow 1_{L_a}$	273.0	6×10^{3}[b]	340,360	1.3×10^{-3}	3×10^{-2}
Anthracene	$1_A \rightarrow 1_{B_b}$	258.7 254.0	5×10^{3}[b] 1×10^{5}[b]	404	8.9×10^{-3} $<4.4\times10^{-3}$	3×10^{-2}
Fluorathene	$1_A \rightarrow 1_{B_b}$	287.0	4×10^{4}[a]	450	1×10^{-3}	1.7×10^{-1}
Pyrene	$1_A \rightarrow 1_{B_b}$	273.0	6×10^{4}[b]	395	0.5×10^{-3}	1.5×10^{-1}

[a] Methanol/water.
[b] Methanol.
[c] Schwarz, F. P. and Wasik, S. P., Fluorescence Measurements of Benzene, Naphthalene, Anthracene, Pyrene, Fluoranthene and Benzo(e)pyrene in Water, *Anal. Chem.* **48**, 524 (1976).

Table 3 summarizes the limits of detection achieved for several biochemicals using laser induced molecular fluorescence [5,6]. Arginine, a nonfluorescent amino acid, was covalently labeled with fluorescamine to produce a highly fluorescent derivative. Aniline and benzene are expected to have similar fluorescence properties, yet the detection limit for the labeled aniline was more than four orders of magnitude lower than that achieved for unlabeled benzene. Finally, Figure 4 illustrates the advantages of temporal resolution in separating out fluorescence impurities for *in vivo* studies.

In summary, laser induced molecular fluorescence has been shown to extend the limits of detection of organics in aqueous solution by one to four orders of magnitude. The resulting analytical working curves are linear for up to seven orders of magnitude, being limited by solubility or inner filter effects at high concentrations.

The optimal excitation source is currently a nitrogen pumped dye laser. Besides offering high peak power and tunability, the temporal resolution can in favorable cases be used to eliminate background or impurities.

TABLE 3. *Limits of detection of biochemicals in water*

Compound	Excitation wavelength (λ_{ex}) nm	Absorptivity at λ_{ex} $L \cdot mol^{-1} \cdot cm^{-1}$	Fluorescence wavelength (λ_{em}) nm	Quantum yield	Limits of detection (μg/L) LIMF	Conventional[a]
Vitamin A acetate	337.1	1.8×10^4	500	---	1.0	100
Vitamin B_2 (riboflavin)	375.0	1.1×10^4	540	0.26	0.47×10^{-3}	0.038
FAD	267.0	3.8×10^4	525	0.04	39×10^{-3}	---
Vitamin B_6 (pyridoxine)	337.1	1.6×10^3	410	0.10	50×10^{-3}	1.5
Vitamin B_{12} (cyanocobalamin)	260.0	1.2×10^4	310		1.4×10^2	---
Tryptophan	270.0	5.4×10^3	358	0.13	50×10^{-3}	3
Aniline/Fluram	390.0	1.7×10^4	500	0.28	3.5×10^{-3}	>40
Arginine/Fluram	390.0	$\sim 1.7 \times 10^4$	500	~0.25	10×10^{-3}	5

[a] Reference [5].

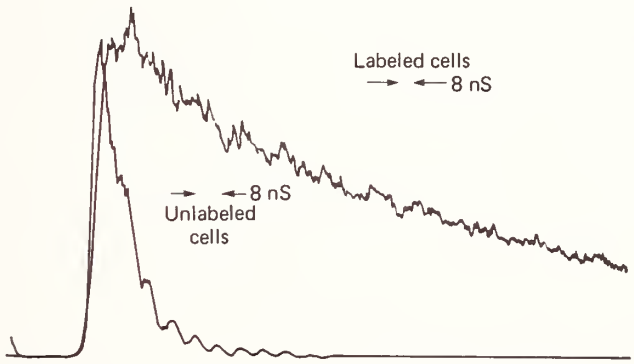

Figure 4. Advantage of temporal resolution in distinguishing unlabeled mouse cells from those labeled with benzo(a)pyrene.

IV. Acknowledgment

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ANALYSIS OF POLYMER OUTGASSING AS STUDIED BY PLASMA CHROMATOGRAPHY-MASS SPECTROSCOPY

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The plasma chromatograph-mass spectrometer has been utilized to analyze the trace level organic species which were outgassed from epoxy polymers. Direct measurements of such compounds as hexahydrophthalic anhydride (HHPA), ethyl cellosolve acetate, and 2,6-ditertbutyl para cresol (BHT) in complex mixtures are demonstrated.

Key words: Epoxy polymer; plasma chromatography-mass spectroscopy; polymer outgassing.

I. Introduction

To protect semiconductor devices from environmental exposure, a wide variety of polymeric materials have been employed in electronic packaging and encapsulation. A major concern which must be addressed in choosing a polymeric encapsulant is its outgassing characteristics. The development of highly sensitive microanalytical techniques is therefore essential to detect and characterize the trace level organic species which are outgassed from encapsulants. Plasma chromatography-mass spectroscopy (PC/MS) is a relatively new ultrasensitive analytical technique which permits the characterization of trace level of organic material on the order of parts per billion and less.

Plasma chromatography [1–5] and the combined plasma chromatography-mass spectroscopy technique [6–7] have been investigated in recent years as methods to detect picogram quantities of organic material. The techniques are capable of detecting both positive and negative ions which are formed at atmospheric pressure as a result of ion/molecular reactions initiated by β-rays emitted from a 63-Ni foil. The phenomenon observed in the ionization process is similar to that observed in chemical ionization mass spectroscopy, with the differences being attributed to the pressure difference [8]. This paper will discuss the application of the combined PC/MS technique to the analysis of trace levels of organic species outgassed from epoxy encapsulants.

II. Experimental

The Alpha-II plasma chromatograph-mass spectrometer manufactured by Franklin GNO Corporation which was used in this investigation has been described previously [9–10]. This instrument consists of a Beta-VII plasma chromatograph coupled to a specially modified Extranuclear Laboratories Spectr-El quadrupole mass spectrometer.

A schematic of the combined PC/MS system is shown in Figure 1. This instrument has the capability of operating in several modes so that ion mobility spectra, mass spectra, and total ion mobility spectra can be obtained. The ionic mobility may be measured in either a one-grid or a two-grid pulsing procedure with the Beta-VII plasma chromatograph mode of operation. Both grids of the drift tube can be held open, allowing all the ions produced in the ionization source to continually drift down the tube and into the quadrupole mass spectrometer, which results in atmospheric pressure ionization mass spectra.

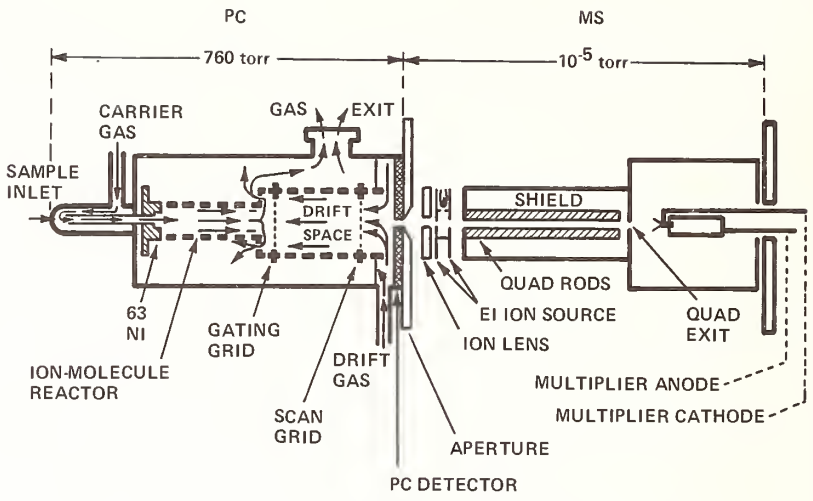

Figure 1. Schematic diagram of Alpha II PC/MS combined system.

TABLE 1. *Operating parameters of the Alpha II plasma chromatograph-mass spectrometer*

Drift gas flow rate	500 mL/min
Carrier gas flow rate	100 mL/min
Applied voltage	±2800 V
Gate width	0.2 ms
Repetition rate	27.0 ms
Temperature	210 °C

Total ion mobility spectra can be obtained by operating the plasma chromatograph in the normal one-grid pulsing mode and the quadrupole mass spectrometer in the total ion mode, which enables the Channeltron electron multiplier detector to measure the ionic distribution as a function of time. By adjusting the mass analyzer to respond only to a single M/E value and by operating the plasma chromatograph in the normal one-grid pulsing mode, the distribution of an individual ion as a function of time can be obtained. The arrival time of the individual ions can then be compared with the arrival time of the ions measured in the total ion mode to produce the mass-identified mobility spectra.

A Nicolet Model SD-72IA integrating ADC mounted in a Nicolet Model 1074, 4096-channel, signal averager was used to digitize the accumulated plasmagrams. Usually 512 scans of 27 ms duration were collected and stored on magnetic tape with a Nicolet Model NIC-28A magnetic tape coupler and Kennedy Model 9700 tape deck. As the data was being accumulated in memory, the information was displayed on a Tektronix Model D10 oscilloscope. The data stored on the magnetic tape was entered into an IBM System/370 computer and was subsequently analyzed using VSAPL under the operating system VM/370. The position and intensity of the peaks in the mobility spectra, along with a plot of the mobility spectra, was then displayed on a graphics terminal from which hard copies could be obtained. The operating parameters used in this study are listed in Table 1.

III. Results and Discussion

The detection and characterization of organic compounds at the subnanogram level has presented a most difficult problem to the materials analyst. The gain in detection limit is often obtained at the expense of other valuable information. For example, chemical ionization mass spectroscopy and, more recently, atmospheric pressure ionization mass spectroscopy offer a tremendous gain in sensitivity over conventional electron impact mass spectroscopy, but only at the expense of sacrificing structural information. The plasma chromatographic technique has shown great promise as being ultrasensitive, but it has its shortcomings.

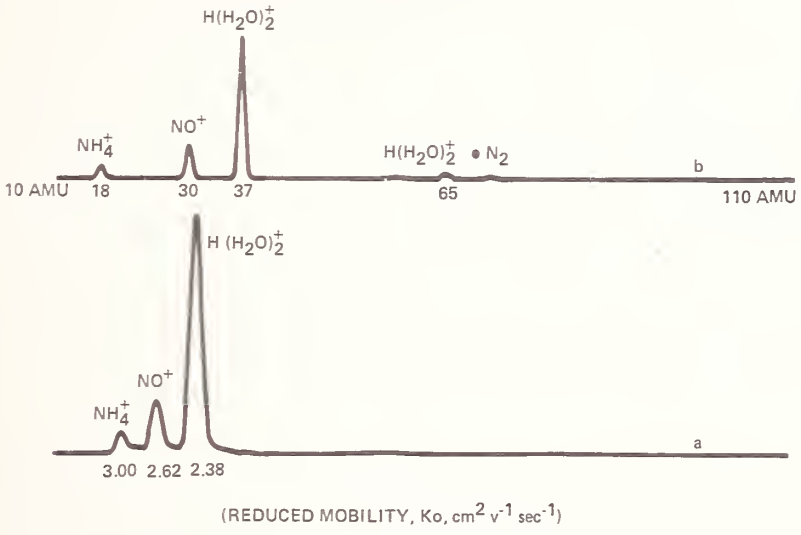

Figure 2. Positive mode (a) ion mobility and (b) mass spectra for nitrogen carrier gas.

Figure 2 shows the positive mode ion mobility and mass spectra obtained from nitrogen carrier gas in the absence of sample. Carroll et al. [11] have identified the positive reactant ions generated in nitrogen carrier gas as being NH_4^+, NO^+, and $H(H_2O)_2^+$. These reactant ions may be involved in several classes of ion-molecule reactions, depending upon the chemical characteristics of the sample molecules. The NH_4^+ and $H(H_2O)_2^+$ ions are generally involved in proton transfer reactions which can be described as simple acid base reactions governed by the relative basicity of the sample molecules:

$$H(H_2O)_2^+ + M \rightarrow MH^+ + 2H_2O \quad (1)$$

The NO^+ reactant ion may be involved in several reactions which can be described as a charge transfer reaction:

$$NO^+ + M \rightarrow M^+ + NO \quad (2)$$

as a hydride extraction:

$$NO^+ + MH \rightarrow HNO + M^+ \quad (3)$$

or as an addition reaction:

$$NO^+ + M \rightarrow MNO^+ \quad (4)$$

In reaction (2) the ionization potential of the sample molecules must be lower than the ionization potential of NO (9.5 eV). Reaction (3) may be described as an acid-base reaction; reaction (4) depends upon the chemical nature of the sample molecules.

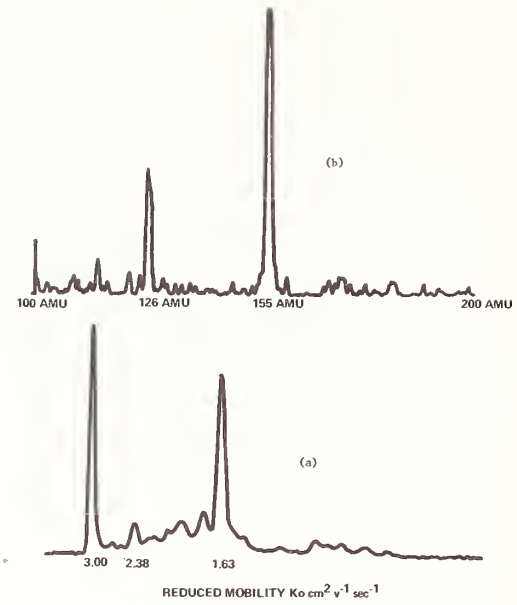

Figure 3. (a) Positive mode ion mobility spectrum obtained from completely cured epoxy polymer; (b) positive mode mass spectrum (100 to 200 amu) obtained from completely cured epoxy polymer.

Figure 3 shows the positive-mode ion mobility and mass spectra obtained from the analysis of the outgassing products from a completely cured epoxy polymer at room temperature. The major component outgassed from this epoxy, the species with the reduced mobility of 1.63 $cm^2\ V^{-1}\ s^{-1}$ and an ionic weight of 155 amu, has been identified as hexahydrophthalic anhydride (HHPA), which is the cross linking agent used in the cure of the epoxy resin. HHPA has a molecular weight of 154 amu and is therefore involved in a proton transfer ionization process.

Figure 4 shows the positive mode ion mobility and mass spectra obtained from the analysis at room temperature of the outgassing products from a similar epoxy cured under slightly different conditions. In addition to the HHPA observed with a reduced mobility of 1.63 $cm^2\ V^{-1}\ s^{-1}$, several other more intense outgassing products are found. The species with mobility 1.26 $cm^2\ V^{-1}\ s^{-1}$ and an associated ionic weight of 219 amu has been identified as 2,6-ditertbutyl-p-cresol (BHT). BHT, a commonly used antioxidant, has a molecular weight of 200 amu; therefore, the most probable ionization mechanism is a hydride extraction reaction with the NO^+ reactant ion. Another species observed to be outgassed from this epoxy polymer is ethyl cellosolve acetate, a commonly used organic solvent. Ethyl cellosolve acetate is characterized by two peaks in the mobility spectrum with reduced mobilities of 1.76 and 2.11 $cm^2\ V^{-1}\ s^{-1}$ and respective ionic weights of 133 amu and 87 amu. Ethyl cellosolve acetate has a molecular weight of 132 amu and is probably involved in a proton transfer ionization reaction leading to the ion of mass 133, the protonation probably occurring at the carbonyl oxygen. The ion of mass 87 is probably formed as a result of fragmentation of the parent molecule. As can be seen from a comparison with the pure ethyl cellosolve acetate as shown in Figure 5, the two ions generated from ECA should be of approximately equal intensity in the mobility spectrum. However, in the ion mobility spectrum of Figure 4, the mobility peak at 2.11 $cm^2\ V^{-1}\ s^{-1}$ is much more intense than the peak at 1.76 $cm^2\ V^{-1}\ s^{-1}$, but a comparison of the peaks in the mass spectrum shows the same ratio observed with the raw material. The reason for the more intense peak with mobility 2.11 $cm^2\ V^{-1}\ s^{-1}$ is that it also contains ions of mass 74. The identity of this species is unknown at the present time. This shows that mobility data by itself can be misleading. The comparison of the negative ion mobility spectra of nitrobenzene and p-chloronitrobenzene further illustrates the problems which can be

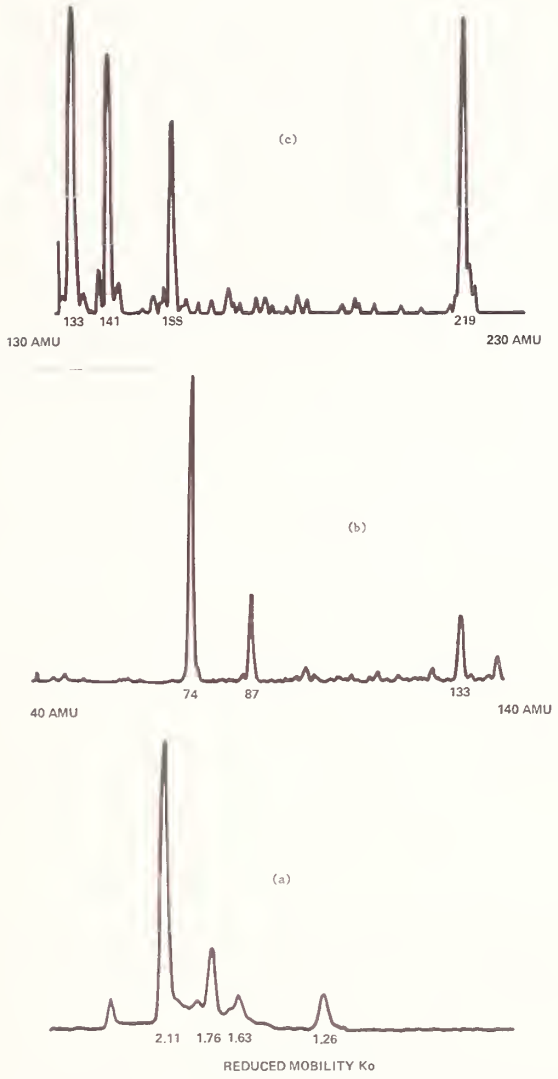

Figure 4. (a) Positive mode ion mobility spectrum obtained from partly cured epoxy polymer; (b) positive mode mass spectrum, 40 to 140 amu, obtained from partly cured epoxy polymer; (c) positive mode mass spectrum, 130 to 230 amu, obtained from partly cured epoxy polymer.

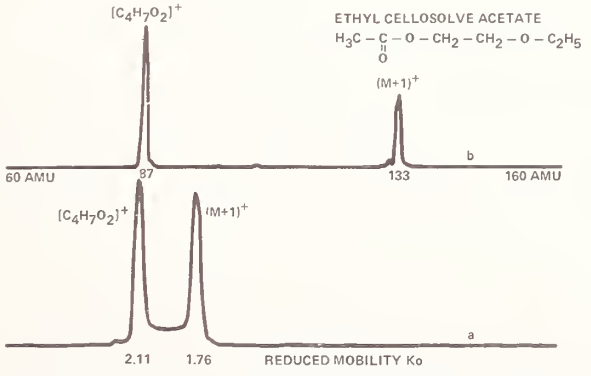

Figure 5. Positive mode (a) ion mobility and (b) mass spectra obtained from ethyl cellosolve acetate.

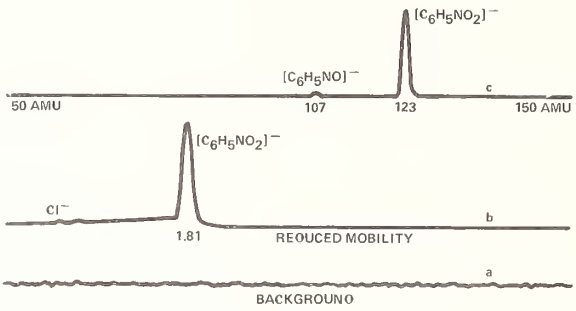

Figure 6. Negative mode ion mobility (a) background, (b) with sample, and (c) mass spectra of nitrobenzene.

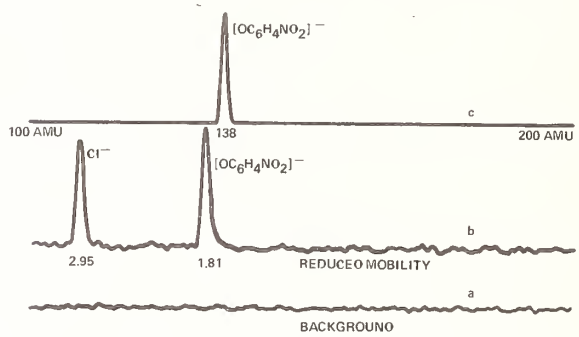

Figure 7. Negative mode ion mobility (a) background, (b) with sample, and (c) mass spectra for para chloronitrobenzene.

encountered in the interpretation of mobility data alone. A comparison of the negative ion mobility and mass spectra for nitrobenzene and p-chloronitrobenzene is shown in Figures 6 and 7. Nitrobenzene is involved in an electron capture ionization process to form $C_6H_5NO_2^-$, which has a reduced mobility of 1.81 $cm^2\ V^{-1}\ s^{-1}$ and an ionic weight of 123 amu. P-chloronitrobenzene, on the other hand, is involved in a substitution reaction with oxygen to yield $OC_6H_4NO_2^-$, the nitrophenoxide ion. The chloride ion is also observed in the mobility as well as mass spectra resulting from p-chloronitrobenzene. It is interesting to note that the $C_6H_5NO_2^-$ and $OC_6H_4HO_2^-$ ions are found to have the same mobility under these experimental conditions. This has also been observed by Dzidic, Horning, et al. [12]. Therefore, extreme caution must be exercised in attempting to elucidate the molecular structure of an ion based upon mobility data alone. However, this problem is not experienced with the combined PC/MS technique.

This technique opens many new areas of research in analytical applications. The direct measurement of organic compounds such as hexahydraphthalic anhydride, 2,6-ditertbutyl-p-cresol, and ethyl cellosolve acetate in complex mixtures without complicated and error-producing concentrations and treatment of the sample provides a great advantage. These applications are for the most part unexplored, but the promise of solution to difficult analytical problems is encouraging.

IV. Acknowledgments

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LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION. STATE OF THE ART AND FUTURE DIRECTIONS

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Over the last 5 years our laboratory has invested a great deal of effort in the development of reliable amperometric detectors for trace organic analysis by liquid chromatography. As a result of this work commercial detectors are now available which exhibit excellent sensitivity and selectivity at minimum cost. Electrochemical detectors provide the instrumentation of choice for a number of important assays where they easily out perform optical detectors.

At present, reverse-phase chromatography offers the greatest compatibility with electrochemical detection due to the polar nature of the mobile phase. The fact that pH, ionic strength, and solvent composition are important variables for *both* chromatography and electrochemistry implies the need for compromise in most cases. Likewise the choice of stationary phase and column length enters into the overall optimization process. The relatively recent use of hydrocarbon phases *intentionally* modified by hydrophobic bonded solvents (e.g., pentanol) and/or polar lipids (e.g., octyl sulfate) brings great flexibility to reverse-phase separations and therefore further enhances the utility of electrochemical detection.

The choice of electrode (glassy carbon, carbon paste, amalgamated gold) and cell geometry play an important role in optimizing the performance of an electrochemical detector for ultimate sensitivity and linear range.

Key words: Cell design; electrochemical detection; liquid chromatography; oxidation; reduction.

I. Introduction

The use of hydrodynamic thin-layer amperometry as a means of detection in liquid chromatography was first realized in response to the need for a sensitive and selective approach to the determination of biogenic amines in the central nervous system [1]. This subject is reviewed elsewhere in this volume [2]. The original studies made use of pellicular cation exchange materials with a silica core. Although such stationary phase materials have fallen into disfavor among many chromatographers, they have excellent properties for use in biological studies with amperometric detection. The fact that it has been possible to routinely use these pellicular materials for determination of injected amounts considerably below 100 pg (1 pg can be "detected" in some cases) speaks for their effectiveness. Over the years amperometric detection has been found to be quite widely applicable to many problems of clinical, pharmaceutical, and environmental interest. The principles and published applications of amperometric and coulometric detection have been reviewed [3]. A frequently updated bibliography of applications is available [4].

Recently we have developed a number of applications based on the use of reverse-phase chromatography with amperometric detection [5–7]. Although many important polar metabolites can be studied using reverse phase columns in conjunction with totally aqueous mobile phases [8], with or without ion-pair reagents [9–10]; many more substances can be studied using the classical mobile phases consisting of water/methanol or water/acetonitrile.

While electrochemical detection using carbon paste electrodes works well for many applications of reverse-phase chromatography, problems can develop when nonaqueous solvents are used due to the combination of a high volume flow rate with the mechanical instability of the

carbon paste matrix. In addition, the relatively high electrical resistance of nonaqueous mobile phases can limit the linear range (on the high end) of thin layer amperometric detectors due to ohmic potential losses along the thin-layer channel. This problem has been qualitatively discussed [11] and mathematically simulated [12] for thin layer cells operated with a stationary sample solution. The iR problem is aggravated in hydrodynamic experiments due to the higher current. The much higher background currents associated with the electrochemical reaction of solvent, electrolyte, impurities, and the electrode material itself all contribute to the problem. When the reference and auxiliary electrode are both downstream from a working electrode positioned in the wall of a thin channel (Fig. 1), the resistance in the channel (often 10^5–10^6 ohms) can cause unacceptable loss of potential control for currents greater than $\sim 10^{-9}$–10^{-8} amperes.

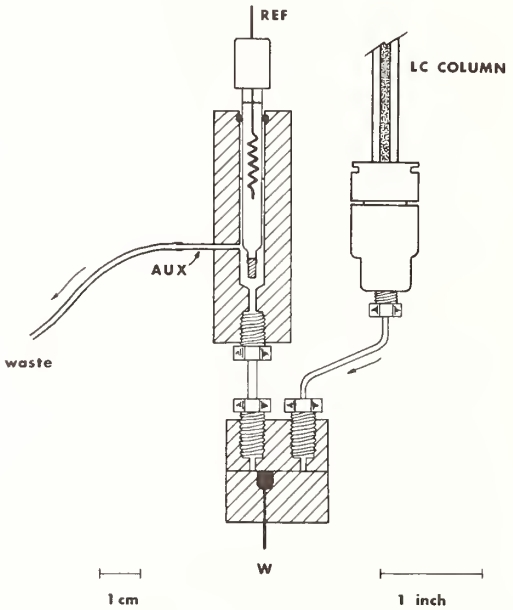

Figure 1. Thin-layer amperometric detector (reproduced by permission from Bioanalytical Systems Inc.).

II. Electrode Materials

While carbon paste can be used in nonaqueous solvents if formulated using high molecular weight waxes, vitreous ("glassy") carbon has been generally found to be far more satisfactory. This material is manufactured by forming the desired shape with a thermosetting resin and then slowly "carbonizing" the product at elevated temperature and (sometimes) pressure [13]. Vitreous carbon has excellent mechanical and electrical properties and is relatively free of impurities. It can be brought to a high surface polish using standard metallographic techniques. Besides its solvent resistance, glassy carbon has a more favorable background current at negative potentials than the usual carbon paste formulations, making it possible to carry out some more difficult reductions. Glassy carbon electrodes have been widely used in electroanalytical chemistry, particularly for anodic stripping voltammetry, a technique in which the carbon is coated with a mercury "film" [14]. Apparently most electrodes used for electroanalytical purposes originate from either Japan (Glassy Carbon, Tokai Electrode Mfg. Co.) or France (Carbone Vitreux, Le Carbone-Lorraine).

It is not yet possible to make detailed comparisons between two electrode materials because organic electron transfer reactions at surfaces are not well understood at a molecular level.

Reactions favorable (fast, reversible) at one surface may be unfavorable at another surface with (apparently) similar properties to the first. Perhaps the most important characteristic of an electrode for hydrodynamic amperometry is "the background current at a potential where the compound of interest reacts at a mass transport limited rate." The fact that one electrode gives a lower background current at a given potential is not necessarily indicative of a better electrode because the electron transfer rates for the analytes of interest might also be lower. At the present time it would appear that both glassy carbon and carbon paste have advantages in given situations. Nevertheless, the superior solvent resistance of glassy carbon will in many cases decide the issue.

Mercury continues to be the electrode of choice for electrochemical reductions, particularly in aqueous solutions. We have explored the use of both small mercury pools and amalgamated gold disks in thin-layer amperometry. Other workers have used pools in a capillary tube [15] and amalgamated platinum wire [16]. Recently Princeton Applied Research introduced a unique approach based on their Model 303 Static Mercury Drop Electrode. Our laboratory and Durst et al. [17,18] have focused on the use of amalgamated gold. This approach results in an inexpensive, easily prepared, and mechanically-rigid electrode which can be used in conventional thin-layer cells (Fig. 1).

It would appear certain that the most important need in LCEC is the development of improved electrode materials. It may be possible in the near future to design an electrode which will give superior performance for certain classes of compounds. Modifying electrode surfaces by covalent attachment of various ligands or electron-transfer catalysts (perhaps even enzymes) may well provide the key to better amperometric devices for all sorts of analytical purposes. Research in the area of chemically modified electrodes (CME's) has been reviewed [19].

III. Cell Design

The iR drop commonly associated with thin-layer cells has not been a significant problem for most applications of the cell illustrated in Figure 1 because aqueous mobile phases of relatively high ionic strength have been used. For the commercial version of this cell a linear range from 10 pg–200 ng injected is easily achieved for the typical separation of small molecules (typ. MW≅200) with capacity factors between about 2 and 5. Obviously many factors can influence the detector response in a given case (volume flow rate, channel thickness, chromatographic efficiency, ionic strength, applied potential, electron transfer rate constant, temperature, and background current (chromatographic "baseline")).

Recently we have begun to use thin-layer amperometric detectors in nonaqueous solvents where the iR drop problem can be very severe due to the poor conductivity. In addition, a number of experiments have been carried out at extremes of potential (positive and negative) where the background current is very great (often far greater than the chromatographic peak heights). Although the background current is always "bucked out" by the electronics and is therefore not "seen" on the chart recorder, it can be the major contributor to the iR drop.

Although electronic compensation for this problem is possible by using positive feedback, the problem cannot be completely solved by this means due to the fact that in cells such as that shown in Figure 1, a significant portion of the iR drop exists along the electrode surface rather than perpendicular to the electrode as in the usual quasi-infinite electrochemical experiment. The current between the working and auxiliary electrodes in Figure 1 passes along the thin-layer channel, including that portion adjacent to the electrode (Fig. 2A). Therefore the potential between the working electrode and the solution is not uniform across the face of the electrode. The potential difference at the downstream edge of the electrode will be closest to the value controlled by the potentiostat, whereas the upstream potential may be insufficient to oxidize or reduce the compounds of interest! The best solution to this problem is to position the auxiliary electrode opposite the working electrode so that the charge passes perpendicular to the working electrode surface (Fig. 2B). This geometry ensures both that the uncompensated iR drop will be extremely

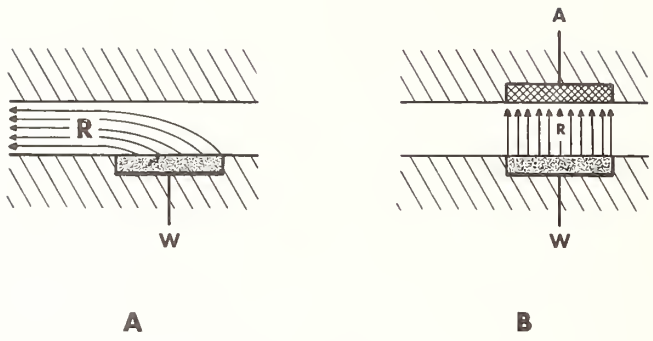

Figure 2. Schematic representation of current passage in thin-layer amperometry (A) auxiliary electrode (not shown) placed down stream from the working electrode; (B) auxiliary electrode (A) placed across the channel from the working electrode (W). (reproduced by permission from Bioanalytical Systems Inc.)

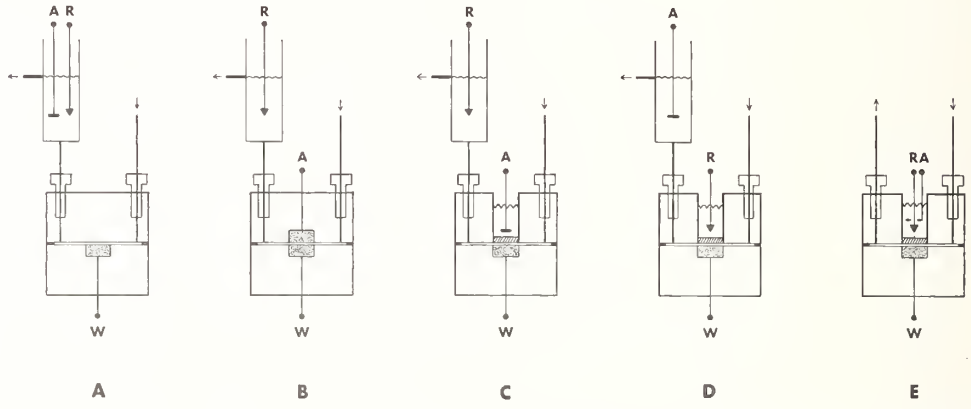

Figure 3. Design options for thin-layer amperometric detector cells. (reproduced by permission from Bioanalytical Systems Inc.).

small (due to the thinness of the layer) and that the potential will be uniform at all points on the working electrode surface. A more detailed treatment of this problem will be published elsewhere [20]; however, the influence on the design of practical detector cells is important and can be easily understood from a qualitative point of view.

Figure 3 illustrates five cell geometries which we have investigated and are currently using for various applications. The cells are all constructed from Plexiglas (machines well, transparent, but not solvent resistant), Kel-F (machines well, not transparent, solvent resistant), and Teflon (machines adequately but does not hold threads, not transparent, solvent resistant, cold flows, large thermal coefficient permits excellent means of mounting gold and glassy carbon electrodes).

Figure 3A illustrates the conventional geometry (Fig. 1) with both the reference (R) and auxiliary (A) electrodes mounted downstream. This design is relatively inexpensive, but can suffer from iR drop problems as described above. It is important to recognize that the three electrode system does not compensate for *any* significant solution resistance but does have the advantage of not drawing current through the reference electrode. At low currents the auxiliary electrode is not needed and the cell can be operated with the reference electrode serving as a classical counter electrode (as in two electrode polarography, or Clark amperometric oxygen electrodes). This cell has the disadvantage that it is not (without simple modification [21]) suitable for collection of fractions (esp. radiolabelled metabolites from metabolism studies).

Figure 3B illustrates a simple modification in which a planar auxiliary electrode is placed opposite the working electrode. This is an excellent design for many applications. The thin-layer

channel serves as a salt bridge to the reference electrode but since no current passes along the thin-layer, there is no resulting iR drop. In most instances the transit time for molecules along the electrode face is short compared to the diffusion time *across* the thin-layer of solution. Therefore no interference occurs between chemical events taking place at the working and auxiliary electrodes. Some attention should be paid to this problem with large electrode areas, slow flow rates, and very thin gaskets, in which case the geometry shown in Figure 3C can be advantageous. The problem here is that cell construction and maintenance becomes considerably more awkward when a porous diffusion barrier must be incorporated in the cell to isolate the auxiliary electrode.

Placing the reference electrode probe opposite the working electrode in the wall of the thin channel can be best accomplished by use of an ionic junction constructed from a porous material which permits ions to migrate but blocks the flow of electrolyte solution (Fig. 3D). Porous Vycor glass ("thirsty glass," Corning 7930 glass) is perhaps the most satisfactory material for this purpose. With the potential-sensing element across the stream from the working electrode the uncompensated solution resistance is small (but not uniform) and the auxiliary electrode can be placed downstream with relatively little ill effect. The compensated resistance is quite large and the current must pass along the layer of solution adjacent to the electrode.

The previous configurations (3B and 3C) are superior on fundamental grounds because the current density across the electrode will not be significantly influenced by iR drop and the potential of the auxiliary electrode will remain low due to the low impedance between A and W.

The geometry illustrated in Figure 3E is perhaps most satisfactory from a fundamental point of view. This design also provides for convenient collection of fractions. Nevertheless, construction difficulties presently make this cell less desirable for routine work. In summary, we find designs A and B to be the most practical approach to rugged cells capable of routine use for many months. With the geometry illustrated by 3B the linear range of the detector extends to over six orders of magnitude even in mobile phases with poor conductivity. In general, we recommend 3A for most trace applications and 3B for those cases where either a high background current or high resistance causes significant iR problems. Cells 3B, 3C, and 3E are also recommended for pulse experiments [3,20].

IV. Applications

While applications of electrochemical detection have been quite diverse [3,4], space only permits brief mention of two applications.

Benzidine and closely related compounds have been of considerable interest with regard to environmental toxicology. These carcinogens are being closely monitored in some factory atmospheres and effluents. It is clear that government regulations will make such measurement mandatory for a significant fraction of the chemical industry. Aromatic amines are readily determined by LCEC and several brief reports have appeared [22–24]. Benzidines are ideally suited to amperometric detection due to their facile two electron oxidation at carbon electrodes. We have initiated a study of benzidine metabolism which required the determination of two levels of benzidine and its principal metabolites (the mono- and di-acetyl derivatives) in urine. Figure 4 illustrates chromatograms for benzidine in an extract of human urine. A 15 cm C_{18} column (Waters 10 μm material) was used with a mobile phase consisting of 0.1 mol/L Ammonium Acetate (75%) and distilled methanol (25%) at a flow rate of 1.0 mL/min. A Bioanalytical Systems LC-50 chromatograph was used with a 20 μL sample loop and a carbon paste detector cell (TL-3) at a potential of 450 mv vs. Ag/AgCl. About 10 pg of benzidine can be readily detected under these conditions in standard solutions. In the example shown, the electrochemical detector was used downstream from a conventional 254 nm UV detector (Bioanalytical Systems). The dead volume in the UV detector broadened the benzidine peak at the electrochemical detector when compared to direct connection of the LCEC cell to the column. Nevertheless, the advantages of electrochemical

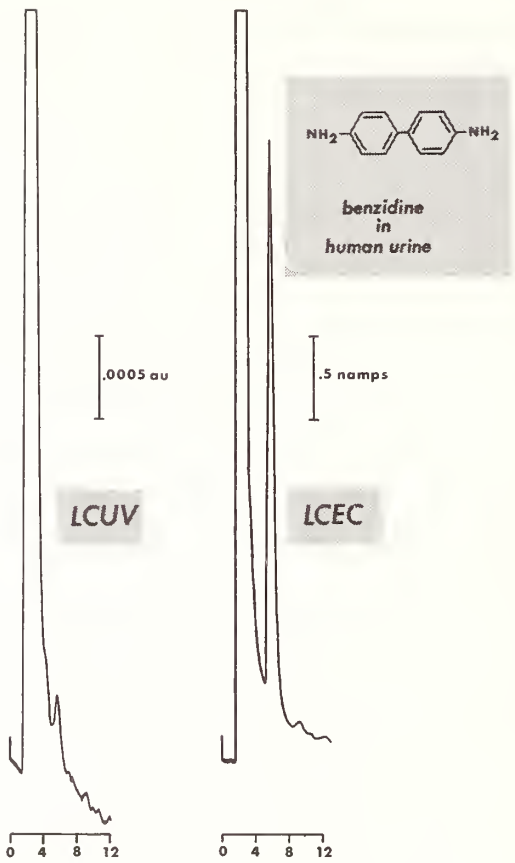

Figure 4. Amperometric (oxidative) and UV detection of benzidine in urine using reverse-phase (C_{18}) liquid chromatography (100 ng/mL in urine; 3 ng injected) (reproduced by permission from Bioanalytical Systems Inc.).

detection (selectivity and sensitivity) are clearly indicated in this example. A detailed report on the determination of benzidine and its acetyl derivatives will be published [25].

In a second example, we wish to illustrate the utility of an electrochemically amalgamated gold disk electrode (3 mm dia.) (Bioanalytical Systems, Model TL-6) for organic analysis. In this case an LC-54 instrument was used with an LC-4 amperometric controller and a PM-20 pumping system. The mobile phase was carefully deoxygenated using nitrogen gas. Figure 5 illustrates the use of this system for detection of p-nitrophenol using a reverse-phase column (Merck RP-2, 10 μm, L=11 cm, i.d.=4.1 mm; Mobile phase: 0.05 mol/L MacIlvaine buffer, pH 3.5, 30% methanol; detector potential E=−0.7 V vs. Ag/AgCl). The chromatogram on the left was obtained after brief deoxygenation (30 s) of the sample with N_2. The chromatograms in the center followed a 90 s deoxygenation. It is interesting to note that O_2 is retained on the reverse-phase column (more so on C_{18} than C_2) and can present a problem in some samples. We also observe an oxygen "vacancy peak" (negative peak at the same retention time) for the situation in which the O_2 concentration in the sample is less than that in the mobile phase.

Figure 6 illustrates another example of reductive LCEC. A number of benzodiazepines and other compounds have been detected with good sensitivity [26] and it is now reasonable to expect that electrochemical detection in the reduction mode will eventually result in a variety of practical applications. It is not yet possible to achieve the low pg detection limit found for oxidative LCEC (because of the reduction of O_2, H^+, and trace metals), however, we are optimistic that routine applications at the level of 100's of pg will be possible in the near future.

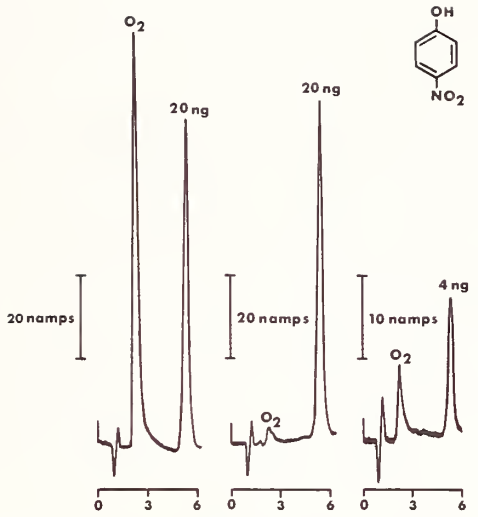

Figure 5. Amperometric (reductive) detection of p-nitrophenol using reverse-phase (C_2) liquid chromatography (reproduced by permission from Bioanalytical Systems Inc.).

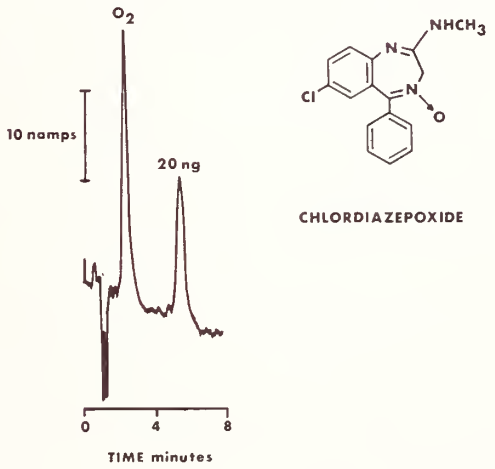

Figure 6. Amperometric (reductive) detection of chlordiazepoxide using reverse-phase (C_2) liquid chromatography (reproduced by permission from Bioanalytical Systems Inc.).

V. Acknowledgment

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MODIFICATION OF CONVENTIONAL GAS CHROMATOGRAPHIC INLETS FOR THE USE OF GLASS CAPILLARY COLUMNS

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A conventional packed column gas chromatographic inlet has been adapted to accommodate glass capillary columns. This simple inlet design requires only five inexpensive, readily obtainable parts, and is applicable to most existing gas chromatographs. The system has performed well with non-polar compounds as well as many polar compounds in both split or splitless injection modes. Temperature programmed operation is routine with the system, and either a manual or automatic sample injection configuration is possible. Reproducibility of individual measurements and linearities of response were determined using internal reference standards. Chromatograms from complex environmental samples demonstrate performance of the system.

Key words: Capillary columns; gas chromatography; hydrocarbons.

I. Introduction

Many gas chromatographers are converting from the use of packed columns to glass capillary columns, particularly because of their efficiency in resolving complex mixtures [1]. Open-tubular capillary columns were first developed over 20 years ago [2], and capillaries made from stainless steel tubing were commercially available a few years later. Glass capillaries, however, have only recently been marketed commercially. Many instrument manufacturers now offer gas chromatographs (GC's) specifically designed to accommodate glass capillaries; retrofit capillary inlet systems for updating older chromatographs are available as well, although at considerable expense. While a state-of-the-art technology is incorporated in some of these products, not every analyst can afford such new equipment. A simple and inexpensive way of modifying conventional GC's for the use of capillary columns would be beneficial. This report describes such a modification, as it has been applied to several different chromatographs. Modification of the packed column inlet requires only five readily available parts which can be assembled in a few hours. The design is suitable for both split and splitless injection modes, and has given excellent performance in the analysis of complex mixtures of non-polar and polar compounds.

II. Experimental

A. Materials

Viton O-rings (0.2 inch o.d., 0.07 inch i.d.) were obtained from Hewlett-Packard (5080-4978). Graphite ferrules (0.8 mm i.d.) were purchased from Quadrex Corp. (GFl) and from Supelco, Inc. (2-0628); Teflon ferrules from Finnigan Corp. (95001-20360); Vespel ferrules from Supelco, Inc. (2-0644); and graphite-impregnated Vespel ferrules from LKB (2101-911). Both 1/4

to 1/16 and 1/8 to 1/16 inch stainless steel Swagelok reducers were used. Pyrex tubing (4.8 mm o.d. and 3.2 mm i.d.) in 8.5 cm lengths was employed for the glass inserts. Glass-lined, stainless steel tubing (GLT), 1/16 inch o.d. and 0.3 mm i.d. (SGE, Inc.), was used for gas chromatograph/mass spectrometer (GC/MS) transfer lines.

B. Apparatus

Hewlett-Packard 5840A and Finnigan 9500 gas chromatographs were used to develop and test the inlet design. The 5840A included an automatic liquid sampler (7671A) and an FID with an enlarged end flame jet (18704-80010); the 9500 GC's were interfaced with a Finnigan 3200 mass spectrometer system for both electron impact (EI) and chemical ionization (CI) operation.

C. Columns

Wall-coated, open-tubular (WCOT) glass columns were obtained from Quadrex Corp. (0.25 mm i.d., 0.75 mm o.d.), J&W Scientific, Inc. (0.25 mm i.d., 0.8 mm o.d.), SGE, Inc. (0.2 mm i.d., 1.0 mm o.d.), and LKB (0.36 mm i.d., 0.8 mm o.d.). Longer columns were cut to 20–30 m lengths. Column ends were straightened to approximately 15 cm in length with a microtorch (Archer) using a 1/2 cm flame, while maintaining a flow of air through the column.

D. Inlet Assembly

The inlet modification is illustrated in Figure 1. The injection port at the septum end should be approximately 5 mm i.d., which may require drilling out the opening. To provide a carrier gas split line, a 1/16 inch stainless steel tube was silver-soldered into a 1/4 to 1/16 inch reducer just

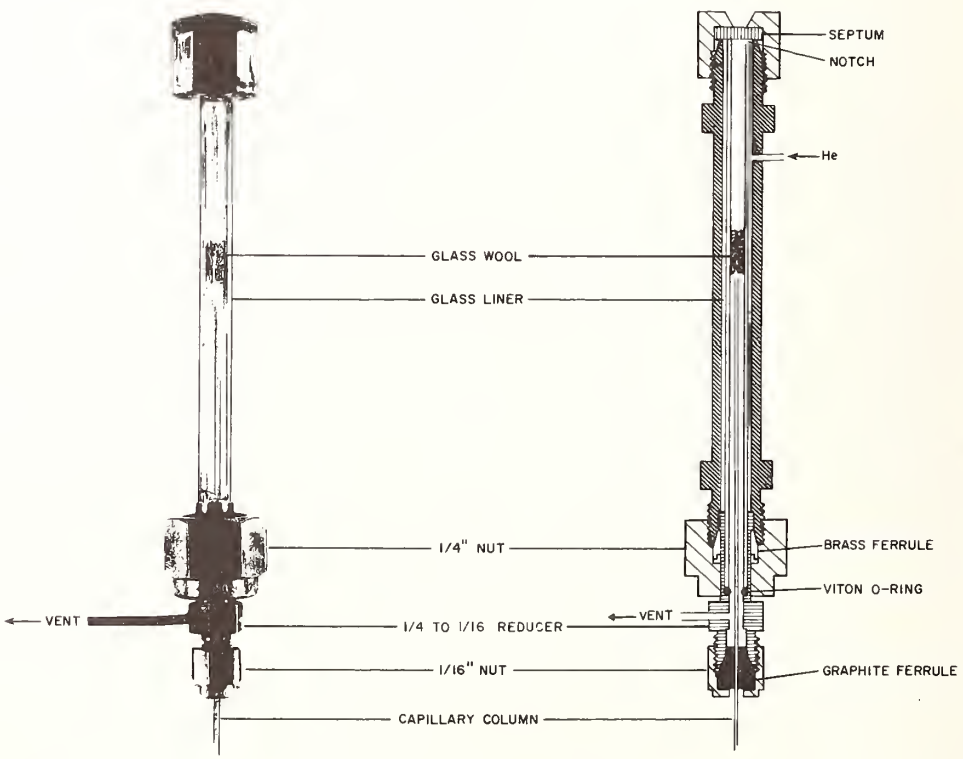

Figure 1. The capillary inlet design.

below the body hex of the fitting. A Viton O-ring was inserted into the 1/4 inch tube of the reducer, and the assembly was then attached to the injection port with a 1/4 inch stainless steel nut and Vespel ferrule.

A glass insert was notched about 4 mm deep at one end, and a silanized glass wool plug was placed in the insert to enhance vaporization of the samples, as well as filter any particulates. The plug was positioned so that the tip of a syringe needle, fully inserted through the septum and septum nut, was immediately above the wool. The insert was then loaded into the injector through the septum end, bottoming at the O-ring in the reducer. When properly positioned and of correct length, the insert protrudes above the top of the injector body *ca.* 0.5 mm. Tightening the septum in place presses the insert against the O-ring, sealing it at the base of the injector.

The capillary column was suspended in the oven on a hook made from steel wire (*ca.* 16 gauge). The capillary column was then attached to the 1/16 inch inlet fitting with a graphite ferrule and a 1/16 inch stainless steel nut. The column was positioned inside the injector with its inlet end 1 cm below the glass wool.

E. Detector Assembly

A 1/8 to 1/16 inch Swagelok reducer was attached to the FID base. The column was inserted through the reducer into the flame jet with the tip as near the flame as possible, and sealed with a graphite ferrule.

In the case of a GC/MS EI interface, the column was inserted approximately 1 cm into a drilled out 1/16 inch GLT running directly to the ion source. The column connection was made with a 1/16 inch Swagelok union using a Vespel ferrule on the GLT and a graphitized-Vespel ferrule on the column. For the CI interface, methane make-up was added at the column effluent end through a 1/16 inch Swagelok union tee, in place of the 1/16 inch union.

F. Operating Conditions

The He carrier gas pressure was regulated to give *ca.* 20 cm/sec linear velocity through the column at the maximum operating temperature. A split ratio of approximately 20:1 (purge: column flow) was normally used for split injections and was achieved with a needle valve in the carrier gas split line. In a splitless injection, an on-off solenoid valve also in the split line is opened (to a 20:1 split ratio) only after a predetermined delay following the injection. A nitrogen make-up flow of 30 mL/min was added to the hydrogen line for the FID, which requires ca. 30 mL/min of carrier flow for optimum efficiency [3]. Samples of 2–3 μL were routinely injected by an auto-sampler, and the oven was temperature programmed from 20 °C below the boiling point of the solvent (*cf.*, GC conditions listed in Fig. 2). Operational techniques for glass capillary GC are discussed in greater detail by Grob [4–6]. Also Kaiser and Reider [7] discuss criteria useful for evaluating performance in capillary GC.

III. Results and Conclusions

The inlet modification described above has been installed in five instruments, including GC and GC/MS systems, which have been in operation for over 2 years. Several of the systems include microprocessor control and automatic sample injection and are in use 24 hours a day, 7 days a week. Although most of the work has involved analysis for aliphatic and aromatic hydrocarbons, samples of more polar compounds (pesticides, phenols, etc.) have been analyzed with equally excellent results. Typical chromatograms obtained with this system are illustrated in Figures 2–4.

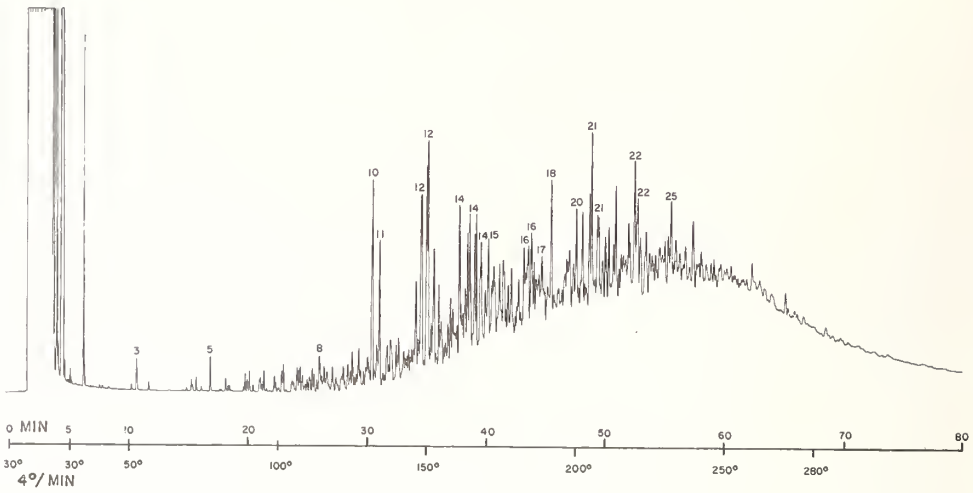

Figure 2. Gas chromatogram of the unsaturated hydrocarbon fraction from an extract of a cod stomach taken in the vicinity of the Argo Merchant oil spill. Hewlett-Packard 5840A GC, with FID. J&W Scientific 30 m×0.25 mm SE-54 WCOT column. Helium carrier pressure 20 psi. 3 μl splitless injection. Split valve opened after 18 sec. Numbered peaks are identified in Table 1.

TABLE 1. *Selected components found in cod stomach and marine sediment extracts*

1. Toluene	18. Phenanthrene
2. Ethylbenzene	19. Anthracene
3. *m,p*-Xylene	20. Methyldibenzothiophene
4. *o*-Xylene	21. Methylphenanthrenes/anthracenes
5. C_3-Benzene	22. C_2-phenanthrenes/anthracenes
6. Dichlorocyclohexane	23. Fluoranthene
7. Chlorobromocyclohexane	24. Pyrene
8. Naphthalene	25. C_3-Phenanthrenes/anthracenes
9. Dibromocyclohexane	26. Methylfluoranthenes/pyrenes
10. 2-Methylnaphthalene	27. 3,4-Benzophenanthrene
11. 1-Methylnaphthalene	28. Benz[*a*]anthracene
12. C_2-Naphthalenes	29. Chrysene/Triphenylene
13. Acenaphthene	30. Benzofluoranthenes
14. C_3-Naphthalenes	31. Benzo[*e*]pyrene
15. Fluorene	32. Benzo[*a*]pyrene
16. Methylfluorenes	33. Perylene
17. Dibenzothiophene	

Figure 2 shows the unsaturated hydrocarbon fraction of an extract from a cod stomach taken in the vicinity of the *Argo Merchant* oil spill [8]. The arene fraction of a contaminated marine sediment from Puget Sound is pictured in Figure 3. A GC/MS analysis of phenols extracted from water after partitioning with Prudhoe Bay crude oil is shown in Figure 4.

To determine reproducibility and efficiency of sample transfer into the column using this inlet design, several aliquots of standard solutions and serial dilutions containing C_{10}–C_{31} *n*-alkanes plus pristane and phytane were analyzed. The dependence of the chromatographic response upon the concentration of the injected standard solutions was established over a range of 0.05–5.0 ng/μL. The linear regression coefficient was determined for each compound by normalizing its response in each solution to the average response for the compound in several analyses of the undiluted solution. The resulting coefficient was 0.96±0.013 (n=12) and ranged from 0.92 to

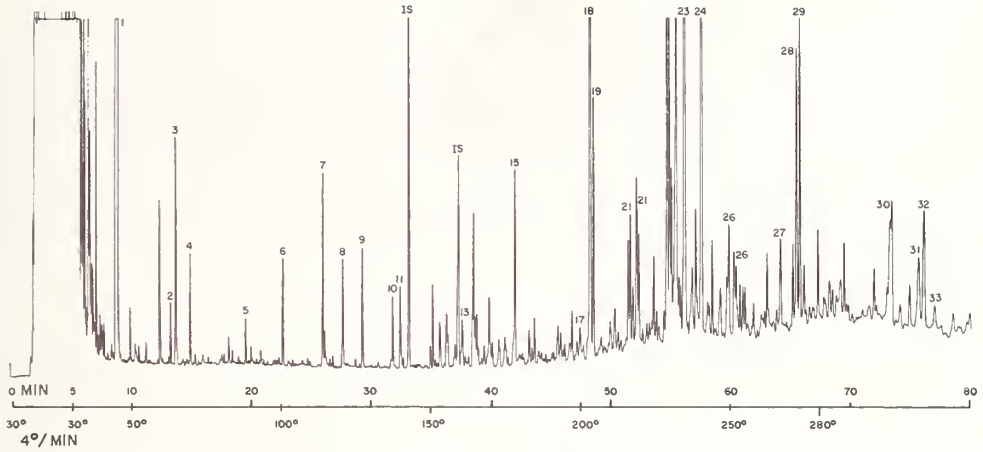

Figure 3. Gas chromatogram of the unsaturated fraction of a contaminated marine sediment near Anacortes, Washington. Parameters and conditions as in Figure 2.

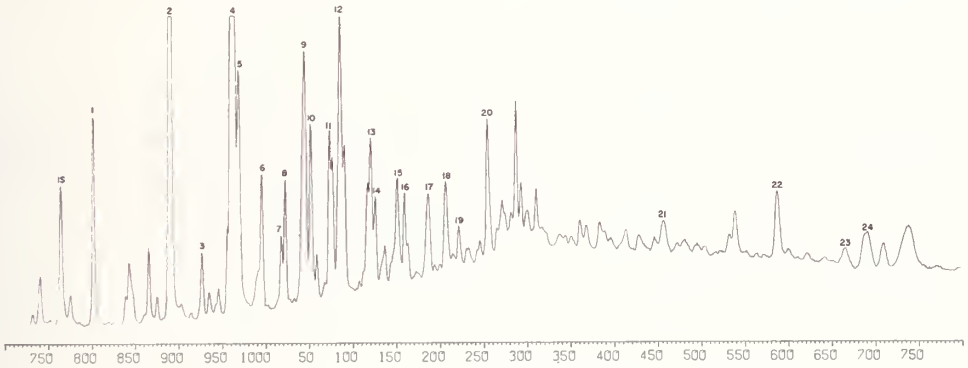

Figure 4. Portion of a reconstructed gas chromatogram of the polar fraction from an extract of the water soluble fraction from a partition of water and Prudhoe Bay crude oil. Finnigan 9500 GC, 3200 MS. J&W Scientific 20 m×0.25 mm SP1000 WCOT column. Helium carrier pressure 8 psi. 3 μl splitless injection. Split valve opened after 20 sec. Numbered peaks identified in Table 2.

TABLE 2. *Selected components of a crude oil, water-soluble fraction*

1. Dimethylphenol	13. Methylethylphenol
2. Phenol + Cresol	14. Methylphenetole
3. Trimethylphenol	15. Dimethylethylphenol
4. Dimethylphenol	16. Methylpropylphenol
5. Cresol	17. C_4-Phenol
6. Isopropylphenol	18. C_5-Phenol
7. Methylethylphenol	19. C_5-Phenol
8. Dimethylphenol	20. C_3-Phenol
9. Methylethylphenol	21. C_2-Bromophenol
10. Dimethylphenol	22. C_2-Bromophenol
11. Thymol	23. Cyclohexylphenol
12. C_2+C_3-Phenols	24. C_3-Bromophenol

0.98. Relative standard deviation of the areas of measured alkanes following repeated injections of the same sample was less than 15% (n=5).

Besides its economy, simplicity, and wide applicability, the design offers several other advantages. It has minimal dead volume and permits operation in either split or splitless injection modes. The glass insert can be easily removed for cleaning or replacement without disturbing the column. Also, the position of the column in the injector can be adjusted relative to the point of vaporization for optimal transfer of sample into the column. Although recommended by some sources [4], a separate septum sweep was not found to be necessary for the types of compounds routinely analyzed.

IV. Acknowledgments

This work was supported by the NOAA Energy Resources Program with funds from EPA.

Reference to a company or product does not imply endorsement by the U.S. Department of Commerce to the exclusion of others that may be suitable.

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

TRACE ORGANIC ANALYSIS USING SECOND-DERIVATIVE UV-ABSORPTION SPECTROSCOPY

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Second-derivative uv-absorption spectroscopy is discussed as a method of trace organic analysis. Various ways of obtaining a second-derivative spectrum are presented, along with examples of improved selectivity between compounds due to the enhanced resolution of the technique. The effect of sample turbidity on direct absorption and on second-derivative absorption spectra is also discussed.

Key words: Absorption spectroscopy; derivative spectroscopy; PNA analysis; trace organic analysis.

I. Introduction

Recent increased emphasis on the production of synthetic fuels from coal-conversion processes, tar sands and shale oils coupled with an awareness of the carcinogenic potential of many of the polynuclear aromatic (PNA) compounds produced by these processes will require the analysis of many environmental and industrial hygiene samples for trace organic compounds. Second-derivative uv-absorption spectroscopy can extend the capabilities of traditional uv-absorption spectroscopy for the trace organic analysis of these samples. Application of the second-derivative technique increases selectivity between compounds and often increases the sensitivity when interfering compounds are present; in addition, this technique permits analysis of turbid samples which may pose problems for direct absorption analysis.

Figure 1 illustrates the increased detail obtained by taking the second-derivative of a water sample containing 10 μg/mL of phenol. Since the second derivative of a function is the curvature of the function, the second derivative of a spectrum will remove features which vary slowly with wavelength, such as broadband absorption, drift in the light source intensity, and absorption due to sample turbidity, while enhancing rapidly varying features such as narrow absorption bands. With this enhanced resolution, improved selectivity between compounds can be obtained. The signal-to-noise ratio (S/N) for a derivative signal generally will be poorer than for the direct signal, leading to a higher detection limit when noise is the limiting factor, but the derivative signal often will give improved sensitivity when the limiting error is due to interference from partially overlapping absorption bands.

II. Methods of Obtaining Second-Derivative Spectra

There are three major approaches to obtaining the second derivative of a spectrum: a) numerical differentiation; b) electronic differentiation; and c) wavelength modulation. For details of the various techniques see references [1–7]. Numerical differentiation requires the direct absorption spectral data to be in digital form. A computer program (or a calculator, if one is persistent) is then used to apply the finite-difference approximation of the second derivative to the direct spectrum. The greater the number of available data points, the closer the finite-difference approximation becomes to the true second derivative. Electronic differentiation is performed by scanning through the spectrum at a constant velocity and taking the time derivative of the direct

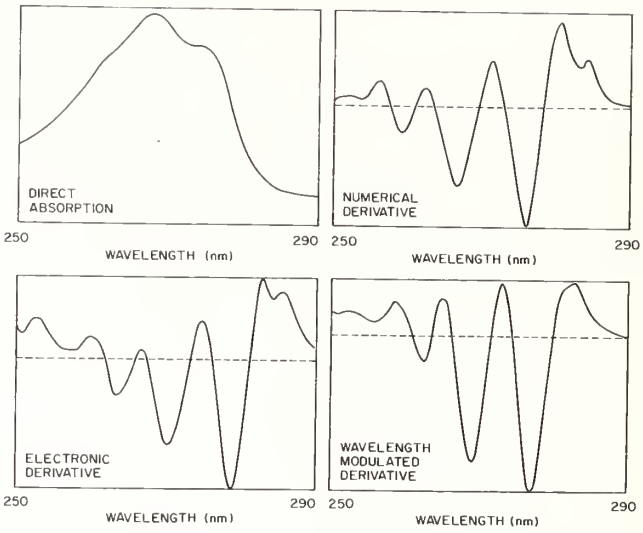

Figure 1. Spectra of phenol in water (10 μg/mL) showing direct absorption spectrum and second-derivative spectra obtained by various techniques.

analog signal using analog circuits. Both numerical differentiation and electronic differentiation can be applied to a standard spectrophotometer without modification. A disadvantage of these techniques is that the second derivative at a specific wavelength can be found only by scanning through that wavelength. The second derivative of a spectrum can also be obtained by modulating, by a few nanometers, the wavelength of the light going through the sample and detecting the second harmonic of the modulation frequency, which is proportional to the second derivative. Modulation is achieved by modification of the standard optic components of the spectrometer. Most often this modulation is provided by a vibrating entrance or exit monochromator slit, or an oscillating mirror, to give a wavelength modulation with a frequency that is fast compared to the scanning rate. In contrast to the previously described methods of obtaining the second derivative, this technique will provide the second derivative at a stationary position, thus allowing continuous monitoring at a given wavelength. Also, since the signal of interest is contained in the ac component with a frequency of twice the modulation frequency, the spectrometer can use an automatic-gain-control (AGC) to maintain a constant dc signal regardless of the sample absorptivity. This allows the electronic components of the system to operate in their optimum ranges. A further discussion of this advancement will soon be available from the authors. Examples of each of the three methods of obtaining the second derivative of a sample of phenol in water are shown in Figure 1, along with the direct absorption spectrum.

III. Analytical Examples

An illustration of the analytical capability of the second-derivative method is the analysis of phenol in waste waters. Figure 2 shows the linearity obtainable with the second-derivative technique. These data were taken using the wavelength-modulation method with a vibrating entrance slit providing a modulation of ±1.5 nm at a frequency of 45 Hz. The samples were measured in a 1-cm quartz cuvette. The detection limit for phenol in water is less than 1 μg/mL. Since the second-derivative signal of a spectrum is larger for narrower bands, the detection limit for a compound with very narrow bands, such as benzene in cyclohexane, may be considerably less than the detection limit of phenol in water. Likewise, for compounds measured in air using a heated multipass sample chamber, the narrower absorption bands observed in the vapor phase product even more enhancement in the second-derivative mode of analysis.

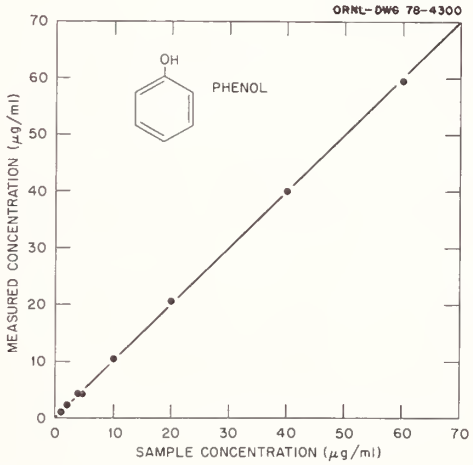

Figure 2. Illustration of linearity in the calibration curve for phenol in water.

The selectivity obtained with the second-derivative technique is illustrated using a mixture of phenol and m-cresol. The main second-derivative peaks for phenol and m-cresol are separated only by slightly more than 1 nm. When a least-squares spectral analysis computer program was applied to the spectrum, the analysis of the mixture yielded 20.2 μg/mL of phenol and 18.7 μg/mL of cresol as compared to the expected values of 20.0 μg/mL of phenol and 20.0 μg/mL of cresol. The analysis is especially good considering the high degree of similarity between the two absorption spectra.

A particular advantage of the second-derivative technique applied to environmental and industrial hygiene monitoring problems is the dramatically reduced effect that sample turbidity has on the spectrum. A turbid sample generally produces an absorption which varies slowly with wavelength so that taking the second derivative tends to remove from the spectrum the contribution due to sample turbidity. The response of a direct spectrum to a turbid waste water sample containing phenol (~25 μg/mL) is compared in Figure 3 with the response of a second-derivative spectrum to the same sample.

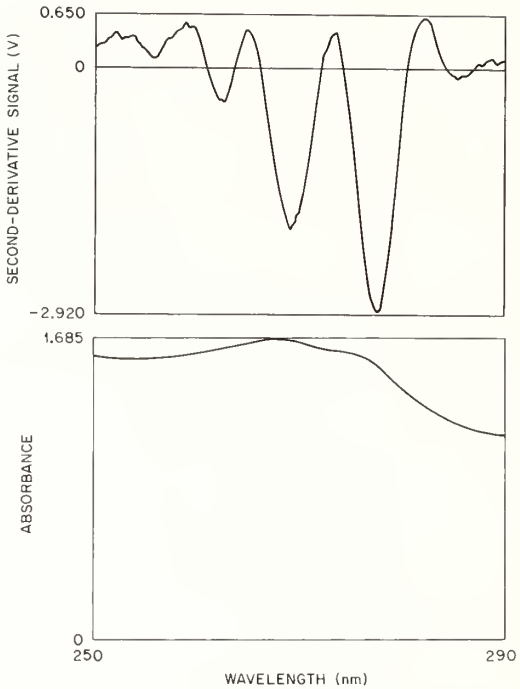

Figure 3. Normal and second-derivation spectra illustrating the effect of sample turbidity on the analysis of phenol in waste water.

IV. Conclusions

Second-derivative uv-absorption spectroscopy offers distinct advantages over direct absorption spectroscopy in increased selectivity between compounds and, when interferences from partially overlapping absorption bands are present, in improved sensitivity. The removal of slowly varying curvature, characteristic of light source drifts or turbid samples, allows the second-derivative technique to be used under less than ideal experimental conditions. The method has been sufficiently promising to design and fabricate a microcomputer-based, wavelength-modulated instrument for use as a field monitor for PNA vapors at coal-conversion facilities.

V. Acknowledgment

Research sponsored by the Division of Biomedical and Environmental Research, U.S. Department of Energy under contract W-7405-eng-26 with the Union Carbide Corporation.

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NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

ON THE ANALYTICAL POTENTIAL OF MICRO-RAMAN SPECTROSCOPY IN THE TRACE CHARACTERIZATION OF POLYNUCLEAR AROMATIC HYDROCARBONS

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The results of a feasibility study are reported aimed at evaluating the analytical potential of micro-Raman spectroscopy (MRS) in the trace characterization of solid organic pollutants. In these experiments, a laser-Raman microprobe developed at the NBS has been used to obtain vibrational Raman spectra of microsamples of polynuclear aromatic hydrocarbons (PAHs). The spectra were obtained with 514.5 nm (green), 568.2 nm (yellow), and 647.1 nm (red) excitation from an Ar/Kr ion laser to specifically examine potential measurement difficulties arising from sample heating (due to radiation absorption) and sample fluorescence. The PAHs were investigated as single microparticles, generally of size 2–10 μm, and include representative compounds of the 3-ring (e.g., anthracene, phenanthrene), 4-ring (e.g., chrysene, pyrene, fluoranthene) and 5-ring (e.g., benzo[a]pyrene) systems. The Raman spectra of these organic solids are highly characteristic of crystal lattice (external) and molecular (internal) vibrations. They can therefore form a basis for the positive identification of the compounds in this class. A detection limit (with the Raman microprobe in its present state of development) of 10–100 pg is indicated for many of these environmentally significant hydrocarbons. These results demonstrate a considerable analytical potential of the MRS technique for application as a sensitive and specific method for the detection and identification of trace level PAHs separated by chromatographic procedures.

Key words: Laser-Raman microprobe; liquid chromatograph-Raman spectrometer interface; micro-Raman spectroscopy; organic microanalysis; PAH microsamples; polynuclear aromatic hydrocarbons; (PAHs); Raman spectra; trace organic analysis.

I. Introduction

The assessment of environmental pollution by trace-level organics requires increasingly sophisticated measurement techniques. In the trace analysis of major polynuclear aromatic hydrocarbons (PAHs)—many of which are either known or suspected potent carcinogens—a fair number of analytical techniques are employed for the determination of these compounds in complex environmental samples. Of the contemporary techniques for the analysis of trace levels of PAHs, high-performance liquid chromatography (HPLC) with combined UV absorption/fluorescence emission detection is probably the most widely used and successful method [1,2]. While fluorescence detection of HPLC-separated fractions of PAHs routinely achieves high sensitivity and good selectivity, there remains a continuing demand for both increased sensitivity and increased selectivity in the determination and measurement of polycyclic organic matter.

We report in this paper on the results of a feasibility study performed to evaluate the analytical potential of micro-Raman spectroscopy (MRS) in the trace characterization of solid organic pollutants. Emphasis in this presentation is on the Raman spectroscopic detection and characterization of picogram quantities of individual PAHs by application of the NBS-developed laser-Raman microprobe [3]. A major aim of this ongoing research is an assessment of the viability of the MRS technique for use as a sensitive and specific detection method for the identification of PAH components in fractions separated by liquid chromatography procedures. The development of

liquid chromatograph-Raman spectrometer interface systems has been suggested as a further advance over conventional analytical chromatography systems [4].

In earlier published work [3,5–6], the capability of performing (qualitative) Raman spectroscopic analysis of discrete microsamples has been demonstrated. A principal area of application of the Raman microprobe has been the chemical identification of airborne particles. It was shown that this new technique of microprobe analysis can obtain information on the molecular composition of microsamples which heretofore have yielded only to elemental analysis. The types of samples analyzed have included single microparticles (of size 1 μm and larger) isolated from urban air particulate dusts, and particles found in stack emissions from coal- and oil-fired power plants. In a related study [6], the carbonaceous material (e.g., "graphitic soot") often associated with pollution particles has been characterized in detailed microprobe measurements.

The work described here is an extension of these earlier Raman microprobe studies on primarily inorganic microparticulate samples to the spectroscopic characterization of picogram quantities of organic compounds that may be isolated from environmental samples.

II. The Raman Microprobe

The spectroscopic measurements carried out in these investigations are performed with the NBS-developed laser-Raman microprobe [3]. This instrument is a non-conventional laser-Raman spectrometer especially designed for microanalytical applications. It permits the acquisition of analytical quality Raman spectra from single particles of size down to 1 μm or other forms of microsamples approaching 1 pg in mass.

In the Raman measurement, a beam of monochromatic, visible laser light is focused on the sample. The radiation scattered by the sample contains weak lines, at frequencies both lower and higher than the exciting radiation. The frequency differences, called Raman shifts, are characteristic of the sample and are independent of the exciting frequency. The spectra obtained with the microprobe are so-called Stokes-Raman spectra. These arise from molecules which scatter photons of lower frequency (Stokes lines) than that of the exciting line. The Raman pattern they represent provides a molecular fingerprint for identification and characterization.

The spectrum is usually excited in a region where the sample does not absorb. Appreciable absorption of the exciting radiation generally leads to sample heating, frequently attended by sample modification or destruction. In microprobe measurements, such problems can be particularly severe because of the high irradiances (power/unit area) that must be employed to excite analytically useful spectra. A major potential limitation in all Raman work is sample fluorescence which may totally swamp the Raman effect. With a choice of laser frequencies, problems of radiation absorption and sample fluorescence often can be minimized, if not virtually eliminated. Thus, it is often possible to select such an excitation frequency that color of a sample is not a limiting factor.

Identification and characterization of molecular species present as major components of microsamples are made by qualitative comparison with reference spectra. If these are not available in the literature from measurements on bulk samples, the information is obtained from microprobe measurements of well-characterized materials. For all cases investigated to this point, the spectra obtained with the Raman microprobe of microsamples of a broad range of compounds are in good qualitative agreement with the spectra of the same compounds measured in the form of bulk samples.

Details on the design and construction of the NBS laser-Raman microprobe have been presented in earlier published work [3,5]. The instrument is a monochannel spectrometer, schematically depicted in Figure 1. For excitation of the Raman spectrum, the light from an argon/krypton ion laser is focused to a small (typically 2–20 μm) spot on the sample. The light scattered by the sample is collected by an ellipsoidal mirror in a 180° back-scattering geometry and transferred into a double monochromator. The signal is detected by a cooled photomultiplier

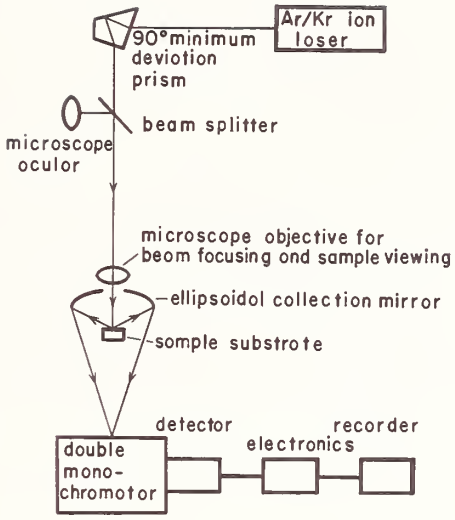

Figure 1. Schematic of the NBS-developed laser-Raman microprobe. Typical measurement parameters employed in the microanalysis of polynuclear aromatic hydrocarbons (PAHs): Excitation, 514.5 nm (green), 568.2 nm (yellow) and 647.1 nm (red); laser power, 5–60 mW (at sample); time constant, 0.2–4.0 s; scan rate, 200–10 cm^{-1} min; spectral slit width, 3 cm^{-1}.

tube and processed by photon counting electronics. The sample is supported by a substrate (typically sapphire or lithium fluoride) mounted on a remotely-controlled sample stage. Irradiance levels employed in routine probe measurements range from several megawatts/cm^2 to several kilowatts/cm^2. Measurement times for solid microsamples of mass 10–100 pg may vary from 20 minutes for (fast) survey spectra to scans requiring 3–5 hours for radiation-sensitive materials.

III. Spectra of Microparticles of PAHs

Raman microprobe measurements were performed on single microparticles (i.e., microcrystals) of a number of selected commercially available PAHs. These PAH standard samples were obtained from several sources and were used without further purification. They include representative compounds of the 3-ring (e.g., anthracene, phenanthrene), 4-ring (e.g., chrysene, pyrene, fluoranthene) and 5-ring (e.g., benzo[a]pyrene) systems. The spectrum of each compound was examined employing green line (514.5 nm), yellow line (568.2 nm) and red line (647.1 nm) excitation. This was to specifically examine effects of sample heating and sample fluorescence. In all cases it has been possible to record analytically useful (with a signal-to-background ratio for the major Raman peaks generally better than 10:1) spectra from discrete PAH microcrystals generally of size 2–10 μm, corresponding to sample masses estimated at well under 1 ng.

In Figures 2–6 are presented the micro-Raman spectra of five PAHs representative of the 4- and 3-membered ring systems. The observed (fundamental) vibrational frequencies of these compounds arise from planar and non-planar vibrations of the molecule [7]. Of interest to the qualitative identification of these compounds are the characteristic Raman shifts corresponding to the lattice vibrations and the internal, molecular vibrations of the crystalline solid. The spectra shown in Figures 2–4 are those of three structurally very similar PAHs in the 4-ring system. These are chrysene, and the isomeric pair pyrene and fluoranthene. Their molecular structures are shown in each figure. The spectrum of a microparticle of chrysene is given in Figure 2. This example is exceptional in that a useful spectrum could be obtained of this PAH using green line excitation. In the case of all other compounds studied, 514.5 nm excitation furnished spectra that were characterized by moderate to high background luminescence levels thought to arise from broad-band fluorescence of the sample. The fluorescence emission in these cases can be so

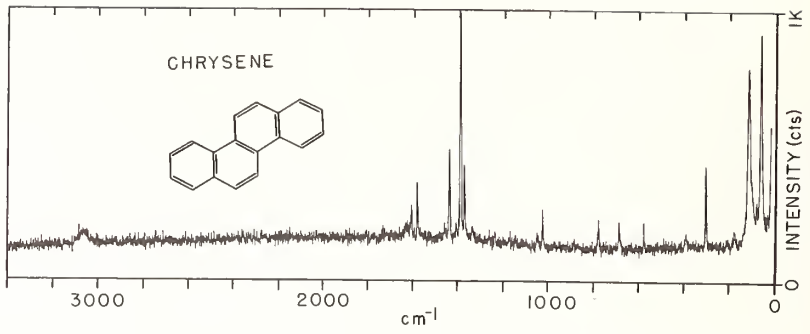

Figure 2. Raman spectrum of a microparticle of chrysene ($C_{18}H_{12}$). Measurement parameters: particle size 7×9 μm; substrate α–Al_2O_3 (sapphire); laser, λ_0=514.5 nm; power, 5 mW (at sample); beam spot, ~18 μm diameter; spectral slit width, 3 cm^{-1}; time constant, 0.25 s; scan rate, 200 cm^{-1} min; intensity, 10^3 counts full scale.

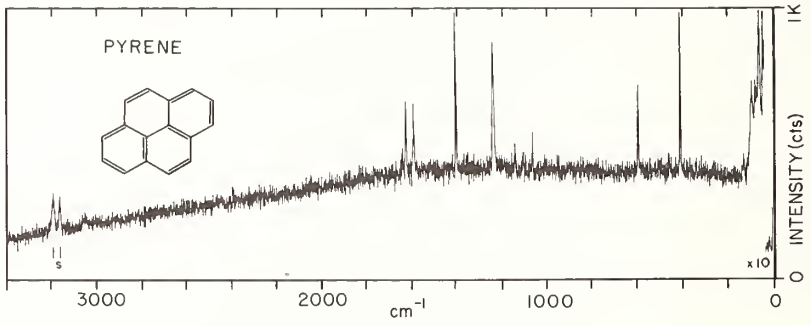

Figure 3. Raman spectrum of a microparticle of pyrene ($C_{16}H_{10}$). Measurement parameters: particle size: 30 μm; substrate α–Al_2O_3 (sapphire); laser, λ_0=568.2 nm; power, 4mW (at sample); beam spot, ~18 μm diameter; spectral slit width, 3 cm^{-1}; time constant, 0.2 s; scan rate, 200 cm^{-1} min; intensity, 10^3 counts full scale. Bands marked S arise from the fluorescence of the substrate.

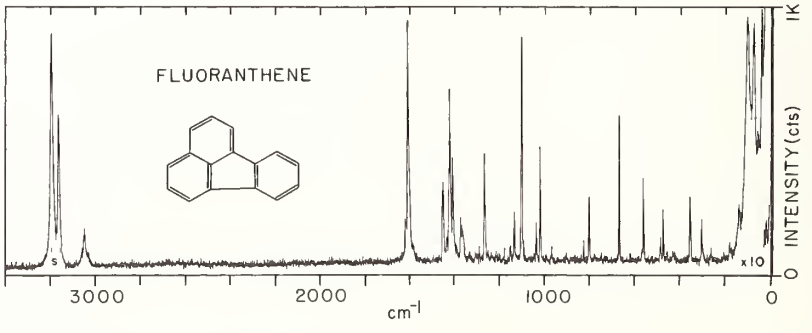

Figure 4. Raman spectrum of a microparticle of fluoranthene ($C_{16}H_{10}$). Measurement parameters: particle size 7×9 μm, substrate α–Al_2O_3 (sapphire); laser, λ_0=568.2 nm; power, 5 mW (at sample); beam spot, ~18 μm diameter; spectral slit width, 3 cm^{-1}; time constant, 0.2 s; scan rate, 200 cm^{-1} min; intensity, 10^3 counts full scale. Bands marked S arise from the fluorescence of the substrate.

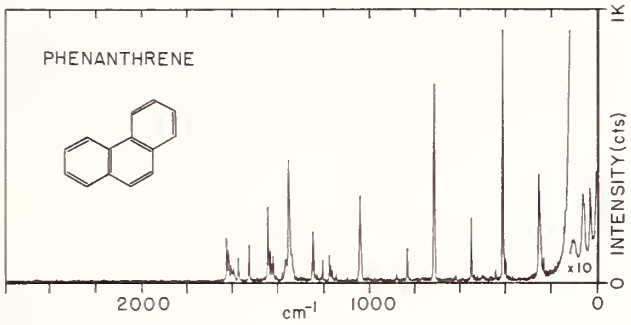

Figure 5. Raman spectrum of a microparticle of phenanthrene ($C_{14}H_{10}$). Measurement parameters: particle size 7×9 mm; substrate LiF; laser, λ_0=647.1 nm; power, 20 mW (at sample); beam spot, ~16 μm diameter; spectral slit width, 3 cm^{-1}; time constant, 1.0 s; scan rate, 50 cm^{-1} min; intensity, 10^3 counts full scale.

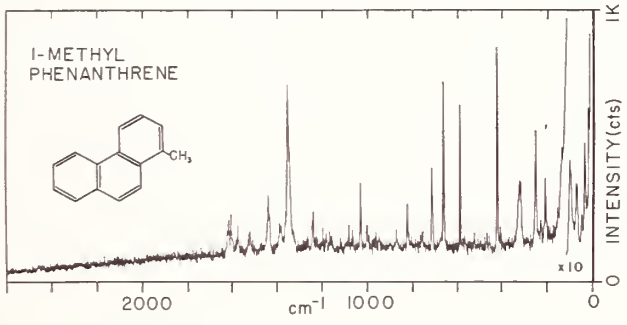

Figure 6. Raman spectrum of a microparticle of 1-methylphenanthrene ($C_{15}H_{12}$). Measurement parameters: particle size 4×6 μm; substrate, LiF; laser, λ_0=647.1 nm; power, 55 mW (at sample); beam spot, ~14 μm diameter; spectral slit width, 3 cm^{-1}; time constant, 3.0 s; scan rate, 20 cm^{-1} min; intensity, 10^3 counts full scale.

predominant as to completely swamp the Raman signal. Sample heating was not observed in these low-irradiance measurements of chrysene microparticles and is not indicated in the spectrum. Excitation with 568.2 nm (yellow line) radiation results in chrysene particle spectra that have appreciably lower background levels (with baseline intensities typically <50 counts), indicating diminished fluorescence emission at this longer wavelength. This has been a general observation made for all the PAHs investigated to this point. In the spectrum of Figure 2, spectral interferences from the weak Raman scattering of the sapphire substrate are not observed above the background luminescence level. The spectrum of a microcrystal of pyrene, shown in Figure 3, was excited with the 568.2 nm line of the laser. Attempts to obtain spectra from microsamples of this compound employing 514.5 nm excitation were unsuccessful due to the overwhelming fluorescence emission from the sample. The spectrum obtained here at rather low irradiance has a tolerable fluorescence background upon which the major Raman bands appear with good intensity. The Raman spectrum and vibrational assignments of pyrene have been reported in the literature, based on a study of single crystal samples [8]. The microprobe spectra obtained in this study are consistent with the results of these bulk Raman measurements. As is the case for all other PAH compounds examined here, many of the lattice modes (in the region below ~200 cm^{-1}) give rise to those peaks in the spectrum that are of greatest intensity. Examination of the frequency positions of these crystal vibrations shows these to be very characteristic of each compound. The doublet at the high frequency end (i.e., at higher wavenumber shifts) is not attributed to the spectrum of pyrene but arises from the ruby (Cr_2O_3, present as a trace impurity) fluorescence in sapphire. This fluorescence doublet (R lines) appears at wavenumber shifts 3167 cm^{-1} (R2 line, 692.9 nm) and 3196 cm^{-1} (R1 line, 694.3 nm), with 568.2 nm spectral excitation.

Subnanogram microsamples of fluoranthene have been examined in the microprobe, and the spectum of a microparticle excited with 568.2 nm radiation is shown in Figure 4. Whereas 514.5 nm excitation stimulated enormous fluorescence emission from this PAH, good spectra could be obtained with both yellow line and red line excitation. The compound is characterized by a larger number of internal vibrational modes than has been observed for the two structural isomers discussed above. Again, several of the crystal lattice modes are the most intense bands in the spectrum. The sapphire fluorescence doublet is pronounced in this measurement and is seen in the 3100–3250 cm^{-1} region.

A qualitative comparison of the spectra (Figs. 2–4) of the three structurally similar PAHs shows that these are sufficiently different to allow unequivocal identification of these closely related molecular solids. Microprobe measurements on selected isomeric compounds of the 5-ring system (e.g., benzo[a]pyrene, perylene) indicate a similarly high specificity of the Raman spectrum.

Figure 5 shows the spectrum of a microparticle of phenanthrene and Figure 6 that of a corresponding microsample of the monomethyl derivative, 1-methylphenanthrene. Here the interest is to characterize spectral differences among the parent compound and its various methyl derivatives. The 2-methyl isomer has also been investigated. The results indicate that within a given series of mono- (or di-) substituted PAHs, the various derivatives can be distinguished on the basis of the characteristic Raman shifts of the predominant, strong bands. The spectra of the two types of microparticles considered here were excited with the 647.1 nm line. For the methyl compound the background level is somewhat higher than is observed for the non-substituted phenanthrene. This may be due to some level of (fluorescing) impurity present in the former but not in the latter sample. Each particle is supported by a lithium fluoride substrate which does not give rise to any spectral interferences. The standard sapphire substrate is much less suitable for measurements employing 647.1 nm excitation, since the R-lines of the trace Cr^{3+} fluorescence appear at shifts 1021 and 1050 cm^{-1} and thereby represent a serious interference in a diagnostic region of the spectrum. With 647.1 nm excitation, Raman spectra can be recorded with the microprobe from near the exciting line to about 2700 cm^{-1} (wavenumber shift). This corresponds to the upper frequency limit (which is 12,750 cm^{-1} absolute or 784 nm) of the spectral domain of the monochromator employed in the instrument. Thus, the CH stretching vibrations for these compounds—whose frequencies fall in the range 3000–3100 cm^{-1}—are not accessible with the present instrument when red line excitation is employed. The phenanthrene spectrum in Figure 5 is consistent with the results of bulk-Raman measurements performed on single crystal samples of phenanthrene [9]. The external (i.e., lattice) and internal vibrational modes of this crystalline solid are seen to give rise to a number of bands of good scattering intensity, with the more predominant internal modes showing shifts at 410, 713 and 1443 cm^{-1}. By comparison, the most intense bands in the spectrum of the closely related (structural isomer) anthracene appear at 396, 748 and 1403 cm^{-1} [7].

IV. Conclusions

We have shown with these examples that the micro-Raman spectroscopy technique can furnish analytically useful vibrational spectra from microsamples of PAHs of interest in environmental pollution studies. The results indicate that this new technique of (qualitative) microanalysis may potentially be applied as a post-column detection method in the identification of trace organics isolated by column chromatography. The measurements on pure compounds indicate a limit of detectability (with the Raman microprobe in its present configuration) of 10–100 pg for various types of PAHs and other classes of polynuclear organics (e.g., nitrogen containing polyaromatics). The technique, therefore, appears to be especially suited as a sensitive and highly specific method of detection for liquid chromatography separations of many classes of organic compounds. These initial studies on the characterization by micro-Raman spectroscopy of PAHs are continued. The scarcity in the literature of Raman data on PAHs makes necessary the

acquisition of a library of reference spectra. Work has been initiated on the characterization of multicomponent synthetic mixtures separated by high-performance liquid chromatography. Procedures are being developed that will allow the analysis of the chromatographic effluent in a static mode whereby a portion of the effluent is trapped and the solid analyte is isolated on the Raman substrate for spectroscopic measurement.

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Section V. GENERAL ANALYTICAL TECHNIQUES

NATIONAL BUREAU OF STANDARDS SPECIAL PUBLICATION 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, Proceedings of the 9th Materials Research Symposium, April 10–13, 1978, held at NBS, Gaithersburg, Md. (Issued April 1979)

COMBINED GLASS CAPILLARY-COLUMN GAS CHROMATOGRAPHY AND MIXED CHARGE EXCHANGE-CHEMICAL IONIZATION MASS SPECTROMETRY OF ISOMERIC POLYCYCLIC AROMATIC HYDROCARBONS

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Glass capillary-column gas chromatography combined with mixed charge exchange-chemical ionization mass spectrometry is used to differentiate between isomeric polycyclic aromatic hydrocarbons found in Utah air particulate matter. A reagent-gas mixture of methane and argon is used to produce unique mass spectra for isomers eluted within the methylphenanthrene/anthracene and the methylfluoranthene/pyrene regions of the chromatogram.

Key words: Capillary-column; charge exchange; chemical ionization; gas chromatography; mass spectrometry; polycyclic aromatic hydrocarbons.

I. Introduction

It has been known for many years that many polycyclic aromatic hydrocarbons (PAH) are carcinogenic, and that this carcinogenic activity is very dependent on the specific structure of the compound [1]. For example there are five structural isomers for the series of four-ring compounds with molecular weight 228. Of these five compounds, benz[*a*]anthracene is a strong carcinogen, chrysene and benzo[*c*]phenanthrene are weak carcinogens, and the other two possess no carcinogenic activity at all. The addition of an alkyl group on the ring can either enhance or decrease the carcinogenic character of the compound. It has been found within the methylchrysene series that only the 3- and 5-methylchrysenes are strong carcinogens while the others are only very moderately carcinogenic [2]. The total carcinogenic potential of a mixture of PAH depends on the exact structural components of the mixture. The mixtures of PAH that are collected from combustion sources such as coal-fired power plants, automobile exhaust, coke production, refuse burning, and tobacco smoking, oftentimes are very complex, containing up to several hundred easily detected compounds. The identification of each of these compounds both qualitatively and quantitatively is important for understanding the possible health hazards of human exposure to these sources. The identification of these mixture components is not an easy task. This can be seen merely by considering the number of possible isomers present in these mixtures. If one considers the number of possible isomers ranging from two to five rings, it is obvious that as one goes to larger compounds, the number of possible isomers greatly increases. While there are only two possible structures for the three-ring compounds molecular weight 178, there are 12 possible structures for the five-ring compounds of molecular weight 278. The picture becomes even more complicated with the consideration of the alkylated species. The number of possible mono-methyl isomers increases from two for naphthalene to 117 for the five-ring isomers of pentacene. The problem becomes even more overwhelming when one considers all of the possible isomeric alkylated species.

In order to determine the complete and detailed composition of a mixture of PAH two basic steps must be accomplished. The first step is to resolve the mixture into its individual components.

In the last few years, glass capillary-column gas chromatography has demonstrated its superiority in the separation of complex mixtures of polycyclic aromatic hydrocarbons. Short columns (10–20 meters) containing over 50,000 theoretical plates can be routinely prepared, and due to the high permeability and inertness of such columns, many PAH encountered in environmental samples and containing from two to seven rings can be resolved in one chromatographic run. This is impressive only if one considers the vast number of structural isomers present in these complex mixtures. The recent separation of over 150 PAH in cigarette smoke [3], air pollution [4], and combustion sources [5], demonstrates the superior resolution attainable.

In addition to improved resolution, the limits of detection are extended with glass capillaries to include trace components present in complex mixtures of interest. Trace compounds that are either unresolved or obscured under baseline noise in conventional gas chromatography can be adequately resolved with glass capillaries and eluted within sharp, narrow bands with high enough intensity for identification. The low volume carrier gas flow rate obtained with capillary-column gas chromatography (1 to 5 mL/min) makes this technique very attractive for direct combination with mass spectrometry. Commonly used separation devices in packed-column work are eliminated and improved mass spectrometer sensitivity obtained.

The second basic step that must be accomplished is the identification of each of the resolved compounds. This is best done by combined gas chromatographic mass spectrometry. The mass spectra of PAH are well-known and, for the most part, are simple, consisting of an intense molecular $(M)^+$ ion for electron impact (EI) ionization or an intense quasimolecular $(M+1)^+$ ion and $(M+29)^+$ ion for chemical ionization, and small ions due to the loss of one to three hydrogen atoms. Low intensity doubly charged molecular ions are also quite common. The abundance of the molecular or quasimolecular ion often facilitates the determination of the molecular weight of PAH, but the lack of fragment ions means that conventional mass spectrometry cannot easily distinguish between isomers.

The use of a mixed charge-exchange chemical ionization reagent gas (5–10% methane in argon) has recently been used to produce distinctly different mass spectra of a variety of PAH isomers [6]. The following sections describe the successful coupling of this ionization technique with glass capillary-column gas chromatography for the identification of a number of isomeric PAH in air particulate matter.

II. Experimental

Five acid washed quartz fiber filters (20.3×25.4 cm) through which Utah County air had been drawn (20,000 m^3 total) at the approximate rate of 90 m^3/h were placed in a Soxhlet apparatus and extracted for 24 hours with 500 mL of methylene chloride. The extract was evaporated to dryness in a rotary evaporator, dissolved in 50 mL of cyclohexane, and washed five times with 50 mL of nitromethane. The nitromethane portions were combined and evaporated to dryness. The residue was dissolved in 2 mL of methylene chloride; 0.5 g of silicic acid (Mallinckrodt, 100 mesh) were added; and the mixture was blown to dryness under a stream of nitrogen gas. The absorbed sample was transferred to a 30 cm×1.5 cm, i.d., glass column packed with 4.0 g of the silicic acid and eluted with 350 mL of *n*-hexane. The hexane eluate was then evaporated to dryness and dissolved in a small portion of methylene chloride prior to gas chromatographic analysis. All solvents used were of high purity. The sample was chromatographed on a 10 m×0.29 mm, i.d., glass capillary column coated with a 0.36 μ film thickness of SE-52 methylphenylsilicone stationary phase. A Varian Series 1400 gas chromatograph equipped with flame ionization detector and modified injection port was used to obtain the chromatogram shown in Figure 1. The oven temperature was programmed from 90 °C to 250 °C at 2 °C/min.

Gas chromatographic-mass spectrometry was performed with a Hewlett-Packard 5982A gas chromatographic mass spectrometer system interfaced to a 5934A data system. The same 10 m×0.29 mm, i.d., glass capillary column and helium carrier gas (approximately 2 mL/min flow

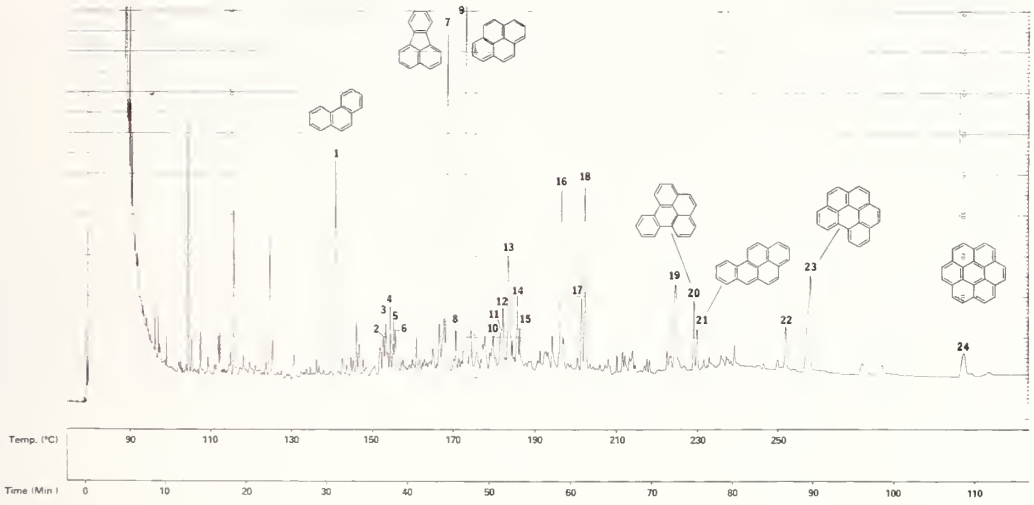

Figure 1. Capillary-column gas chromatogram of the PAH fraction of Utah County air particulate matter. Numbered peaks are listed in Table 1.

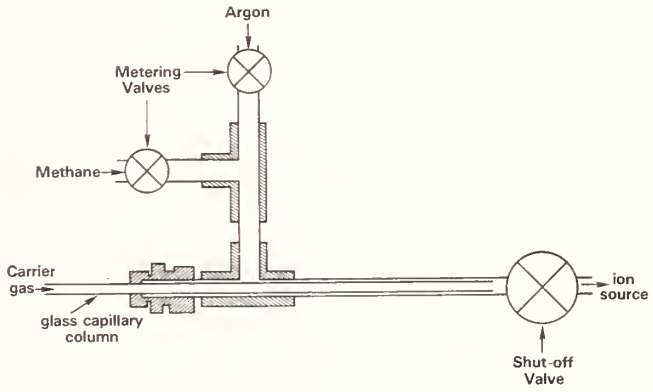

Figure 2. Schematic diagram of the capillary column/mass spectrometer interface.

rate) were used to separate mixtures prior to mass spectral analysis and to introduce samples into the ion source. The oven temperature was programmed from 100 °C to 250 °C at 2 °C/min for each run. For electron impact mass spectrometry, masses from 50 to 350 a.m.u. were scanned continuously at the rate of 140 a.m.u./sec. and the mass spectrometer was operated at 70 eV ionizing energy. For chemical ionization mass spectrometry reagent gases and capillary column effluents were introduced as shown in Figure 2. Metering valves allowed for the adjustment of the flow rates of argon and methane. When using only methane as reagent, the ion source pressure was adjusted to 0.7 torr by opening only the methane metering valve. For mixed argon and methane reagent, the argon flow rate was adjusted first to provide a constant pressure of 0.4 torr in the ion source. Methane was then introduced until the ratio $(M+1)/M$ of the abundance of the $(M+1)^+$ and M^+ ions of naphthalene, which was introduced through the direct probe, equaled approximately 1.6. Tuning in this manner led to results comparable with previous work [6]. It was found necessary to periodically check and adjust the tuning to naphthalene during operation. The mass spectrometer was operated at 350 eV ionizing energy. For acquisition of complete mass spectra, masses from 50 to 350 a.m.u. were scanned at the rate of 140 a.m.u. per second as before. Otherwise, since only the abundances of the M^+ and $(M+1)^+$ ions were needed, the mass

spectrometer was operated in the SIM mode. The dwell time on each mass was 200 ms. Ratios of (M+1)/M were reproducible (±5%) down to a sensitivity level of 10 ng of compound injected into the gas chromatograph.

III. Results and Discussion

Figures 3 and 4 show the mass spectra obtained for anthracene and phenanthrene under electron impact and methane chemical ionization conditions, respectively. It is obvious that neither of these techniques can distinguish between the two isomers. When a charge exchange-chemical ionization reagent gas such as an argon and methane mixture is used under chemical ionization conditions, a number of reactions occur. The argon is first ionized to Ar^+ by the electron beam (eq. 1). Charge transfer then occurs between the Ar^+ and PAH molecule to

$$Ar + e^- \rightarrow Ar^+ + 2e^- \quad (1)$$

give an abundant molecular ion as in electron impact (eq. 2). Ionization of

$$M + Ar^+ \rightarrow M^+ + Ar \quad (2)$$

methane also occurs by the electron beam (eq. 3) or by the Ar^+ ions (eq. 4).

$$CH_4 + e^- \rightarrow CH_4^+ + 2e^- \quad (3)$$

$$CH_4 + Ar^+ \rightarrow CH_4^+ + Ar \quad (4)$$

Further reaction with another methane molecule gives rise to the reactant ion CH_5^+ (eq. 5) which then reacts with the PAH molecule to produce an abundant

$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3 \quad (5)$$

$(M+1)^+$ ion (eq. 6). The relative rates of the charge exchange (eq. 2) and

$$M + CH_5^+ \rightarrow MH^+ + CH_4 \quad (6)$$

proton exchange (eq. 6) reactions, and hence the ratio of the abundance of the $(M+1)^+$ ion to the abundance of the M^+ ion will vary according to the proton affinity and/or ionization potential of each PAH. Since the ionization potentials of PAH isomers are dependent on the specific structure of the molecule, the argon-methane reagent can produce quite different spectra for different isomers. This is seen by comparison of the argon-methane CI spectra for anthracene and phenanthrene (Fig. 5).

In order to test the feasibility of using this new technique of producing unique mass spectra for PAH isomers under capillary-column gas chromatographic conditions, the PAH fraction of a sample of air particulate matter was analyzed as described in the experimental section. The capillary-column gas chromatogram which was obtained is shown in Figure 1. There are well over 100 detectable compounds in this fraction. Many of these compounds have been identified in previous work by gas chromatographic mass spectrometry and retention comparisons with

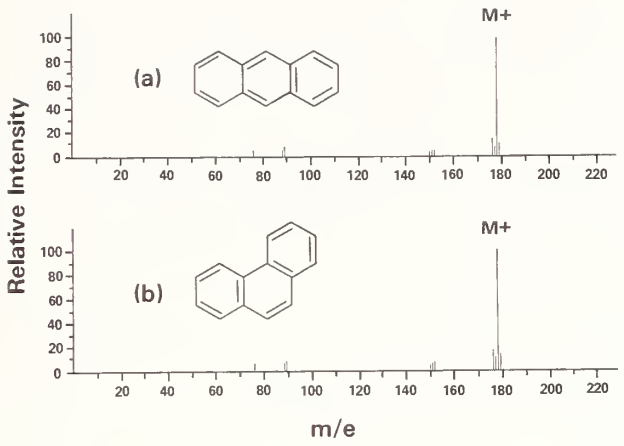

Figure 3. Electron impact mass spectra of (a) anthracene and (b) phenanthrene.

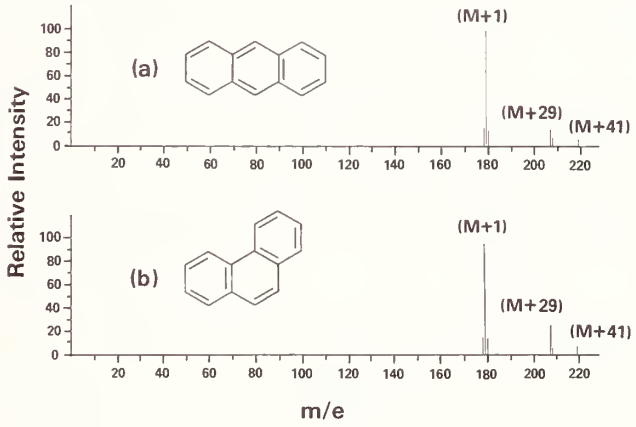

Figure 4. Methane chemical ionization mass spectra of (a) anthracene and (b) phenanthrene.

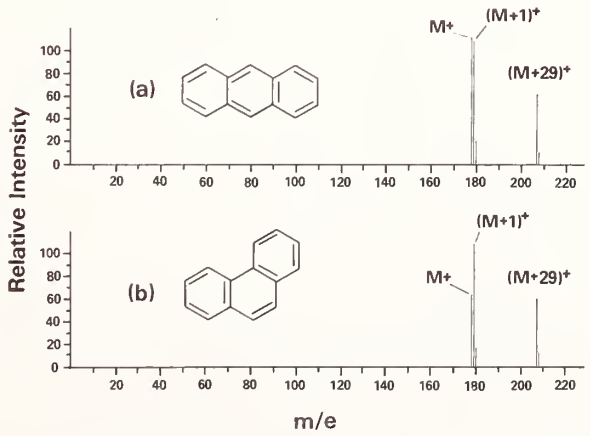

Figure 5. Argon-methane chemical ionization mass spectra of (a) anthracene and (b) phenanthrene.

TABLE 1. *PAH identified in Utah County air particulate matter*

Peak No.[a]	Compound
1	Phenanthrene
2	3-Methylphenanthrene
3	2-Methylphenanthrene
4	4*H*-cyclopenta[*def*]phenanthrene
5	9-Methylphenanthrene
6	1-Methylphenanthrene
7	Fluoranthene
8	Benzacenaphylene
9	Pyrene
10	Methylfluoranthene
11	Methylfluoranthene
12	Benzo[*a*]fluorene
13	2-Methylpyrene and benzo[*b*]fluorene
14	4-Methylpyrene
15	1-Methylpyrene
16	Benzo[*ghi*]fluoranthene
17	Benzo[*a*]anthracene
18	Chrysene antriphenylene
19	Benzofluoranthene
20	Benzo[*e*]pyrene
21	Benzo[*a*]pyrene
22	Indeno[1,2,3-*cd*]pyrene
23	Benzo[*ghi*]perylene
24	Corenene

[a] Peak numbers refer to chromatographic peaks in the chromatogram in Figure 1.

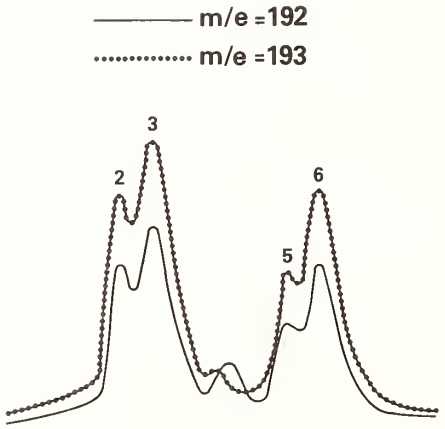

Figure 6. Selected ion plots of m/e 192 and 193 of Utah County air particulate matter. Peak numbers refer to peaks in Figure 1.

available standard compounds. Table 1 lists 24 of the major PAH which have been identified. The areas of particular focus in this study are the methylanthracene/phenanthrene region (peaks 2–6) and the methylfluoranthene/pyrene region (peaks 10–15).

Figure 6 shows the selected-ion plots for m/e 192 as compared to m/e 193 for the air particulate PAH sample. These masses represent the molecular and quasi-molecular ions, respectively, for either methylanthracenes or methylphenanthrenes. In this case the quasi-

molecular ion is more abundant than the molecular ion. Table 2 gives the values of (M+1)/M for these four peaks along with several standard compounds. Notice that the addition of a methyl group on the ring reduces the (M+1)/M ratio for phenanthrene about 20% while the ratio for anthracene is reduced only slightly. It is obvious from these data that the four peaks are all methylphenanthrenes.

Figure 7 shows similar plots of m/e 216 and m/e 217. These compounds could be methylfluoranthenes, methylpyrenes, or benzofluorenes. The calculated ratios of (M+1)/M for each peak is given in Table 3 along with several standard compounds. From comparison of these data there is good evidence that peaks 10 and 11 are methylfluoranthenes and peaks 14 and 15 are methyl pyrenes. Either peak 12 or 13 could be benzo[a]fluorene according to this data. Retention data of standard compounds suggests that peak 12 is benzo[a]fluorene and that both benzo[b]fluorene and 2-methylpyrene have the same retention as peak 13. A mixture of the two could certainly lead to a (M+1)/M ratio of 0.81.

TABLE 2. *Abundance ratios for several anthracenes, phenanthrenes, and selected peaks from air particulate matter*

Compound[a]	M+1[b] / M
Anthracene	0.82
2-Methylanthracene	0.77
9-Methylanthracene	0.79
Phenanthrene	1.57
1-Methylphenanthrene	1.20
9-Methylphenanthrene	1.20
Peak No. 2	1.26
Peak No. 3	1.20
Peak No. 5	1.24
Peak No. 6	1.24

[a] Peak numbers refer to peaks in Figures 1 and 6.
[b] The ratios have been corrected for the natural abundance of ^{13}C.

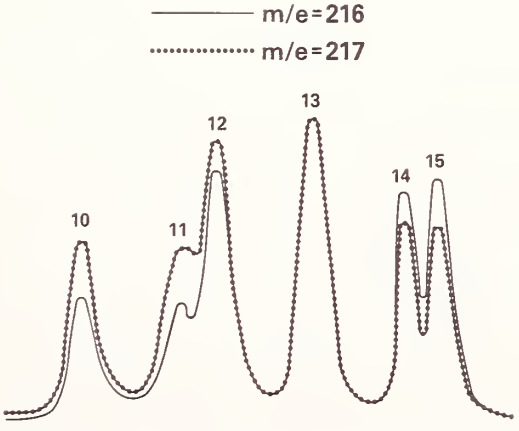

Figure 7. Selected ion plots of m/e 216 and 217 of Utah County air particulate matter. Peak numbers refer to peaks in Figure 1.

TABLE 3. *Abundance ratios for several fluoranthenes, pyrenes, benzofluorenes, and selected peaks from air particulate matter*

Compound[a]	M+1[b] / M
Fluoranthene	1.55
Pyrene	0.73
1-Methylpyrene	0.64
4-Methylpyrene	0.69
Benzo[*a*]fluorene	0.81
Benzo[*b*]fluorene	1.05
Peak No. 10	1.20
Peak No. 11	1.24
Peak No. 12	0.89
Peak No. 13	0.81
Peak No. 14	0.68
Peak No. 15	0.62

[a] Peak numbers refer to peaks in Figures 1 and 7.
[b] The ratios have been corrected for the natural abundance of ^{13}C.

These examples show both the power and limitations of using a mixed charge-exchange-chemical ionization reagent gas for analysis of mixtures of PAH isomers. Although one can distinguish between methylanthracenes and methylphenanthrenes or between methylfluoranthenes, methylpyrenes and benzofluorenes in general, the position of methylation on the ring cannot be determined. Retention data of standard compounds on efficient gas chromatographic columns must be used to complete the exact identifications. On the other hand, the ability to determine the parent-ring structure by mass spectrometry is one step further toward understanding the detailed composition of complex PAH mixtures.

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NEW CONCEPTS OF QUANTITATION IN HEADSPACE GAS ANALYSIS BY STRIPPING AND TRAPPING COMPONENTS IN A CLOSED CIRCUIT

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A theoretical analysis of the method of stripping and trapping components in a closed circuit was carried out. Alternatives involving the discontinuation of the stripping and trapping process either before the frontal zone of a component under determination starts breaking through the trapping column or after the component reaches a state of equilibration within the system are specified. Equations relating the amount of a solute component accumulated in the trapping column and the initial total amount of the component in the system analyzed were derived for both of the above alternatives. The standard-addition technique is shown to be a universal means of quantitation in headspace gas trace analysis by the method studied.

Key words: Equilibration trapping; gas chromatography; headspace gas stripping; standard-addition calibration; trace analysis; trapping column.

I. Introduction

The method of stripping and trapping components in a closed circuit has been described by Grob et al. [1–4]. In this method the gaseous phase of a gas-liquid system is recycled through the liquid phase and a column with a sorbent by means of a pump, and the components entrapped in the column are determined by gas chromatographic analysis. From the methodological point of view the above procedure is interesting in that it makes it possible to combine the process of stripping in an on-line arrangement with the method of chromatographic equilibration [5]; this aspect and its analytical implications with regard to quantitation by the standard-addition method were discussed earlier [6].

The authors of the method of stripping and trapping components in a closed circuit employed for quantitation the internal-standard method and the method of comparison with a model reference system. Both methods undoubtedly are applicable in the given arrangement if all the substances to be determined are completely stripped out of the system and trapped in the column. The second method can be employed even if the components are not stripped out and trapped completely, but it is necessary in this case that the composition of the matrix material of the reference system be at least approximately the same as that of the matrix material of the system under analysis. In case of the determination of hydrocarbons in water both above-mentioned requirements can relatively easily be fulfilled, but in other cases it may be very difficult. Components which show a high affinity towards the matrix material cannot be stripped out completely. On the other hand, if the matrix material is constituted by several components the identities and proportions of which are unknown, then this material cannot be modelled. Hence,

the problem of reliable quantitation in headspace gas analysis by stripping and trapping the components being determined essentially remains open.

In this paper we specify different experimental variants of the method, and for each variant there are derived relations between the amount of the substance under determination accumulated in the trapping column and the initial total amount of this substance in the system analyzed. By virtue of these relations the use of the standard-addition method as a universal means of quantitation is discussed.

II. Physical Substance and Experimental Alternatives of the Method

The experimental arrangement for stripping components of a liquid material and trapping them in a column of sorbent in a closed circuit represents generally a three-phase multicomponent system in which two condensed phases, the liquid and the sorbent, are separated from each other by a common gaseous phase. By recycling the gaseous phase in one direction the whole system can be brought into thermodynamic equilibrium. At the initial stage of the process the components transported by the gas are subject to frontal chromatography in the trapping column. If the concentrations of the components in the gas were constant the column would be saturated by all the components just after the front of the most sorbed one has broken through, and the contents of the components trapped in the column would not change on further continuing the process. However, in the above-specified arrangement the concentration of a component in the gas entering the trapping column gradually decreases as the component accumulates in the sorbent, which results in a drop of the amount of trapped component across the column in the direction opposite to the flow of the gas. Hence, the frontal zone prior and after breakthrough will contain an amount of the trapped component larger than that corresponding to equilibrium with the instantaneous concentration of the component in the gas entering the column. This excess amount will be the larger the lower the capacity of the gas-liquid system and the higher the sorption capacity of the trapping column. Thus, in the closed-circuit arrangement, at breakthrough of the frontal zone the amount of the trapped component does not represent a true state of equilibrium. In order to attain equilibrium, it is necessary that a volume of gas equal to several multiples of the retention volume of the component, as measured on the filling of the column under the given conditions, be drawn through the trapping column. If there are in the system several components to be stripped and trapped, the whole system becomes equilibrated only after a state of equilibration has been attained for the most strongly sorbed component. A schematic illustration of the system at different stages of the process is shown in Figure 1, where the shaded areas represent the amounts of solute component in the gas-liquid system and in the trapping column.

It is evident from the above considerations that the relations describing the mass balance of a component in the entire system will differ according to the way of carrying out the experiment.

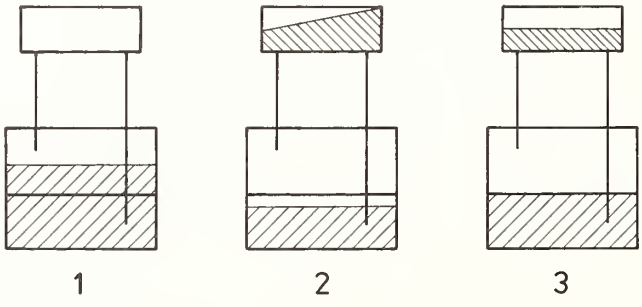

Figure 1. Schematic representation of the distribution of solute between the gasliquid system and the trapping column at different stages of the process. 1–initial state, 2–a state when the frontal zone has just broken through the column, 3–state of equilibration.

In some cases it is possible to arrange the experiment so as to strip and trap the component completely before breakthrough. In such a case the mass balance of the component is trivial. However, in most cases, a substantial part of the component remains in the gas-liquid system at the moment of breakthrough. In such cases it is expedient to consider two limiting situations for the mass balance of the solute components. One such situation (Alternative I) occurs if the process of stripping and trapping is stopped before the edge of the frontal zone of the component under determination reaches the column outlet; if several components are concerned, the moment at which the process has to be stopped is determined by that one which is least sorbed in the column. Under these circumstances the proportions of the components trapped in the column will be identical with the mean proportions the components assumed in the gas during the process. The other situation (Alternative II) occurs if the process of stripping and trapping is stopped only after the component being determined reaches a state of complete equilibrium; in case of several components the process is stopped after the most sorbed one comes to equilibrium. In this version the proportions of the components trapped in the column differ from the proportions the components have assumed in the supplied gas, the relative degree of accumulation of the individual components in the column being dependent on their distribution constants in the given gas-sorbent system.

Let us denote the volume of the gaseous phase having passed through the column during the process of stripping and trapping by V. As the concentration profile represented by a frontal zone conforms to the integrated concentration profile of the corresponding elution zone, the first alternative can be specified by the condition $V \leqslant V_R - 2\ \sigma_V$ where V_R is the gross retention volume as measured for the component in question on the trapping column at its temperature and outlet pressure and σ_V is the volumetric standard deviation of the elution zone that would develop in chromatography of the component under the above conditions. The volume necessary for the frontal zone to be completely purged out of the column is $V = V_R + 2\ \sigma_V$, so that the condition for the component to reach a state of equilibrium can be written as $V >> V_R + 2\ \sigma_V$. The relations between the situation in the trapping column and the position of the edge of the frontal zone with respect to the column outlet are illustrated in Figure 2. According to the definition of the theoretical plate it holds $\sigma_V = V_R/\sqrt{N}$ where N is the number of theoretical plates of the trapping column. Hence, the conditions that qualify the procedure as Alternative I or Alternative II are given by

$$V \leqslant V_R[1 - (2\sqrt{N})] \tag{1}$$

and

$$V >> V_R[1 + (2\sqrt{N})] \tag{2}$$

respectively. For V_R it applies that

$$V_R = V_{Gt} + K_{SG}V_S \tag{3}$$

where V_{Gt} and V_S are the void volume and the volume of the sorbent in the trapping column, K_{SG} being the distribution constant defined as the ratio of equilibrium concentrations of solute in the sorbent and in the percolating gas at the temperature of the trapping column.

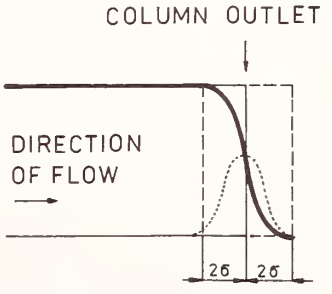

Figure 2. Diagram of a frontal concentration profile breaking through the outlet of the trapping column.

III. Relations Between the Amount of a Component Accumulated in the Trapping Column and the Initial Total Amount of the Component in the System

When employing the procedure discussed the analyst is confronted with the problem of establishing the total concentration of component i, W_i, in the gas-liquid system from the determination of the amount of this component contained in the trapping column, W_{it}. Hence it is expedient first to search for the relation between W_{it} and W_i. Obviously, for the two alternatives this relation will be different.

A. ALTERNATIVE I

For a given system, at any stage of the stripping and trapping procedure, the conservation equation is valid

$$W_i = W_{iL} + W_{iG} + W_{it} \tag{4}$$

where W_{iL} and W_{iG} are the amounts of component i in the liquid and in the gaseous phase. Under the conditions specified for Alternative I all three quantities on the right-hand side of equation (4) are functions of time during the process and are interdependent such that

$$dW_{it} = -d(W_{iL} + W_{iG}). \tag{5}$$

Component i is transported from the gas-liquid system into the trapping column by the gaseous phase only, hence

$$-d(W_{iL} + W_{iG}) = c_{iG}\, F\, dt \tag{6}$$

where c_{iG} is the instantaneous concentration of component i in the gaseous phase, F is the volumetric rate of the circulation of the gaseous phase, and t is the time. The quantity c_{iG} is defined by

$$c_{iG} = W_{iG}/V_G \tag{7}$$

while it is assumed that

$$\frac{W_{iG}}{V_G} = \frac{1}{K_{LG}} \frac{W_{iL}}{V_L} \tag{8}$$

where V_G and V_L are the total volumes of the gaseous and the liquid phase in the system and K_{LG} is the distribution constant of component i in this system, so that c_{iG} can also be expressed by

$$c_{iG} = \frac{W_{iL}+W_{iG}}{V_G+K_{LG}V_L}. \tag{9}$$

Combining equations (9), (6), (5), and (4) we obtain

$$\left[\frac{dW_{it}}{dt} = \frac{(W_i-W_{it})F}{V_G+K_{LG}V_L}\right]. \tag{10}$$

Equation (10) is a differential equation describing the stripping and trapping process under non-steady state conditions (Alternative I) and applies to any variation of the experiment, i.e., both for

work in closed and open circuits. By integrating equation (10), with the initial conditions of $t=0$ and $W_{it}=0$, we obtain

$$W_{it} = W_i \left[1-\exp\left(-\frac{F\,t}{V_G+K_{LG}V_L}\right)\right]. \tag{11}$$

Equation (11) has been derived under the assumption that at any time the concentration of component i in the stripping gas entering the trapping column is in equilibrium with the concentration of this component in the liquid phase.

B. ALTERNATIVE II

Again starting from equation (4), the form

$$W_i = W_{iL} + W_{iG} + W_{iS} \tag{12}$$

where W_{iS} denotes the amount of component i in the absorbent of the trapping column. Since

$$W_{it} = W_{iS} + W_{iGt} \tag{13}$$

and

$$W_{iGt} = \frac{V_{Gt}}{K_{SG}V_S}\, W_{iS} \tag{14}$$

where W_{iGt} is the amount of component i contained in the void space of the trapping column (the meanings of V_{Gt}, V_S, and K_{SG} have been specified with equation (3)), we can write

$$W_{iS} = W_{it}\,\frac{K_{SG}V_S}{V_{Gt}+K_{SG}V_S}. \tag{15}$$

In this alternative the quantities W_{iL}, W_{iG}, W_{iS}, and W_{it} have constant values. If the entire system is in equilibrium then

$$W_{iG} = \frac{V_G}{K_{SG}V_S}\, W_{iS} \tag{16}$$

and

$$W_{iL} = \frac{K_{LG}V_L}{V_G}\, W_{iG} = \frac{K_{LG}V_L}{K_{SG}V_S}\, W_{iS} \tag{17}$$

so that by substituting W_{iL} and W_{iG} from equations (16) and (17) into equation (12) and combining the latter with equations (15) and (4) we obtain

$$W_{it} = W_i\left[\left(\frac{K_{SG}V_S}{V_{Gt}+K_{SG}}\right)\left(\frac{V_G}{K_{SG}V_S} + \frac{K_{LG}V_L}{K_{SG}V_S} + 1\right)\right]^{-1}. \tag{18}$$

Equation (18) applies only for a closed circuit, as it is only in this arrangement that the whole system can be brought into equilibrium. In a normal stripping procedure where a stream of gas is employed to carry the components to the trapping column, equilibrium can not be obtained because concentrations in the stripping gas continuously decrease during this process.

In most cases one can assume that $K_{SG}V_S >> V_{Gt}$ and thus $W_{iS} >> W_{iGt}$ and $W_{iS} \cong W_{it}$. Hence the factor $[K_{SG}V_S/(V_{Gt} + K_{SG}V_S)]$ in equation (18) approximately equals unity.

IV. Quantitation by the Standard-Addition Method

Equations (11) and (18) can be written briefly in the form

$$W_{it} = W_i f \tag{19}$$

where f is a system factor corresponding to the respective experimental alternative. By employing the standard-addition method the system factor can be eliminated and the value of W_i can be conveniently calculated by virtue of the determined value of W_{it}. This concept is based on the assumption that by adding to the system a defined small amount of a substance that already is a constituent of this system one does not alter appreciably the values of distribution constants K_{LG} and K_{SG} nor the volumes of the phases, provided the other conditions are kept constant. Equation (11) shows that if also F and t are maintained constant, it is possible to eliminate the system factor in the Alternative I case.

In headspace gas analysis the standard-addition method can be applied in two different ways [6].

A. Two Systems Operation

Two identical stripping and trapping systems containing analyte are prepared, a defined amount (W_s) of the component under determination (i) is added into one of them, and both systems employ the same procedure of stripping and trapping with separate trapping columns at identical conditions. The amounts of the component trapped in both columns are determined. For the system containing the analyzed material as such W_{it} is given by equation (19), and for the system containing the added standard we can express the amount of the trapped component, W'_{it}

$$W'_{it} = (W_i + W_s)f. \tag{20}$$

Dividing equation (20) by equation (19) and solving for W_i yields

$$W_i = \frac{W_s}{(W'_{it}/W_{it})-1}. \tag{21}$$

If W'_{it} and W_{it} are determined chromatographically at the same conditions, the ratio W'_{it}/W_{it} is given by the corresponding ratio of peak areas, A'_i/A_i, in the chromatograms, and equation (21) can be rewritten as

$$W_i = \frac{W_s}{(A'_i/A_i)-1}. \tag{22}$$

B. Single System Operation

One system containing the analyte is prepared, the process of stripping and trapping is carried out, and the amount of trapped component i, W_{it} is determined. Then a defined amount of component i, W_s, is added to the same system, and the whole procedure is repeated. In this variant, it is possible to express the amount of component i, W'_i, entrapped in the second run

$$W'_{it} = (W_i + W_s - W_{it})f. \tag{23}$$

Dividing equation (23) by equation (19) and solving for W_i (with $W'_{it}/W_{it}=A'_i/A_i$) yields

$$W_i = \frac{W_s - W_{it}}{(A'_i/A_i)-1}. \tag{24}$$

With the single-sample variant it is possible to work without any addition of standard; *viz.*, in order to determine the initial total amount of a given component in the system analyzed it is sufficient to know the amounts of this component that are trapped in two subsequent stripping and trapping runs carried out with one and the same system. Let us designate the amounts of component i trapped in the first and in the second run again by W_{it} and W'_{it}, respectively. W_{it} can be expressed in terms of equation (19) and W'_{it} can be expressed as

$$W'_{it} = (W_i - W_{it})f \tag{25}$$

By dividing equation (25) by equation (19) and solving for W_i we arrive at

$$W_i = \frac{W_{it}}{1-(A'_i/A_i)} \tag{26}$$

The variant without addition of standard is based on the same philosophy as the method of replicate equilibrations, described by McAuliffe [7]. Equation (26) could also be obtained readily from equation (24) by substituting $W_s=0$. The value of W_{it} can be determined by external calibration.

It follows from equations (11) and (18) (the presence of distribution constant K_{LG}) that with both alternatives one must control the temperature of the gas-liquid system; equations (22) and (24) are valid only if the temperature of the gas-liquid system in both runs is the same.

The concentration of the analyte in the bubbles leaving the surface of the liquid phase can differ somewhat from the true equilibrium concentration. The extent of this deviation depends on a number of experimental parameters, such as the size of the bubbles, their amount per unit volume of the liquid, height of the liquid layer being bubbled through, etc. In addition, spurious effects may occur in the system, such as the adsorption of the component on the inner walls of the system and the formation of concentration excesses at the phase interface, the extent of which depends on the size and shape of the system. Owing to the above nonidealities it is expedient that the stripping and trapping systems be both geometrically and physically congruent. In this way the consequences ensuing from the nonideality effects can largely be eliminated.

The effect of the temperature of the trapping column is different with each alternative. If all the conditions specified by equation (1) must be fulfilled, then in the case of Alternative I the temperature of the trapping column does not need to be controlled. However, in the case of Alternative II (the presence of constant K_{SG} in equation (18)) the control of the temperature of the trapping column is as essential as the control of the temperature of the gas-liquid system; however, these two temperatures need not necessarily be the same.

If the values of F and t in the analyses with and without the added standard are maintained constant, then the applicability of equations (22), (24), and (26) is not limited to any of the above alternatives; these equations apply to all stripped and trapped components regardless of the situation given by the state of the development of their zones in the trapping column. Unless F and t are kept the same in the runs with the original and the standardized systems, and/or if they are not controlled at all, equations (22), (24), and (26) apply merely to those components which become equilibrated during the stripping and trapping process (Alternative II). Finally, it is necessary to recall that all the considerations concerning the elimination of the system factor are valid only if it can be assumed that the addition of the standard does not alter substantially the values of K_{LG} of the components under determination; with Alternative II it is also necessary that the values of K_{SG} remain invariant. These requirements can be fulfilled if the amounts of the components and, consequently, the amounts of the standards added are small.

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ERROR ESTIMATES IN TOTAL ORGANIC ELEMENTAL CONTENT PASSIVE PERSONAL SAMPLERS

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The development of specific passive personal samplers for each volatile organic form of an element will become more complex, ultimately leading to a common monitor which can detect any or all organic forms of that element. Such a device is introduced here as a total organic elemental content passive personal sampler. The goal of this paper is to show how to compute and limit the ambient concentration error attributed to molecular diffusion approximations for total organic elemental content passive samplers. Some of the implications of data collected by this approach are discussed. For example, calculations of the anticipated error in determining total organic chlorine levels (TOCl) at a vinyl chloride plant in known workplace situations indicated the maximum error between monitor and ambient concentration values due to diffusion assumptions would be less than 11%. The total organic elemental content sampler error depends upon the value of the weighted diffusion coefficient used to convert the mass of element collected to ambient concentration. The error is calculated from the following equation derived for n species:

$$C_r^{\%} = \left[\frac{\sum_{j=1}^{n} D_i x_i C_i}{W_\sigma D_j \left[\sum_{i=1}^{n} x_i C_i \right]} - 1 \right] 10^2$$

where $C_r^{\%}$ = error in ambient total organic elemental content, D_i = diffusion coefficient of the i form of the element, x_i = number of equivalents per molecule for the i species, C_i = ambient total organic elemental concentration of the i form of an element, W_σ = weighting factor of the occupational situation on the diffusion coefficient of the j species of the element, and D_j = diffusion coefficient of the j species. Unfortunately, the value of the weighted diffusion coefficient tends to be nonideal in occupational exposure situations, because the relative concentrations of the different organic volatile forms of an element diffusing into the sampler are variable. Since it is not possible to calculate an exact weighted diffusion coefficient, an error will exist between sampler and ambient concentration values. These calculations are examples of what could be accomplished with total elemental content passive samplers. The methods of approach should also be applicable to other occupational situations where there is a need for more comprehensive exposure data. It is hoped that this paper will stimulate development of this novel concept in personal sampling.

Key words: Ambient concentration; compute and limit; error; sampler response factors; total elemental content passive personal samplers; total organic chlorine; weighted diffusion coefficient.

I. Introduction

The concept of threshold limiting values requires the measurement of time-weighted-average (TWA) concentrations in workplace atmospheres. A passive personal sampler used in a manner analogous to a radiation safety badge offers a simple, accurate means to measure TWA concentrations because the device can sample the breathing zone and requires no air pumps. The air contaminant being sampled enters the sampler via molecular diffusion and is fixed/immobilized by a collection element. Assay of the collection element gives the mass of material collected. This is converted to ambient concentration by utilizing the sampler geometry factors, exposure time, and molecular diffusion coefficient.

An alternative approach to measure polypollutants in the workplace environment is to develop a passive sampler which can sense any or all forms of an element in a summation mode and revert to species-specific detection only as necessary. In this approach, pollutants are grouped into classes of compounds containing a common elemental precursor. The example developed in this paper is that of a total organic chlorine (TOCl) personal sampler at a vinyl chloride plant. The rate limiting step in ambient air sampling via the TOCl passive sampler is diffusion within the device. To determine the TOCl ambient burden it is necessary to compute a weighted diffusion coefficient representative of the organic chlorine species entering the monitor. The inexactness in the diffusion calculation introduces an error in the resultant TOCl value. In this paper, generalized equations and methods are developed to compute and limit the error, and predict the sampler response in important occupational situations. The vinyl chloride plant calculations illustrate the concept.

II. Derivations

Figure 1 is a simplified schematic of the monitor, which consists of a porous draft shield, diffusion path, and collection element. The purpose of the draft shield is to eliminate convective air flow inside the device. The rate limiting step is diffusion across the static path length to the collection element. Fick's first law [1] can be integrated over n species, $\sum_{i=1}^{n}$, to describe the transport of a volatile element and its compounds within the stagnant air layer at isothermal and

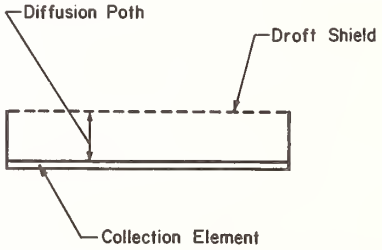

Figure 1. Simplified passive sampler schematic.

isobaric conditions, assuming 100% collection efficiency. Let the jth form of the volatile element entering the monitor be of interest. Assume that the number of elemental equivalents of the jth form (x_j) is the same for all forms. The equation obtained is:

$$C_\tau = M_n\lambda/tAx_jD_\sigma = M_n\lambda/tAD_\sigma = M_n\lambda/tAW_\sigma D_j \quad (1)$$

where

$D_\sigma = W_\sigma D_j$

C_τ = total elemental concentration obtained via the passive sampler (mol/cm^3)

M_n = mols of the element of interest obtained by assay of the collection element

λ = diffusion path length (cm)

t = exposure time (s)

A = diffusion path cross-sectional area (cm^2)

D_σ = weighted diffusion coefficient for an occupational situation expressed in terms of the j component (cm^2/s)

D_j = diffusion coefficient of the j species (cm^2/s)

W_σ = weighting factor of the environmental situation on D_j (dimensionless).

The numerical value of the x_j term in the denominator of eq. (1) is a constant, always equal to one. A weighting factor of $W_\sigma = 1$ implies that the diffusion coefficient of the j species is sufficient to represent that of the occupational system. Value of $W_\sigma \cong 1$ will normally be encountered for polypollutants diffusing into the sampler.

The percent error in C_τ, relative to the true total elemental ambient concentration is given by

$$C_\tau^{\%} = \left[\frac{\sum_{j=1}^{n} D_i x_i C_i}{W_\sigma D_j \left[\sum_{i=1}^{n} x_i C_i \right]} - 1 \right] 10^2 \tag{2}$$

Optimization of W_σ will minimize $C_\tau^{\%}$ resulting from mixed diffusion effects. Two methods of approach have been developed to solve for W_σ and yield limiting [theoretical and refined (operational)] values.

Limit operations on the C_τ error function in eq. (2) give lower, median, and upper limit theoretical values for the weighting factor and environmental system diffusion coefficient:

$\lim_{i \to \infty} \sum_{i=1}$	$\frac{W_\sigma}{D_l/D_j}$	$\frac{D_\sigma (= W_\sigma D_j)}{D_l}$
$l=i=n$	$(D_l+D_n)/2D_j$	$(D_l+D_n)/2$
$i=n$	D_n/D_j	D_n

The notation $l=i=n$ implies the limit operations with $C_l = C_n$ as both approach infinity; $i=1$ refers to the species of lowest molecular weight whereas $i=n$ represents the species of highest molecular weight. For the $l=i=n$ median limit operations, the range of maximum error in C_τ is given by

$$C_\tau^{\%R} = \left[\frac{2(D_l - D_n)}{D_l + D_n} \right] 10^2 \tag{3}$$

and the error distributed about C_τ is $\pm C_\tau^{\%R}/2$.

The operational or refined value for W_σ can be found by setting $C_\tau^{\%} = 0$ in eq. (2) and solving for W_σ. The equation obtained is coded for simplicity so that

$$W_\sigma = \alpha/\beta \tag{4}$$

where

$$\alpha = \sum_{i=1}^{n} D_i x_i C_i \tag{4}$$

and

$$\beta = \sum_{i=1}^{n} x_i C_i$$

Note that term in the larger brackets in eq. (2) is dimensionless; the calculations are greatly simplified by normalizing all D_i and C_i values relative to the j species.

The response of a total elemental content passive personal sampler to any occupational situation (C_τ^{OS}) can be expressed relative to a referenced situation (e.g., the B situation):

$$C_\tau^{OS}/C_\tau^{B} = \alpha_x/\alpha_B \tag{5}$$

where $\alpha_X = \alpha$ value for the "test" occupational situation and $\alpha_B = \alpha$ value for the comparison B situation.

III. Total Organic Chlorine Passive Personal Sampler For Vinyl Chloride Plant

A suggested protocol for computing W_σ and $C_\tau^{\%}$ applicable to TOCl and other total elemental content passive samplers is as follows: obtain historical data (ambient species and concentrations) for intended workplace environment; identify important job activities or occupational situations; choose one species to represent the j component and normalize relative to j; compute the maximum error range given by $C_\tau^{\%R}$ and the error distribution, $\pm C_\tau^{\%R}/2$, compute projected operational W_σ, $C_\tau^{\%}$, and $C_\tau^{\%R}/2$ limits; and compute ("test") the sampler response to the important occupational situations.

Table 1 summarizes historical parameters and important exposure situation data. The data were obtained via collaboration with EPA [2] and the B.F. Goodrich Company [3]. Diffusion coefficients were computed from Graham's law. The j component is represented by vinyl chloride (VC). Currently, VC is the only organic chlorine species regulated in the workplace environment at vinyl chloride plants. With regard to exposure situations A and C, it is assumed that transient "burst" in organochlorine species might occur as a result of opening a pipe, etc.: for A, 5-min duration at 100X the VC level in B, and for C, 5-min duration at 90X the VC level in B.

TABLE 1. *Historical and parameter data for vinyl chloride plant environment*

Species	x	MW	$D \cong \sqrt{1/MW}$ (cm^2/s)	D, Normalized	C, Normalized
Vinyl chloride	1	62.5	0.126	1.000	1.00
1,1-Dichloroethylene	2	97.0	0.102	0.810	<0.01
1,2-Dichloroethylene	2	97.0	0.102	0.810	<0.01
1,1-Dichloroethane	2	99.0	0.101	0.802	<0.01
1,2-Dichloroethane	2	99.0	0.101	0.802	<0.03

Code	Occupational Exposure
A	Vinyl chloride only (i.e., tank car loaders, maintenance workers)
B	Overall plant (i.e., plant operators, maintenance workers)
C	EDC only (i.e., tank/line maintenance)

The computed maximum error range for $C_{TOCl}^{\%R}$ is 22% and $\pm C_{TOCl}^{\%R}/2 = \pm 11\%$. A breakdown of $C_{TOCl}^{\%}$ obtained with the lower, median and upper W_σ values is shown in Table 2. The projected operational $C_{TOCl}^{\%}$ limit with a refined W_σ value of 0.929 is only $\pm 6.5\%$, as shown in Table 3.

TABLE 2. *Vinyl chloride plant dependence of $C^{\%}_{TOCl}$ on limiting occupational situations*

Limiting exposure situation	Exposure time (min)	α	β	α/β	$C^{\%}_{TOCl}/W_\sigma$ (a)	(b)	(c)
A	5	1.042	1.042	1.000	0.0	+11.0	+25.0
(VC only)					1.000	0.901	0.802
B	470	1.074	1.098	0.978	-2.0	+8.5	+22.0
(Overall plant)					1.000	0.901	0.802
C	5	1.504	1.875	0.802	-20.0	-11.0	0.0
(EDC)					1.000	0.901	0.802
A·B·C (5:470:5)	480	3.620	4.015	0.902	-10.0	0.0	+12.5
					1.000	0.901	0.802

(a) Lower $W_\sigma = D_{VC}/D_{VC}$
(b) Median $W_\sigma = (D_{VC}+D_{EDC})/2D_{VC}$
(c) Upper $W_\sigma = D_{EDC}/D_{VC}$

TABLE 3. *Vinyl chloride plant predicted operational $C^{\%}_{TOCl}$ limits*

Operational exposure situation	Exposure time (min)	α	β	α/β	$C^{\%}_{TOCl}$ ($W_\sigma = 0.929$)
AB	(1:95) 5-A 475-B	2.127	2.152	0.988	+6.5
B	480-B	1.097	1.121	0.979	5.5
BC	(95:1) 475-B 5-C	2.589	2.985	0.867	-6.5
AB·B·BC	(1:1:1) (1:95)-AB (95:1)-BC 480	1.938	2.086	0.929	0.00

The final equation for calculating TOCl via the total organic chlorine passive sampler is

$$C_{TOCl} = M_{TOCl}\ \lambda/tA\ \ (0.929)D_{VC} \tag{6}$$

In eq. (6), DVC is the actual value, in contrast to a value of unity for the error calculations.

The TOCl sampler response to the other known occupational situations at a vinyl chloride plant relative to the B exposure is

$$C^{BC}_{TOCl}/C^{AB}_{TOCl}/C^{B}_{TOCl} = 2.36/1.94/1.00$$

Note that the passive sampler response factors to the BC and AB 5-min "burst" situations are significantly greater than the 8-hr B response.

IV. Conclusions

Equations were derived and a protocol suggested for computing the ambient concentration error attributed to molecular diffusion approximations for total elemental content passive personal samplers. An example error calculation was described for a total organic chlorine passive sampler at a vinyl chloride plant. Sampler sensitivity in known occupational situations may be useful in correlation studies. It is likely that all organic chlorines should be of concern in the current search for carcinogenesis correlation, though vinyl chloride is the only one now regulated as a carcinogen.

After exposure, all samplers could be rapidly screened for TOCl, without resolving the individual organic chlorines contributing to the assay value. Only when higher than acceptable levels are observed would it be necessary to determine the individual organic chlorines contributing to the TOCl burden. This dual summation and species-specific detection mode might be incorporated into the TOCl sampler by using two different collection elements and/or assay techniques. Total organic elemental content passive samplers need not be restricted to TOCl, but could include total organic bromine, total aromatic amines, etc.

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CONCERTED TRACE ORGANIC ANALYSIS IN INDUSTRIAL RESEARCH

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The three primary analytical fields utilized in the conduct of trace (submicrogram) organic studies are spectroscopy, chromatography, and thermal analysis. It is within each of these specialities, developed with state-of-the-art computer capabilities, that the field has advanced to its present level. Complex problem-solving is seldom accomplished with a single analytical tool, even with the sophisticated instrumentation available to most researchers. Thus, the interaction of the organic analysts with organic and physical chemists, chemical and instrument engineers, and computer specialists provides a wide range of cooperative efforts in basic and applied industrial chemical research.

Illustrations in polymer characterization and degradation, as well as trace organic detection and identification in pollution-related fields, represent approaches that use significant technology transfer between instrumentation manufacturers and industrial research teams. A trace organic analytical system will be discussed which was cooperatively developed to provide information in difficult areas of polymer characterization and pollution analysis.

The modular unit incorporates a variety of sample injection modes (gas, liquid, solids, pyrolyzer) with an on-line concentrator trap, and an analytical separation column with stop-flow valving to direct effluent peaks into an on-line functional group or elemental reactor system. The simultaneous determination of C,H,N,O and certain heteroatoms is achieved by microchemical conversions to diagnostic products which are separated on a second internal chromatographic column to aid in peak identification. Outline of the equipment design and engineering will be presented and its adaptation to diverse applications in trace organic analysis.

Key words: Fourier transform infrared; gas chromatography; modular organic analysis system; pyrolysis; trace organic analysis.

I. Introduction

Within the past few years the need to develop a concerted approach to trace organic analysis was evident in two major areas of research and development: (1) polymer characterization and degradation and (2) air and water pollution analysis. We shall focus upon these areas of research in the discussion of the development of a modular organic analysis system that emphasizes gas chromatography and microchemistry, with concentrator/trap and stopflow capabilities. The diversity of sample types and problem orientations within the industrial research effort necessitated such a concerted, cost-effective instrumental capability.

Illustration of this modular system, manufactured by the Chemical Data Systems after cooperative development over several years, will be given in six functional categories:

(1) Use of a GC #1 separation column combined by appropriate stopflow valving with a GC #2 "small molecule" separation column.

(2) Use of programmed pyrolysis accessory and GC #1 separation column for "time-resolved" pyrolysis GC studies.

(3) Use of on-line concentrator/trap for pyrolysis-GC and air or water pollution studies.

(4) GC #1 effluent identification using stopflow and functional group (noncatalytic) pyrolysis for individual peaks.

(5) GC #1 effluent identification using stopflow and on-line simultaneous CHNO elemental analysis by microchemical (catalytic) conversions.

(6) Direct introduction (syringe, pyrolysis) or thermally-desorbed volatiles from remote sampling tubes identified by Fourier transform infrared spectroscopy (FT-IR) with or without prior GC separation.

II. Discussion

(1) Since the Analyzer has incorporated two independent column ovens, each with its own heating and cooling controllers (Fig. 1), one may utilize them in tandem to provide retention information. Injection into GC #1 separation column from syringe, pyrolysis, etc., allows a normal separation to be conducted over a full programmed temperature range of 80° to 350 °C. Generally, there is a trade-off in resolution within portions of the chromatogram; therefore, it is advantageous to rechromatograph on a second separation column any peaks that develop. The stopflow arrangement allows this to be performed by a simple valve change. In the illustration shown as Figure 2, the GC #1 column, SE-30, was temperature-programmed to elute high retention time components in a reasonable time, but is provided poor separation for the earlier components. Hence, as the "composite" peak emerged from GC #1, the stopflow valve was turned and subsequent transfer and analysis took place on the Porapak GC #2 column. The stopflow valve was then returned to the original position and completion of the GC #1 separation took place.

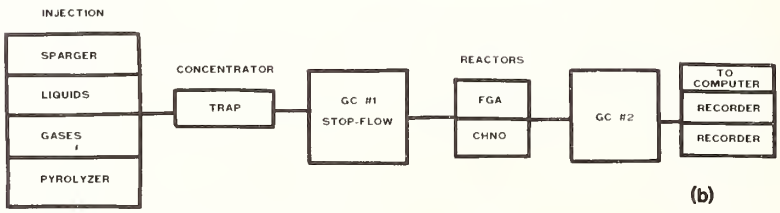


Figure 1. Pyrochrom™ Elemental Reaction Analyzer—a modular organic analysis system.

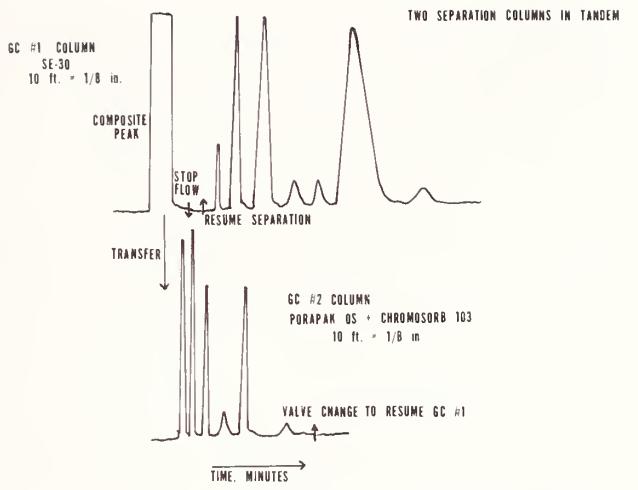


Figure 2. Complex mixture separation using two analytical columns in tandem and stopflow valving.

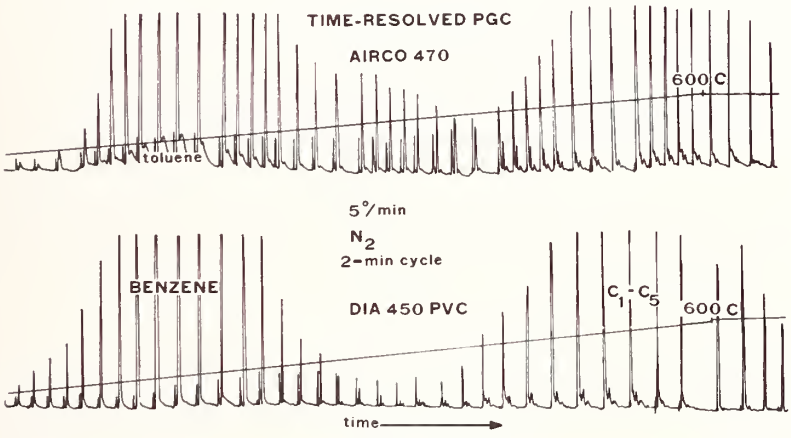


Figure 3. Programmed pyrolysis and time-resolved pyrolysis-GC analysis.

(2) Controlled degradation has been shown to provide details of polymer microstructure in characterization studies [1]. Using a programmable pyrolyzer to heat about a milligram of PVC powder at 2 °C/min and valving the volatiles into an appropriate separation column gave the results seen in Figure 3 to demonstrate a "time-resolved" pyrolysis GC application [2]. The column was chosen to specifically separate only the desired benzene, toluene, and C_1–C_5 volatiles in a 2-minute repetitive cycle. In this manner, the generation and decay "envelopes" of these degradation products were monitored throughout the pyrolysis. The temperatures of maximum production of benzene, toluene, and chain-scission products are thus directly measured. Such time/temperature-resolved GC studies permit delineation of degradation kinetics in polymer analysis, as well as detailed influences in organic mechanism studies.

(3) One of the most significant advances in the concerted trace analysis approach has centered upon the on-line concentrator/trap capability within the Analyzer. Either direct sparging onto the concentrator filled with an appropriate adsorbent or remote sampling with an OSHA-type charcoal sampling tube may be handled in a cost-effective manner. Sampling tubes taken for personnel or area monitoring may be inserted directly into the Analyzer interface and thermally-desorbed onto the GC #1 column. Alternatively, a pyrolyzer or a sparger may be utilized to load

the concentrator/trap directly within the Analyzer. These applications are illustrated in Figures 4–6. Tenax or Chromosorb 102 adsorbents have been used successfully for a variety of problem-solving air or water pollution analyses. A new and promising adsorbent, "Ambersorb" (Rohm & Haas), is currently being evaluated.

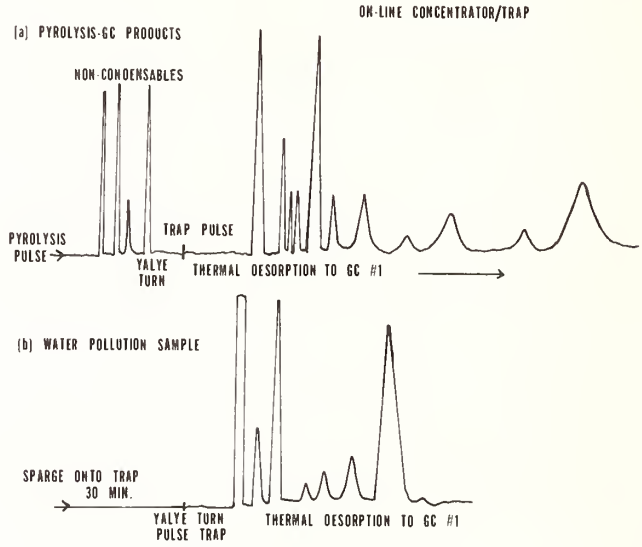


Figure 4. On-line concentrator/trap used with complex mixtures and thermal desorption into GC #1. (a) Pyrolysis products; (b) Sparger used with water pollution sample.

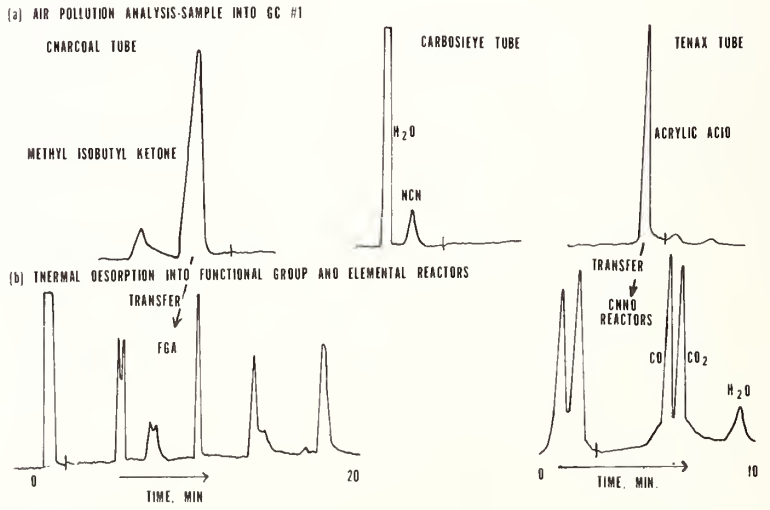


Figure 5. On-line concentrator/trap using remote sampling tube and programmed interface module. (a) Thermal desorption into GC #1; (b) Thermal desorption into functional group and elemental reactors.

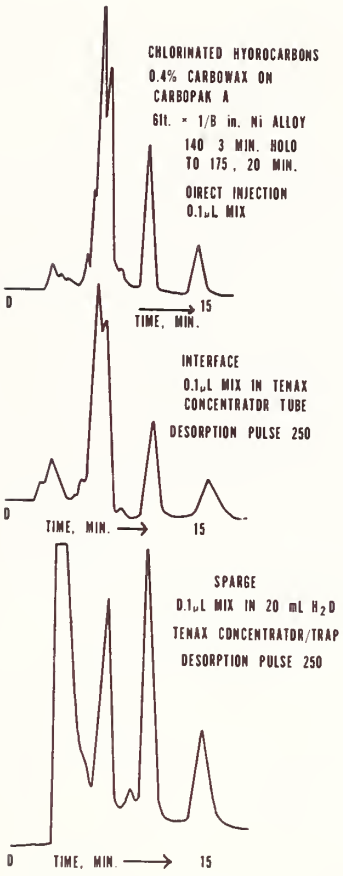


Figure 6. Comparison of injection modes.

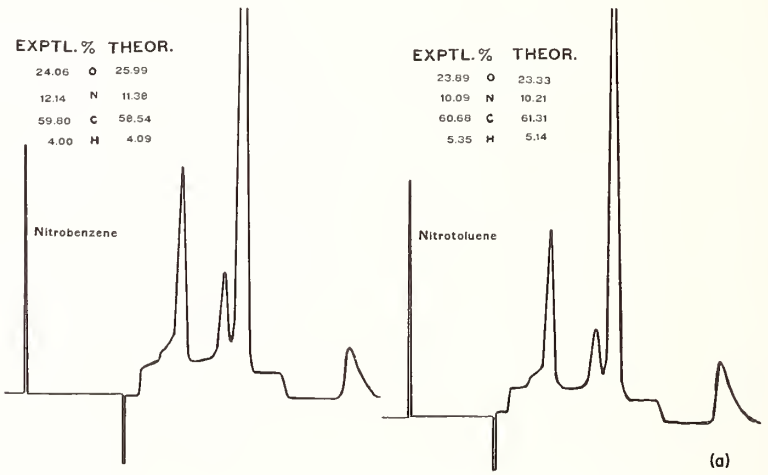
(4) The features discussed thus far have been concerned with diverse sample introduction and manipulation for pyrolysis, sparging, concentration and trapping, thermal desorption, and multiple-column utilization. However, a further stage of analysis is generally necessary to complete the characterization: chemical or spectroscopic identification of the GC #1 column effluents. The functional-group (noncatalytic) analysis capability and the elemental (catalytic) analysis capability of the Analyzer have been demonstrated over the past few years in many laboratories [3]. Fragmentation patterns on the "small molecule" functional group separation column (GC #2) provide differentiation of isomeric and chemical components.

The effluent from GC #1 (or from any direct injection mode) is valved into the noncatalytic reactor held at 650 °C to provide diagnostic fragment ratios of CO, CO_2, H_2O, C_1–C_5 hydrocarbons, as well as possible HCl, HCN, and H_2S from the respective heteroatom-containing effluents. Applications of the noncatalytic reactor with added Pt fillings, operated at 900 °C under hydrogen, have been made for the reductive pyrolysis mode. This is used for analysis of highly-resistant halogenated materials or heterocyclic sulfur compounds.

(5) The microchemical conversions conducted in the catalytic reactors allow the simultaneous elemental analysis of C,H,N,O to be performed on any or all of the effluent peaks to supplement the above functional group information. This analyzer is the only commercially available system that provides simultaneous CHNO data and thereby represents a unique instrumental tool for trace organic analysis [4]. During the development of the system, certain compromises were made in order to maximize the efficiency of the analysis and availability of chemically significant information. Thus, the absolute precision of the full CHNO determination

may fall below the classical 0.1% level used by analysts for standard CHN determinations performed on milligram-size samples. However, the ability to provide better than 1 to 2% precision on samples in the submicrogram range for the full simultaneous CHNO determination more than offsets this lower accuracy (Fig. 7).

(6) Finally, for the most demanding combination of trace organic studies, the above data from GC retention behavior, and functional group and elemental microchemical modes may still be insufficient to identify a component in a mixture. The time-proven advantage of infrared spectroscopy in identification of chemical structures has recently been extended to the field of trace organics by means of commercially available Fourier transform IR units. These systems may be equipped with GC accessories containing light pipes designed to provide spectral detection of GC effluents in the nanogram range (Fig. 8a) [5]. Again, special valving allows on-line analysis or trapped-mode studies of volatiles, whereby excellent signal-to-noise advantages may be obtained by co-adding multiple scans. Figures 8b and 9 illustrate the combustion studies of a composite material undergoing controlled degradation in air with the volatiles being swept directly into a Digilab FTS spectrometer system equipped with a GC accessory [6]. Simultaneous detection and identification of many gases in the complex mixture were recorded throughout the degradation by the rapid-scan FT-IR capability and co-adding of spectra at 2-minute intervals. The infrared



MOLECULAR FORMULAE PROGRAM

Nitrobenzene
$C_6H_5NO_2$

C	H	N	O	
58.54	4.09	11.38	25.99	Theoretical %
59.80	4.00	12.10	24.10	Exptl. %
2.00	2.00	2.00	2.00	Exptl. Error

C_6	H_5	N_1	O_2	X_0	Mol. Wt.
-1.26	0.07	-0.72	1.92		123.
C_7	H_7	N_1	O_2		
1.51	1.11	-1.88	-0.74		137.
C_{10}	H_4	N_2	O_3		
0.20	-2.00	1.90	-0.10		200.

Search Complete Through C 10

MOLECULAR FORMULAE PROGRAM

Nitrobenzene

C	H	N	O	
59.80	4.00	12.10	24.10	Exptl. %
2.00	1.00	2.00	2.00	Exptl. Error

C_6	H_5	N_1	O_2	X_0	Mol. Wt.
-1.26	0.07	-0.72	1.92		123.

Search Complete Through C 10

(b)

Figure 7. (a) On-line CHNO analysis for nitrobenzene and nitrotoluene; (b) Typical output from molecular formula search program for experimental error 1–2%.

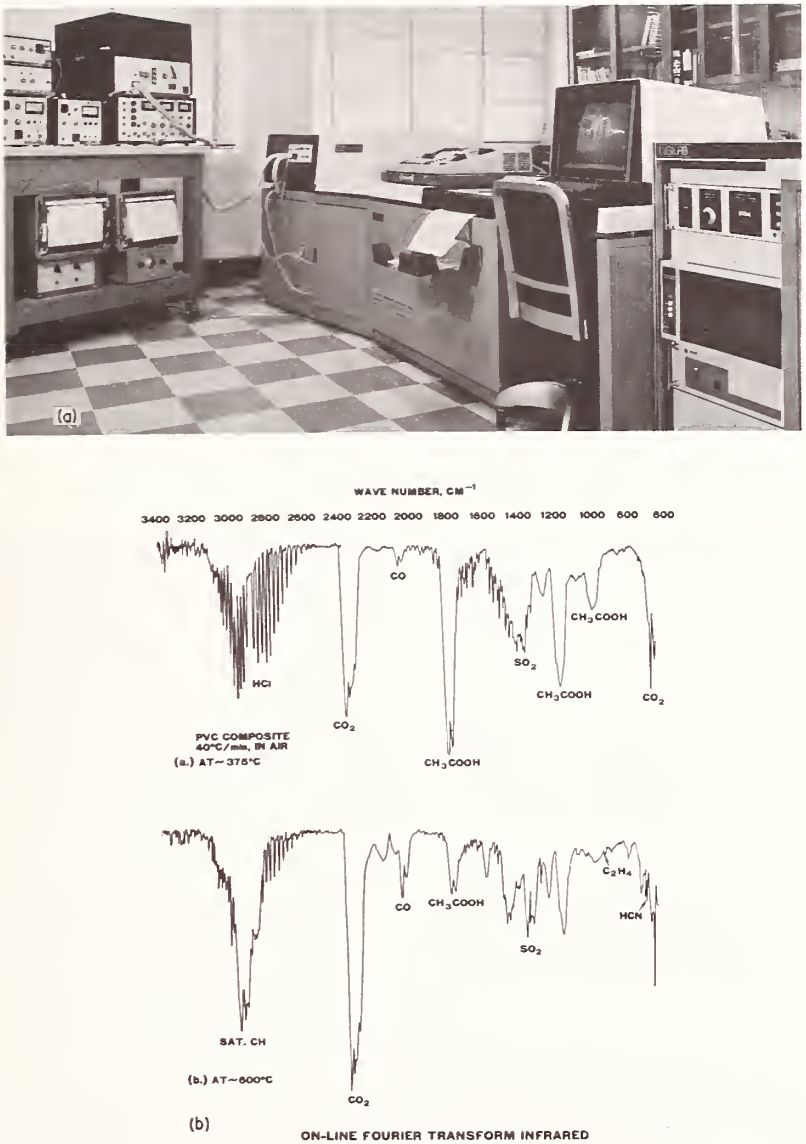


Figure 8. GC-IR analysis of combustion gases from PVC composite with programmed heating.

interpretation is reinforced by known patterns and spectral correlations with chemical structures established by experienced analysts over several decades for upwards of a quarter of a million organic substances. The ability to obtain this indispensible spectroscopic data for trace levels now is combined with the chromatographic and microchemical data discussed above.

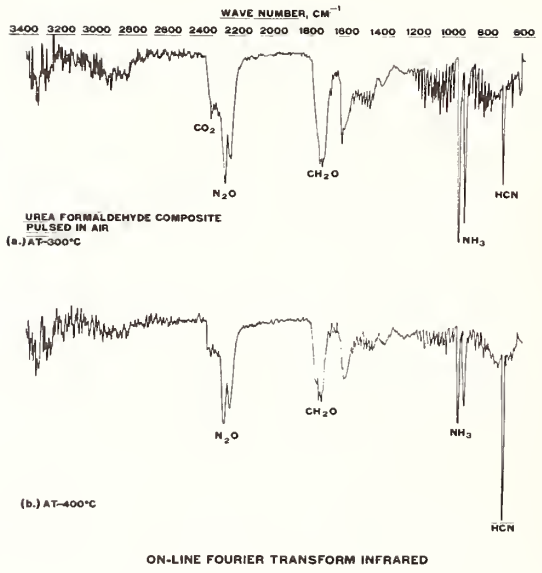


Figure 9. GC-IR analysis of combustion gases from urea-formaldehyde composite with programmed heating.

III. Summary

In conclusion, we have demonstrated the capability to perform trace organic analysis on a wide variety of materials in several problem-solving situations common to industrial research and development efforts. The modular unit developed to provide handling, detection, and identification of organics in solids, liquids, and gases has emphasized case and versatility. Frequently, the qualitative and quantitative analysis of organics involve the interplay of several data bases for a given sample. The three key sources of information developed in this approach have focused upon gas/liquid chromatography, microchemistry, and IR spectroscopy. In this manner, structurally significant data on trace levels of organic substances have been developed with realistic interpretative skills and minimal instrumental expertise.

IV. Acknowledgment

The invaluable contributions to the design and development of the system by Dr. D. H. Ahlstrom, Armstrong Cork Company, and W. Bell, Chemical Data Systems, are gratefully acknowledged.

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DIFFUSION TUBES AS A PRIMARY STANDARD FOR OSHA-TYPE CALIBRATION

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The technique of diffusion tubes is presented as a means of generating dynamically a primary standard of a material in a flowing air stream that can be used for calibration. The entire technique of diffusion tubes is discussed including both the theoretical and empirical data obtained from typical diffusion tubes. An instrument is described to incorporate diffusion tubes in the development of these dynamic standards. The use of dynamic standards generated from diffusion tubes is used for the calibration of charcoal tubes. This mode of calibrating charcoal tubes is compared with the recommended way of calibration.

Key words: Diffusion tubes; OSHA; primary standards.

I. Introduction

Dynamically generated gas standards have proven to be the best method for calibration of instruments in the measurement of various gas constituents at the ppm level. Permeation tubes, as developed by O'Keefe and Ortman [1], have found widespread use in the field of air pollution for the dynamic development of ppm level standards. The major advantages of dynamic standards are that adsorption problems are reduced to a minimum and the concentration developed can be varied by a simple flow variation. An additional advantage of devices, such as permeation tubes, is that they allow independent calibration by weight loss such that the standard developed dynamically can be of primary standard quality.

The permeation tube technique is not applicable to common solvents in OSHA-type analysis due to the higher boiling materials and, thus, lower vapor pressure. Permeation rates are extremely low. Standards are desired near the TLV levels, which may be 100 ppm or more. Permeation tubes, even at higher temperatures, have not been successful except for materials where the TLV is at the ppm level. Diffusion tubes can be used to generate dynamic standards for solvent vapors at the TLV levels. Diffusion tubes retain the gravimetrically calibrated feature and the concentration can be changed by flow changes.

II. Diffusion Tubes

A diffusion tube consists of a liquid-filled reservoir with a long neck of precision bore glass tubing. A typical diffusion tube is shown in the line drawing in Figure 1. Material is placed in the reservoir of the diffusion tube. Nominally, it contains approximately 3 to 4 mL of liquid. The diffusion tube is then held at a constant temperature. The material diffuses through the precision bore capillary neck of the diffusion tube at a constant rate following its thermal equilibration. The amount of material that diffuses per unit time is determined by weight loss on the diffusion tube itself. If, while the diffusion tube is being thermostated; a known flow of air passes across the diffusion tube, the concentration of the material in the air can be calculated. This then provides a known dynamic standard for that particular material in air.

Diffusion tubes can be used with different bore sizes. The length of the diffusion path can certainly be altered. Finally, the temperature of the diffusion tube can be changed. These variables

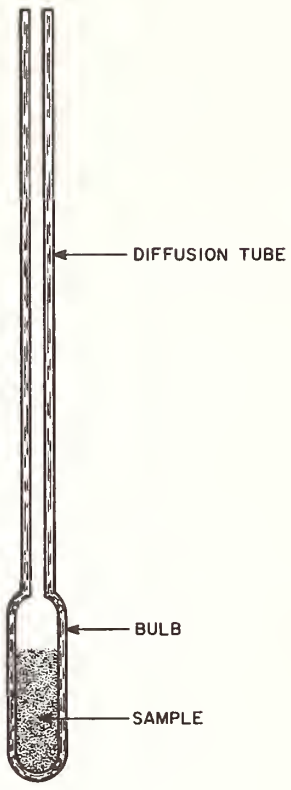

Figure 1. Line drawing of a diffusion tube. Reservoir capacity is approximately 4 mL and overall length is 12 cm maximum.

provide means of obtaining a wide range of concentrations. The fundamental equation used to calculate the concentration obtained in the flowing gas stream is given below.

$$C = \frac{R \times K}{F} \quad (1)$$

Where

C = Concentration in ppm (vol.).
R = Diffusion rate in ng/min.
F = Air flow in mL/min.
K = Reciprocal vapor density of material in nL/ng.

The diffusion rate, R, is measured by weight loss. The constant, K, that appears in the eq. (1) is dependent on the material in the diffusion tube. This converts the weight of the material diffusing per unit time to a gas volume of the material so that the final concentration appears as a volume-to-volume concentration. This K term is temperature and pressure dependent. The temperature and pressure used should be the same temperature and pressure at which F, the gas flow across the diffusion tube, is measured. This ensures that when the two volumes are ratioed for the ppm concentration, the volumes are at the same temperature and pressure. If the temperature of air flow measurement is 25 °C and 760 torr, K becomes 24:45 divided by the molecular weight of the material.

The basic theory of diffusion tubes is discussed by Altshuller and Cohen [2], especially as it relates to the dynamic generation of known standards. Their theoretical equation, in more useful units, is given below.

$$R = 2.216 \times 10^6 \frac{DMPA}{TL} \log\left(\frac{P}{P-p}\right) \tag{2}$$

Where

R = Diffusion rate in ng/min.
D = Diffusion coefficient at T & P in cm^2/s.
M = Molecular weight.
P = Total pressure in torr.
A = Cross sectional area of diffusion path in cm^2.
T = Temperature in °K.
L = Length of diffusion path in cm.
P = Vapor pressure of material at T in torr.

The diffusion coefficient is also temperature and pressure dependent as follows:

$$D = Do \left(\frac{T}{To}\right)^m \left(\frac{Po}{P}\right) \tag{3}$$

Where

Do = Diffusion coefficient at the standard conditions of To=273°K and Po=760 torr.
m = Constant—usually 2, but may be 1.75.

If eqs. (2) and (3) are combined and the values of To and Po are placed in the numerical constant, the following equation results.

$$R = 6.169 \times 10^6 \, DoM \left(\frac{A}{L}\right)\left(\frac{T}{To}\right)^{m-1} \log \left(\frac{P}{P-p}\right) \tag{4}$$

The importance of temperature control can be approximated by assuming an average of about 50% change of vapor pressure for a 10 °C temperature change, a reasonable average for organic materials. This will cause an approximate change in the log term of 50%. The 10 °C change will cause approximately a 3% change in the temperature term in the same direction. Overall then, a 1° temperature change causes about a 5% change in diffusion rate; or temperature should be controlled to within 0.2 °C to maintain 1% accuracy in the developed standard.

The geometry term indicates probably the best way to alter the concentration range with a specific material. With a given diffusion tube, the flow rate is then the best way to alter the concentration that is developed.

A typical example of the use of the above eq. (4) would be for toluene. The conditions are as follows:

Do = 0.0709 cm^2/s
M = 92.13
A = 0.0314 cm^2 (for 2.0 mm bore)
L = 6.8 cm
T = 303°K
P = 752 torr
P = 36.7 torr @ 30 °C.

Then:

R = 4490 ng/min.

The actual rate on a diffusion tube of the dimensions used on the calculations above, operating at those conditions; was 4550 ng/min determined by weight loss. The calculated and measured diffusion rates for toluene are listed in Table 1 along with corresponding data for a number of other common solvents. The error is the error of the calculated value relative to the measured value which is assumed correct. Most of the error will be due to the area used in the calculated value. The area was calculated assuming the diameter of the diffusion path to be the nominal diameter of the precision bore tubing. The length was measured to the nearest 0.1 cm. The rest of the error will probably be in the values used for the diffusion coefficients and for the temperature exponent, m. The important point to be gained from this information is simply that the most reliable and accurate method of obtaining the diffusion rate is by weight loss as opposed to relying on the theoretical calculated rates. The theoretical equation can be used to initially size a diffusion tube for an approximate value.

TABLE 1. *Diffusion rates at 30 °C for 2.0 mm tube i.d.*

Material	Diffusion length cm	Measured rate ng/min[a]	Calculated rate ng/min[b]	Error % rel.
Benzene	6.6	13,500	14,300	+ 6.2
Toluene	6.8	4,550	4,490	- 1.3
Ethyl benzene	6.8	1,650	1,710	+ 3.6
n-Hexane	6.3	24,620	22,400	- 9.0
n-Octane	7.0	2,160	1,917	-11.2
Methanol	6.8	15,100	13,700	- 9.1
Ethanol	7.0	6,950	6,840	- 1.4
Ethyl acetate	7.0	15,100	14,200	- 5.7
Propyl acetate	6.8	5,740	5,330	- 4.3
Methylene chloride	7.0	127,300	113,600	-10.7
Chloroform	6.3	45,600	52,200	+12.8
Carbon tetrachloride	6.3	29,200	33,000	+14.5
Trichloro ethane	7.0	27,600	26,400	- 4.3
Acetone	7.0	34,500	32,100	- 6.9

[a] Measured by weight loss over extended period of time.
[b] Calculated by diffusion equation.

III. Standards Generator

To properly use the diffusion tubes in the generation of dynamic standards, two important parameters must be considered. The temperature control of the diffusion tube must be such that, at the operating temperature of the diffusion tube, the temperature variation is no more than 0.1 °C. This ensures the diffusion rate of the tube to be within 1%. The second major parameter is that of the flow of gas. This must be measured accurately to ensure proper accuracy in the standard. A Calibration System designed to use, to the fullest extent, the technique of diffusion tubes is the AID Model 350.

The flow system of the Model 350 is illustrated in Figure 2. It is designed to provide the operator of the instrument maximum flexibility while providing flow control and readout with an accuracy of ±1%. Flow meters are provided for reading the chamber and the diluent flows. In general, it is best to maintain a low, constant flow through the chamber to pick up the material exiting the diffusion tube. This then can be diluted with a diluent flow following the chamber to provide the desired concentration. With proper manipulation of the flows available in the Model

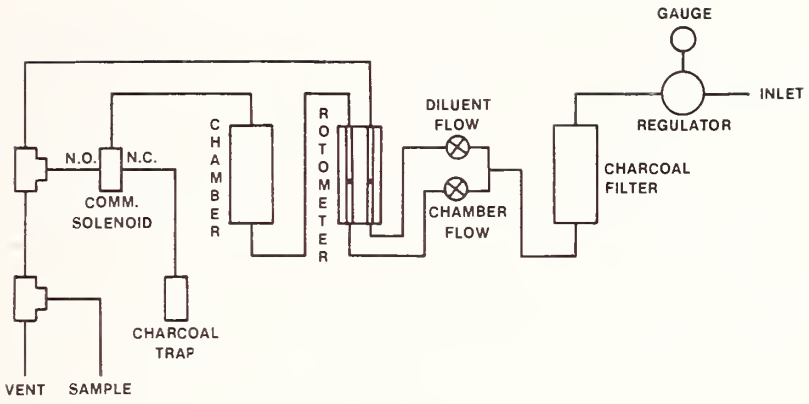

Figure 2. Flow schematic of the Model 350 Standards Generator.

Figure 3. The Model 350 Standards Generator showing the gas flowmeters and temperature readout.

350, a 10-fold change in output concentration can be obtained. The output flow can be varied between 150 mL/min and 1.5 L/min. Through the use of an internal solenoid valve, the operator can select the calibration stream or a zero air stream. In the case of zero air stream, the material coming from the chamber is vented through a charcoal filter trap. In use, a flow in excess of that required for the standard is used in the 350. The excess is vented out the vent port. The sample port provides the calibration gas available at atmospheric pressure, identical to the environment sampling that would be used.

The Model 350 uses a unique temperature readout system to ensure that the selected chamber temperature is well within 0.1 °C. This utilizes a thermistor sensor with proper amplification and presentation to a null-type meter. This system design provides the user with a thermal system and display with an accuracy of ± 0.025 °C at each of the selectable temperatures of 30°, 50°, and 70 °C. A 40 °C change in temperature, the maximum range of the Model 350, represents approximately a 5-fold increase in diffusion rate for diffusion tubes.

Figure 3 is a picture of the Model 350 showing specifically the front panel. Here the two rotometers for both the diluent and chamber flows can be seen as well as the temperature readout meter. As long as the meter is within the black center band of the meter movement, the

temperature is within 0.1 °C of the set temperature. Both the chamber and diluent flows are separately adjustable. Gas (generally air) is provided to the rear of the instrument at approximately 35 psig. It can be provided by house air lines or cylinder gas. It may even be provided by a small pump, external to the Model 350.

As can be seen in Figure 2, the inlet air passes through a charcoal filter within the instrument to clean the air, thus, ambient air can indeed be used to generate the standard. Figure 4 shows the instrument with the cover removed. The oven is seen on this side of the instrument. The chamber has been removed showing the thermistor within the chamber that is actually providing the temperature readout. The chamber can accommodate up to 4 different diffusion tubes. This allows a multiple standard to be generated such that interferences can indeed be checked on the sampling and analytical methods.

The Model 350, when used with diffusion tubes, is capable of generating dynamic standards for a large number of common organic solvents at the TLV levels for these materials. Adsorption and reaction problems of static standards are completely avoided. In addition, a large amount of standard material can be developed over an extended period of time. Concentration may be changed by a simple flow change.

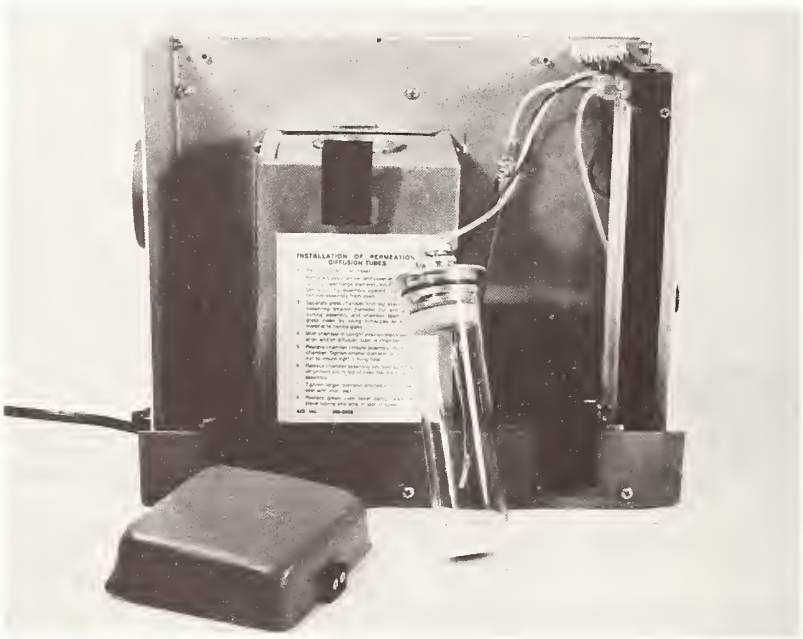

Figure 4. The Model 350 with the chamber assembly out of the oven. The small probe inside the glass chamber is the temperature readout sensor.

IV. Charcoal Tube Calibration

A very common technique in industrial hygiene for personnel sampling, is the integrated sampling technique known as charcoal tubes. These charcoal tubes are small glass tubes approximately 5 cm in length $\times$ 0.6 cm in diameter. They are packed with two separate sections of 100 mg and 50 mg of activated charcoal. In use, the sealed tips on both ends of the tube are broken open. The air sample to be analyzed is pulled through the tube at a known flow rate such that it encounters the 100 mg section of carbon first and then through the 50 mg. The air sample is pulled through these tubes for a fixed period of time at this constant flow. The charcoal adsorbs the organic materials of interest in the sample. Following the sampling, the glass tube is broken and each of the two separate charcoal sections are dumped into small vials. The organic materials that are adsorbed on the charcoal are desorbed from the charcoal generally using carbon disulfide.

The desorbing liquid is then analyzed by gas chromatography for the organic materials of interest.

For quantitation, the amount of desorbing liquid has to be known accurately as does the sample that is injected into the gas chromatograph. One of the disadvantages of charcoal tubes is the fact that the desorption of the material is not quantitative. Efficiencies vary widely depending on the particular chemical that is being desorbed. It can indeed vary from batch to batch for the activated charcoal used to pack the tubes. For this reason, it is absolutely necessary to calibrate the charcoal tubes with known standards. This generally must be done for each individual batch of charcoal tubes. Determining the desorption efficiencies of the charcoal tubes and the calibration of the gas chromatograph itself, can be combined in one step. Known samples are pulled through a charcoal tube at a known flow for a given period of time. These samples are desorbed and the desorbing liquid injected into the chromatograph. The size of the peaks for the organic vapors and the standard samples then determine the calibration not only for the gas chromatograph, but for the entire sampling system as well.

The Model 350, when used with diffusion tubes, can provide ideally the standard needed to calibrate the sampling system and the gas chromatograph. In addition, the desorption efficiencies of the charcoal tubes can be determined independently as well. To illustrate this, three diffusion tubes were placed in the Model 350. These tubes contained benzene, ethyl acetate and methyl isobutyl ketone. A standard was then generated containing each of these materials at known concentrations. The diffusion tubes were calibrated by weight loss. The standard so generated was pulled through charcoal tubes at known flow rates of approximately 50 mL/min for approximately 60 minutes. The actual amount of each of the three materials adsorbed on the charcoal tube was calculated from the diffusion rate of each diffusion tube and the known time and flow rates. The Gas Chromatograph was standardized using standards of each of the three materials prepared in carbon disulfide. The data from these tests are given in Tables 2, 3, and 4. The desorption efficiencies determined for benzene appear close to the average published desorption efficiency, however, for ethyl acetate and for the methyl isobutyl ketone; the desorption efficiencies are higher in general than the published desorption efficiencies. In many cases, the published desorption efficiencies have been determined by loading small quantities of liquid directly into the charcoal tube. The tube is then allowed to sit for a period of time and is desorbed with carbon

TABLE 2. *Charcoal tube desorption studies*

Benzene

Recommended tube loading
Published desorption efficiency 96%
Tube flow rate 50 mL/min for total time 60 minutes

Actual loading by diffusion tube mg	Concentration ppm (vol)	Amount desorbed mg	Desorption efficiency %
0.123	14.7	0.112	91
0.0398	4.1	0.0374	94
0.0234	2.2	0.0204	87

Liquid loaded into tube with syringe

Actual loading mg	Amount desorbed mg	Desorption efficiency %
0.876	0.799	91
0.438	0.413	94
0.088	0.079	90

TABLE 3. *Charcoal tube desorption studies*

Methyl isobutyl ketone

Recommended maximum tube loading	10 mg
Published desorption efficiency	81 ±5%
Tube flow rate 50 mL/min for total time	60 minutes

Actual loading by diffusion tube mg	Concentration ppm (vol)	Amount desorbed mg	Desorption efficiency %
1.24	114	1.18	95
0.401	32	0.367	92
0.236	17	0.183	77

Liquid loaded into tube with syringe

Actual loading mg	Amount desorbed mg	Desorption efficiency %
1.21	0.986	82
0.603	0.475	79
0.121	0.086	71

TABLE 4. *Charcoal tube desorption studies*

Ethyl acetate

Recommended maximum tube loading	12.5 mg
Published desorption efficiency	89 ±5%
Tube flow rate 50 mL/min for total time	60 minutes

Actual loading by diffusion tube mg	Concentration ppm (vol)	Amount desorbed mg	Desorption efficiency %
4.41	467	4.15	94
1.43	131	1.41	98
0.841	109	0.790	94

Liquid loaded into tube with syringe

Actual loading mg	Amount desorbed mg	Desorption efficiency %
4.47	3.89	87
2.24	1.97	88
0.447	0.371	83

disulfide in the usual fashion. For comparison, this type of tube loading was done for each of the three materials as well. Again, in the case of benzene, there is good agreement, however; in the case of the ethyl acetate and the methyl isobutyl ketone, desorption efficiencies are less and are close to what has been published in the literature. It would appear that there is a significant variation between the methods of determining the desorption efficiencies of charcoal tubes and that the dynamic standard approach is by far preferable simply because this is the manner in which the charcoal tubes will be used to sample the work environment. At least in our limited experiments, it would appear that there is no interference of the materials on each other as far as the desorption efficiency is concerned. Virtually the same desorption efficiencies were obtained for each of the materials separately.

V. Conclusion

The old technique of diffusion tubes when coupled with state of the art instrumentation, can indeed be used to provide badly needed dynamic standards of a wide variety of organic chemicals in air at basically the TLV levels. Variation of flow rates can provide variation in concentrations. The diffusion tubes themselves can be standardized gravimetrically, providing effectively primary standard quality. Multiple standards can be prepared by using more than one diffusion tube in the same flowing system. In addition to the use of dynamic standards prepared from diffusion tubes for the calibration of charcoal tubes; other forms of sampling, such as sample bags, evacuated cylinders, and midget impingers can also be checked and sample integrity verified by the use of dynamically generated standards from diffusion tubes.

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METHODS FOR THE ORGANIC ANALYSIS OF THE MIGHEI; A REPUTEDLY CONTAMINATED METEORITE

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Of the various classes of meteorites, the Type I and II carbonaceous chrondrites are the least metamorphosed and contain the largest fraction of extractable organic compounds. Since recently fallen Type I and II carbonaceous chondrites are rare, occurring at ~5–10 year intervals, it is desirable to be able to use older fallen meteorites to add to the specimens available for analysis. However the older specimens, usually preserved in museum collections, were considered contaminated and unsuitable for organic analysis.

A sampling technique was developed and applied to a fragment of the supposedly contaminated Mighei Type II carbonaceous chondrite which fell in Russia in 1889. Using GC and GC-MS methods, protein and nonprotein amino acids were identified. Equal amounts of the diastereomers of alanine, α-aminobutyric, norvaline, β-aminoisobutyric, aspartic and α-aminoadipic acids were found. This distribution is not found in naturally occurring terrestrial materials, thus the asymmetric amino acids are shown to be indigenous to the meteorite and not the result of contamination. The analysis of the Mighei demonstrates that older fallen meteorites can be used to study indigenous organic compounds. This makes additional and varied specimens of these rare meteorites available for prebiotic studies of organic processes of the early solar system which may have led to chemical evolution.

Key words: Abiotic synthesis; carbonaceous chondrite; chemical evolution; meteorite; Mighei.

I. Introduction

In studies to detect evidence of the early abiotic synthesis of organic compounds from which life may have evolved [1] lunar rock samples, ancient sediments and meteorites have been analyzed. The lunar specimens contained small amounts of carbon, 50–200 ppm, which did not appear to be in the form of organic molecules. Microscopic evidence indicates life has been present on Earth over 3 billion years. It is unlikely, therefore, that unaltered terrestrial evidence of prebiotic organic processes will be found. Therefore the 4.5–4.7 billion year-old meteorites provide the only samples available for the study of the natural prebiotic synthesis of organic molecules.

Specifically, the organic rich Type I and II carbonaceous chondrite meteorites (CCI and CCII) are considered to be low temperature condensate of the solar nebulae and contain extractable organic compounds [2]. They condensed from the gas phase to the solid phase and accreted into larger bodies presumably at the same time the planets were forming and provide a unique record of organic processes of the early solar system.

Although it is agreed that carbonaceous chondrites contain extractable organic compounds, the probability of terrestrial contamination of the meteorites has long been recognized. The minute quantities of organic compounds analyzed make the separation and identification of contaminants difficult. Thus the organic analysis of meteorites presents a formidable challenge. The samples are small, rare and irreplaceable. Sophisticated analytical methods are required to separate and identify complex suites of organic compounds in submicrogram amounts. Interpretation of the results requires knowledge of the distribution of compounds that would indicate earthly contamination as well as the distribution of compounds produced in abiotic synthesis reactions indicative of the extraterrestrial origin of compounds indigenous to the meteorite.

Since 1950, investigators have reported amino acids, purines, pyrimidines, and sugars as well as hydrocarbons, acids, alcohols, phenols and porphorins in meteorites [3]. However, after comprehensive efforts to identify sources of laboratory contamination as part of the preparation for the analysis of the lunar samples and comparison of these findings with published results of meteorite analysis, strong similarities were noted which cast doubt on all organic analyses of meteorites prior to 1970. In that year, an analysis of the newly fallen Murchison meteorite at a NASA lunar analysis laboratory found the first unequivocable evidence of indigenous organic compounds in meteorites [4]. Nearly equal amounts of D and L amino acids were analyzed in the Murchison in contrast to the singular L configuration of almost all terrestrial protein amino acids. Additionally, the amino acids found were mixtures of both protein and non-protein amino acids which is additional evidence of extraterrestrial abiotic synthesis. The Murchison, a CCII meteorite, fell as a large shower of stones in Australia in 1969 and has permitted investigators to examine the organic fraction in detail and various classes of organic compounds have been reported [5].

To extend the knowledge of primordial organic processes, it is important to examine other carbonaceous chondrites with the techniques developed for lunar samples. The carbonaceous chondrites have significant differences which could reflect formation in different regions of the condensing solar nebulae. An analysis of a carefully preserved specimen of the Murray, a CCII meteorite that fell in 1950 [6], showed amino acids similar to those obtained for the Murchison but at one-half to one-third the quantity and the proportion of individual amino acids varied. The analysis of Orgueil [7], a CCI meteorite, which fell in 1864 indicated contaminant amino acids. The results of this analysis cast doubt on the use of older fallen meteorites for the study of primitive organic processes.

A study of the Mighei, a CCII meteorite, was undertaken to determine if older fallen meteorites could provide information on indigenous molecules to extend the knowledge of extraterrestrial organic processes. The analysis of the Mighei presented a challenge. It fell in Russia in 1889 as a single 8 kg stone. Soon after recovery, it was coated, probably with fat, while a mold was made [8]. Later it was cut and pieces are in a number of museums. Examination of the published results of 1963 [9] and 1970 [10] analyses indicate that a significant portion of the compounds attributed to the Mighei suggest terrestrial origin. In the present analysis of the Mighei a different sample preparation method was used and rigorous precautions were employed to eliminate known sources of contamination.

II. Samples

The Mighei meteorite sample specimens were obtained from the Smithsonian Institution from a 500 g slab measuring approximately 7.6 cm$\times$10.2 cm$\times$2.5 cm. Two sections of the meteorite slab appeared to have broken off in the past and been glued into place. The samples used in this analysis were from the edge opposite the repaired area and included some fusion crust. The first sample analyzed weighed 9 g; later a 21 g sample was obtained to confirm the results of the initial investigation.

III. Experimental

All sample preparation and wet chemical procedures were carried out in a class 100 clean room and ante-clean room. Filtered air at positive pressure was maintained in both rooms. Clean outerwear, hoods and gloves were worn in these areas. To minimize cross contamination of the interior, a stepwise sampling technique was employed. While holding the meteorite specimen with solvent-cleaned surgical glove-covered hands, the outside (exterior) was scraped off. After changing gloves, knife and glassware, a second deeper layer (middle) was removed. Again gloves and glassware were changed, and the inner sample core was successively rinsed in benzene-methanol (4:1) and air dried. A hand-picked sand blank, the interior core, the middle layer and the exterior

layer were separately and successively ground to fine powders in an agate mortar, then stored in all glass vials.

Special care was required to purify water. Although multiple distillation was tested, the most organic-free water was obtained with the addition of 30 mL of phosphoric acid to a 10 liter boiler of an all glass still which was operated for several days to remove all traces of amino acids. All organic solvents were redistilled. The HCl solutions were prepared by bubbling HCl through H_2SO_4 into water or alcohol. Acid cleaned glassware was loosely wrapped in aluminum foil, heated at 450 °C for at least 5 hours and stored in covered glass containers in the clean room. Liquid transfers were accomplished with glass pipettes connected with Teflon tubing to leur syringes.

One to 4 grams of the meteorite powder was refluxed with H_2O for 20 hours in sealed vials. The H_2O extract was evaporated to dryness under an infrared lamp and refluxed with 6 mol/L HCl for 20 hours. The hydrolyzate was evaporated to dryness, redissolved in H_2O and charged on a Dowex 50 (H+) ion exchange column. The column was successively eluted with H_2O and 2 mol/L NH_4OH. The NH_4OH eluate was evaporated to dryness and the N-trifluoroacetyl-D-2-butyl ester of amino acids was prepared [11]. The amino acid derivative was analyzed by gas chromatography (Perkin Elmer 900) using a flame ionization detector and 46 m×0.25 mm i.d. metal capillary columns, wall coated with UCON 75-H-90,000 or MBM (Perkin Elmer). The amino acids eluted in different order and the resolution of individual diastereomer pairs varied on the two columns which aided in the identification. Procedural blanks showed no evidence of laboratory contamination.

The same UCON column was used in the GC-MS analysis. A Varian 2700 GC was interfaced through a 1 cc Cangal CG5 membrane separator to a CEC 21-491 mass spectrometer. Mass spectra of compounds were obtained using 70 eV electrons and successive scans were taken at 4 s per decade. The amino acids were quantitated with a Durrum D500 amino acid analyzer.

IV. Results and Discussion

A. Exterior and Middle Portion

The 9 g original fragment of the Mighei meteorite was separated into 2.4 g exterior, 1.6 g middle and 4.2 g interior portions. The gas chromatogram of the exterior layer indicated the presence of predominately L protein amino acids superimposed on what appeared to be indigenous protein and non-protein amino acids. Two less abundant non-protein amino acids, α-aminobutyric acid and β-aminoisobutyric acid were found to have essentially equal quantities of D & L isomers.

The gas chromatogram of the middle portion of the meteorite fragment indicated that layer also contained significant peaks of L protein amino acids. However, there were less L contaminants found in the middle portion than were found in the exterior layer.

B. Interior Portion

The gas chromatogram of the interior fragment core that had been washed in benzene-methanol appears in Figure 1. The D and L diastereomers of the protein amino acids alanine (2,2′) and aspartic (18,18′) as well as the non-protein amino acids α-aminobutyric, (4,4′), norvaline (8,8′), β-aminoisobutyric acid (10,10′) and α-aminoadipic acid (20,20′) are present in essentially equal amounts. The relative peak heights of several of the amino acid diastereomers in gas chromatograms of the exterior, middle and interior solvent washed portions of the Mighei fragments appear in Table 1.

Initial efforts to resolve the D and L isomers in the mass spectrometer were unsuccessful. It was determined that the low flow rate of <2 mL/min from the 0.25 mm i.d. capillary column necessitated a low volume separator. A splitless injection system was required since the amount of some amino acids was under 50 ng for the sample injected. When the GC-MS system was optimized, excellent spectra of the meteorite diastereomers were achieved with the membrane

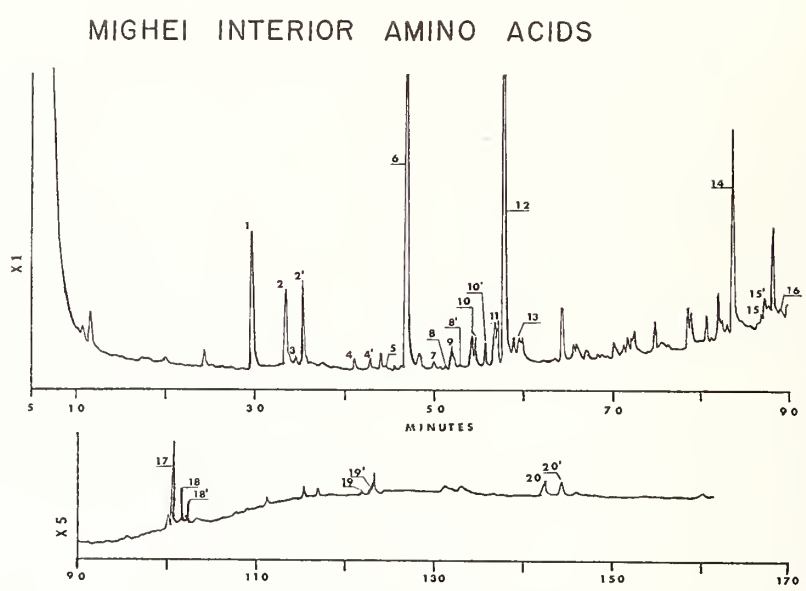

Figure 1. Gas chromatogram: D-2-Butanol-N-TFA derivative products of interior portion of the Mighei meteorite. MBM column programmed at 80 °C (18 min)–1°/min–175°(hold). He 14 psi, attenuation Xl.

TABLE 1. *Ratio of disastereomer peak heights for Mighei samples*

	Exterior layer	Middle layer	Interior layer Fragment 1	Fragment 2
Alanine L/D	3	2	1.0	1.1
Aspartic L/D	10	5	1.0	1.1
Glutamic L/D	22	18	2.7	5.7
β-Aminoisobutyric L/D	1	1	1	1

TABLE 2. *Amino acids in interior samples of the Mighei, Murchison, and Murray Meteorites*

	Mighei	Murchison [4]	Murchison [12]	Murray [12]
Aspartic acid	240 ng/g	3000 ng/g	1700 ng/g	1600 ng/g
Glycine	780[a] ng/g	6000 ng/g	61000 ng/g	3000 ng/g
Alanine	150 ng/g	3000 ng/g	3500 ng/g	1300 ng/g
Glutamic acid	250 ng/g	3000 ng/g	3100 ng/g	1600 ng/g
Theronine	48[b] ng/g			
Serine	24[b] ng/g			

[a] Includes α-aminoadipic acid.
[b] Identified only by AAA retention times.

separator and the amino acids were verified by comparison to elution time and spectra of derivatives of authenticated standards.

Amino acids previously identified in the Murchison [4] and the Murray [6] meteorites were found in the Mighei meteorite; however, the quantities were reduced. About 1/10 of the amount of individual amino acids identified in the Murchison were present in the Mighei meteorite (Table 2).

V. Conclusion

The presence of nearly equal amounts of the D,L diastereomers of alanine, α-aminobutyric, norvaline, β-aminoisobutyric, aspartic and α-aminoadipic indicate that these protein and non-protein amino acids are indigenous to the Mighei meteorite and not the result of contamination from the terrestrial L protein amino acids. Additional evidence of the extraterrestrial origin of the meteorite amino acids is the presence of large suites of non-protein amino acids.

Since the exterior layers of the Mighei had large amounts of terrestrial amino acids, organic analysis of small fragments of meteorites would not appear prudent. Significant amounts of L amino acids remained in the middle 20 percent layer after removing the outer 25 percent of the Mighei meteorite fragment. A combination technique of removal of the exterior layers and benzene-methanol washes of the interior core however was successful in removing terrestrial amino acids.

This study demonstrates that an intact 9 g specimen of an older supposedly contaminated meteorite did provide evidence of extraterrestrial chemical processes. Thus, the analysis of older fallen meteorites should be undertaken to extend the knowledge of primordial organic chemistry.

VI. Acknowledgments

The Mighei meteorite specimens were obtained from Mr. Roy Clarke of the Smithsonian Institution, Washington, D.C. The experimental work was carried out at the Laboratory of Chemical Evolution, University of Maryland, College Park, under the direction of Dr. Cyril Ponnamperuma.

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THE USE OF A NOVEL GAS CHROMATOGRAPHIC DETECTION SYSTEM FOR THE ANALYSIS OF TRACE HALO-ORGANICS

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A novel ionization (electron capture) gas chromatographic detector is described. The new design features an open cell, an anode-to-cathode gas flow and a porous anode. These features greatly reduce recirculation in the detector which in turn means that polar solvents may be injected without loss of the ability to detect trace quantities of halo-organics. The new detector design is also remarkably tolerant to highly impure samples and can be used at low flows. The desirable properties of electron capture type detectors, such as selectivity, linearity of response and extreme sensitivity are retained. A number of practical applications are described.

Key words: Electron capture detector; gas chromatography; halo-organics; ionization detector; porous anode.

I. Introduction

It will be recalled that the principle of electron capture detection is based upon the selective ability of compounds having a high electron affinity to strongly influence the flow of current in an ionized gas stream. The ionization is provided to the carrier gas as it flows past a source of beta emission. If a fixed potential is applied to the detector, this ionization creates a standing current the alteration of which produces a signal whose strength is dependent on the nature and the amount of the compounds passing through the detector. Other factors which influence the changes in the standing current produced by a given amount of a given material are: the nature of the carrier gas, the pressure of the carrier gas, the voltage applied, the distance between the electrodes and the interelectrode capacitance.

Early detectors of this type featured a wire anode surrounded by a radioactive cathode containing a source of beta emission. As the practical utility of such detectors became more widely appreciated due to their extreme sensitivity and high selectivity for halogenated organic compounds, detector design began to evolve toward the current state of the art. Wire anodes gave way to rods which in turn gave way to tubular designs with the gas either entering or leaving via the tube.

The Basic Lovelock detector [1] (Fig. 1) is characterized by an enclosed system and a tubular anode through which the chromatographic effluent enters the system. The cell walls serve as both the cathode and the beta source. Variations of this design such as that instituted by D. C. Fenimore [2] feature a tubular anode with the insulators moved away from the source of radiation so that they would be less likely to acquire a charge which might contribute either to the standing current or the noise. Gas flow proceeds from the cathode to the anode. These currently popular designs are extremely useful and have proven their value for the analysis of picogram levels of halogenated materials in a wide variety of samples.

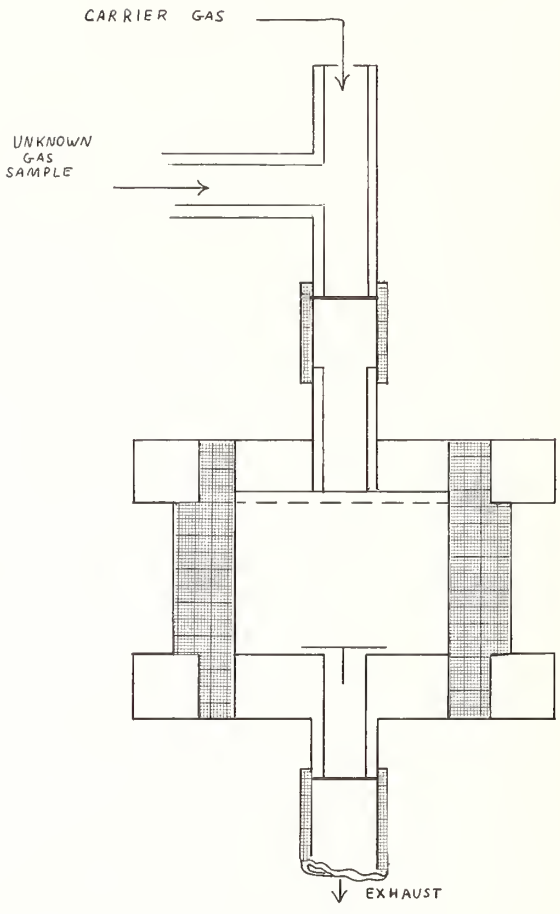

Figure 1. Basic Lovelock detector.

II. The Porous Anode Detector

Evolution is an on-going process, however, and some problems do remain which limit the usefulness of currently available ionization detectors. It is the writers' belief that the porous anode detector [3] reduces such problems as recirculation, dirt buildup inside the detector chamber, internal electronic shorting between the elements of the detector, the inability to perform well at low flow rates and a variable response during temperature programming. The design of this detector is shown in Figure 2.

Unlike presently popular electron capture type detectors, it should be noted that the porous anode detector features an open cell design. The desirable high gas pressures at the point of detection are achieved by virtue of the fact that considerable pressure is required to force the gas through the pores of the anode. Since the detection phenomenon takes place just inside the surface of the anode, little is sacrificed in the way of sensitivity in spite of the fact that the gases are at atmospheric pressure as soon as they enter the space between the anode and the cathode. This fact confers a number of special advantages upon the system with the most significant being concerned with recirculation. Because the point of detection is at a higher pressure than the gases on their way out of the detection chamber, there is a greatly decreased opportunity for sample molecules to reenter the zone of detection once they have left the anode. Furthermore, this same factor greatly decreases the amount of column bleedout and other dirt which can deposit on the interior surfaces of the detector chamber and interfere with normal operating conditions.

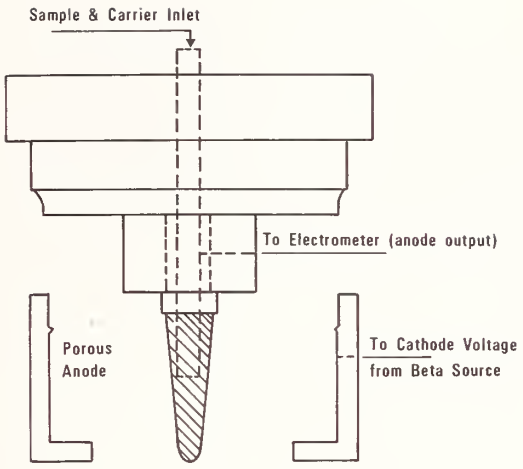

Figure 2. Basic Lupton detector.

Since the open design permits a more remote placement of insulators and other elements of the detector, internal electronic shorting is greatly reduced. Although interior dimensions of the detector cell itself do not appear to be as important as is the case for closed systems, it may be desirable at some time in the near future to reduce the size of the anode itself in order to make it more compatible with the dimensions of capillary columns. This latter item is a matter of immediate interest to the authors since it has already been established that the detector will perform acceptably at unusually low flow rates (i.e., 3–5 ml/min). It has been further demonstrated that the porous anode detector is not as susceptible to drift as are some types of electron capture detectors when subjected to rapid temperature programming. More thorough comparisons will be required to fully document this point.

III. Operating Characteristics

The response of typical ionization detectors has been reported to be sensitive to the applied voltage [4]. In this respect, the response of the porous anode detector to changes in cell potential is quite similar to that of other electron capture detectors except that the optimum cathode voltage is less than 10 volts. An initial rapid rise in detector response is followed by a slight decrease and a long plateau as shown in Figure 3.

The response of the detector to varying quantities of bromodichloromethane is shown as a logarithmic plot on the left side of Figure 4. A similar, but linear plot is shown on the right side of Figure 4 for lindane. Thus it can be seen that the detector response is linear over a useful range in both cases. The standing current employed during the course of this work was 10 nanoamps. It should be pointed out that the open cell design minimizes the degradation of the standing current by allowing column bleedout to leave the detection chamber more effectively.

As is typical of electron capture detectors, the detector response is dependent upon the flow rate of the carrier gas. Within the range of 5 to 50 mL/min, an acceptable response (i.e., ppm in an extract) can be achieved for such compounds as lindane and the halomethanes. Detector response was observed to increase with flow up to 50 mL/min. A more complete exploration of this parameter is planned. Sensitivities for the trihalomethanes have been established in the 50 picogram range. The authors fully expect to lower this value by at least an order of magnitude as developmental efforts continue.

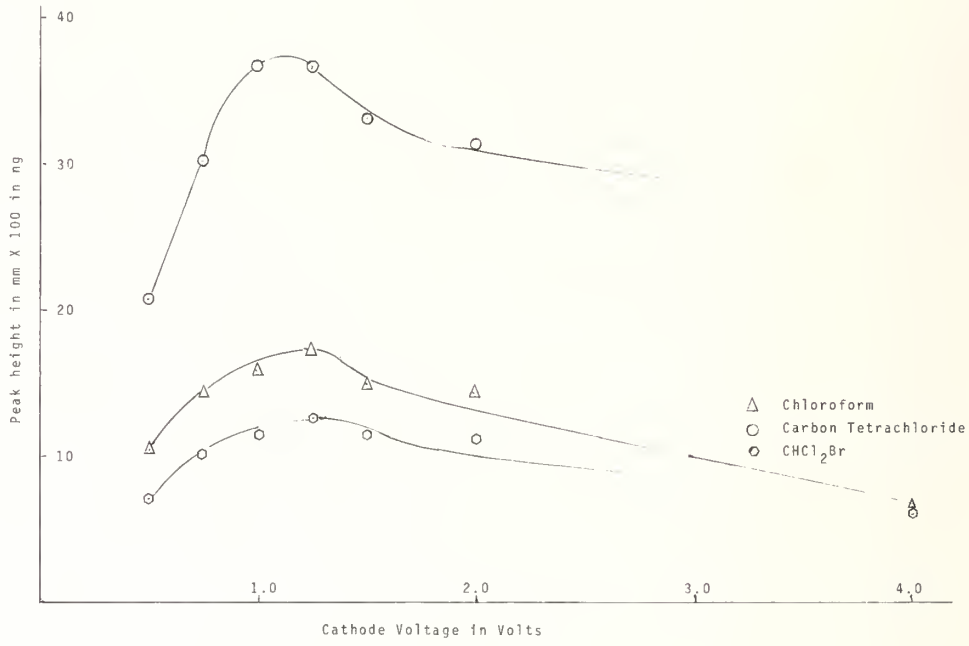

Figure 3. Detector response as a function of applied voltage.

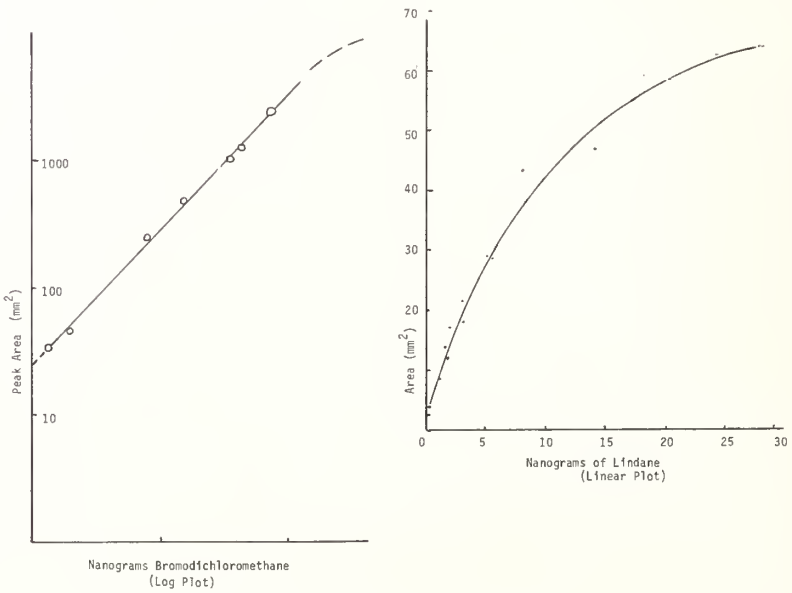

Figure 4. Linearity of response.

IV. Practical Applications

The "unswampable" nature of the porous anode detector is summarized in Figure 5 in which case successive injections were made with pure chloroform. The return to baseline even in the most severe case required less than two minutes. The practical utility of this capability is evidenced by the fact that the laboratory has been able to determine lindane by chloroform extraction followed by direct injection into the gas chromatograph. The ability of the detector to return quickly to baseline should also prove to be useful in the analysis of halomethanes as evidenced by the separation achieved in Figure 6 using a 6′×2 mm Ultrabond 20 M column. The first three peaks all eluted within a minute thus making the quick return to baseline an important property. The final example of a practical application is provided by Figure 7 which illustrates the analysis of what must be regarded as an extremely dirty sample. It is the direct extract of an industrial sludge which was heavily contaminated with pesticides. The only pretreatment employed was drying over anhydrous sodium sulfate. Nevertheless, there was no difficulty involved in estimating that there was a considerable quantity of dieldrin present. Ordinarily a 3 day's run with such materials could be expected to ruin an ordinary detector. In this case, it was not necessary to clean the detector at all. In fact the data shown in Figure 6 were taken immediately afterward. Thus it is apparent that lengthy cleanup procedures may be omitted in certain cases without suffering a corresponding increase in detector down-time. In any event, the detector is not difficult to clean in the unlikely event that cleaning should be required.

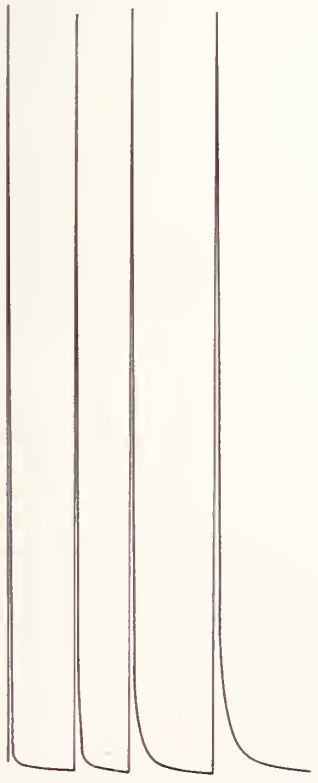

Figure 5. Rapid recovery of detector.

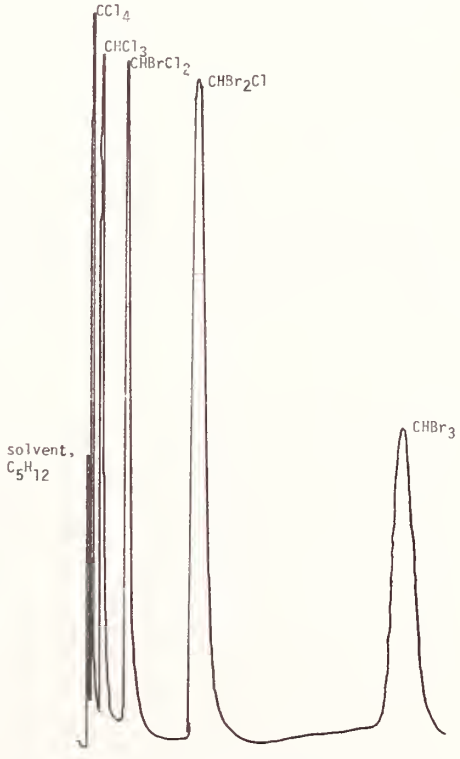

Figure 6. Analysis of halomethanes.

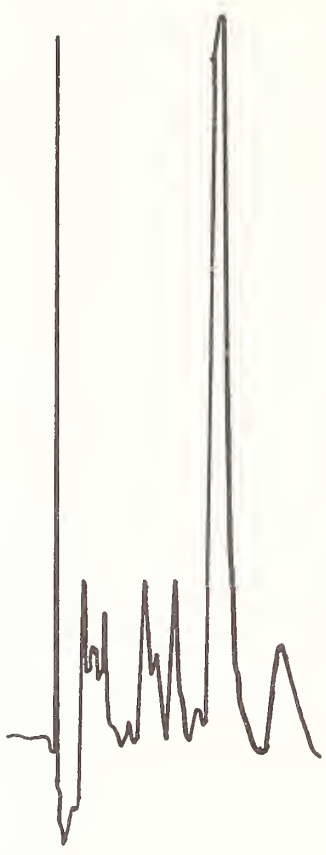

Figure 7. Dieldrin in an industrial sludge.

V. Acknowledgment

The authors gratefully acknowledge the partial support of the U.S. Environmental Protection Agency, Office of Water Supply, Criteria and Standards Division, Contract No. 68-01-4480.

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USE OF POLYMER SUPPORTED FUNCTIONAL GROUPS FOR THE SELECTIVE CONCENTRATION OF ORGANIC COMPOUNDS

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The objective of this research has been the preparation and characterization of chemically modified solid supports containing specific functional groups for the separation and/or concentration of selective classes of organic compounds. These resins derive their high selectivity and strong affinity from specific chemical reactions between the polymer supported functional group and the solute.

Key words: Affinity chromatography; environmental analysis; functional group analysis.

I. Introduction

Analysis of functional groups in multicomponent organic samples is of interest in a number of disciplines. Identification and quantitation of individual components in ecological, pharmaceutical, biological, and organic reaction systems are routinely required. Most analyses dealing with matrices of such complexity require a chromatographic separation followed by identification of individual fractions. This often involves a series of extractions followed by an analytical column separation. The procedure can be tedious, require large sample sizes, and at times be inefficient or ineffective.

An ideal isolation step would be one that removes only the compound containing the functional group of interest from the matrix. Accordingly, the objective of this research has been to synthesize and to characterize chemically modified solid supports containing specific functional groups for the selective separation and/or concentration of several important classes of organic compounds. Polymer supported functional groups presently under investigation are ones specific for the determination of phenolics, aromatic amines, aromatic nitro compounds, mercaptans, azo dyes, and polyaromatic hydrocarbons. These resins derive their high selectivity from specific chemical interactions between the polymer supported functional group and the solute.

II. Discussion

A. Phenolics and Aromatic Amines

The ability of diazonium salts to couple with phenolics and aromatic amines to form highly colored azo derivatives has formed the basis for numerous methods for the determination of these compounds due to the general applicability and high sensitivity of this technique. Immobilization of the diazonium functionality onto a solid support provides a resin capable of selectively coupling with, and sequestering from solution, phenolics and aromatic amines. Quantitation of the immobilized azo derivative may be effected in one of two ways: (1) The coupled resin can be subjected to hydrolysis conditions causing release of the immobilized azo dye which can be quantitated spectrophotometrically, or alternately, (2) the coupled resin can be treated with a small volume of a reducing agent, such as titanous chloride or sodium dithionite, to cleave the azo bond,

thus releasing the amino derivative of the analyte which can also be determined spectrophotometrically. In practice, hydrolysis of the coupled resin is preferred due to the high extinction coefficient of the released azo derivative and to the minimization of optical interferences.

As diazonium salts are inherently unstable and decompose slowly even in the solid state, the resin is stored as its aryl amine precursor and activated to the diazonium salt only before use. Synthesis of the immobilized aryl amine involves the attachment of an aminosilane to a silica gel support, followed by derivatization with p-nitrobenzoyl chloride and reduction of the nitro group [1–3] (Fig. 1). Derivatization of the aryl amine to its active diazonium form is accomplished by treatment with fluoroboric acid and sodium nitrite.

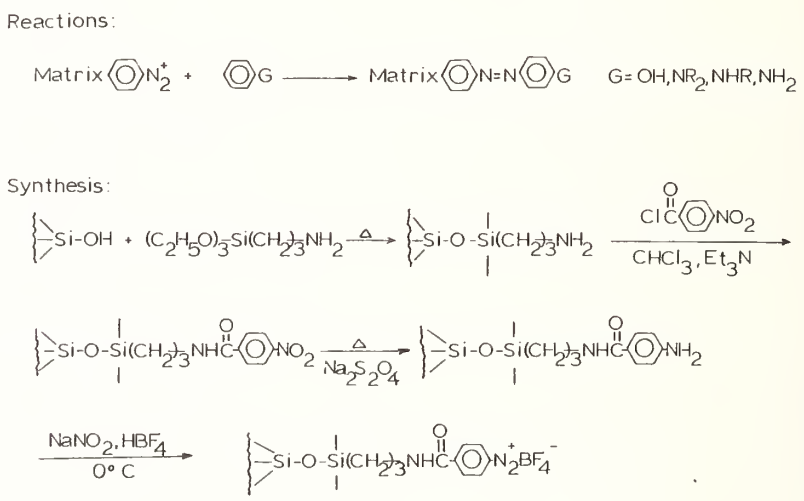

Figure 1. Synthesis and reactions of an immobilized diazonium salt functional group resin.

In order to optimize conditions for the coupling reaction, it was important that several competing side reactions be investigated in addition to the coupling reaction. These were:

(1) Decomposition of the diazonium functionality; (2) reaction of the diazonium salt as a dibasic acid; and (3) hydrolysis of the resin's siloxane bonds.

Kinetic data for each of the above reactions, as well as for the coupling reaction, were collected and plotted against temperature and pH to determine the optimum experimental conditions for each coupling component investigated. From these specific conditions, general experimental conditions were formulated.

pH: The coupling reaction should be carried out in a medium such that the equilibria of the diazo and coupling components favor as much as possible the diazonium ion and the phenolate ion or the free amine, respectively. Experimentally, phenolics were found to couple best at or about pH 6, amines at or about pH 8.

Temp: Diazo decomposition reactions and hydrolysis reactions possess greater energies of activation and hence a larger temperature gradient than azo coupling. Accordingly, the coupling reaction was carried out at depressed temperatures, usually between 5–10 °C.

To date, the following phenolics and aromatic amines have been successfully studied: phenol, resorcinol, 1-naphthol, aniline, N,N-dimethyl aniline, and N-l-naphthylethylene diamine.

B. MERCAPTANS

The reaction of mercaptans with heavy metal ions such as Ag(I), Cu(I), and Hg(II) is well known and has formed the basis for a number of analytical techniques [4–6]. Various organomercurial resins, used for the separation of thio containing enzymes, proteins, and other

Reactions:

Matrix-HgOAc + RSH ⟶ Matrix-HgSR + HOAc

Matrix-HgOAc + RC≡CH ⟶ Matrix-HgC≡CR + HOAc

Synthesis:

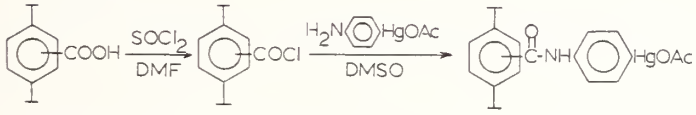

Figure 2. Synthesis and reactions of an organomercurial-polystyrene resin.

biological compounds, have been reported in the literature [7]. These resins were prepared by covalently bonding an organomercurial functionality to an agarose substrate by means of a water soluble carbodiimide or by activation of the substrate with cyanogen bromide. Such resins have capacities of 1–8 μmol/mL of packed resin. Accordingly, an organomercurial resin of much higher capacity and efficiency was prepared and used for the sequestering of mercaptans. Synthesis of such a support is illustrated in Figure 2. Organomercurialpolystyrene resins prepared in this manner possessed capacities of 1.2–1.9 mequiv/mL of packed resin.

Mercaptans were separated from the reaction medium through formation of a resin-mercaptide derivative. Recovery of the mercaptan could be effected by elution with strong acid. To date the following mercaptans have been studied: benzenethiol, *p*-chlorobenzenethiol, *p*-toluenethiol, *n*-butanethiol, and thioglycolic acid. In addition, this technique should be useful for the analysis of acidic alkynes.

C. AROMATICS

The pyrrolidone moiety has received considerable attention by our workers in the past as a sequestering resin for phenolics [8] and azo dyes [9]. A number of workers have used crosslinked, water insoluble poly(vinyl pyrrolidone) (Polyclar AT) in low pressure chromatography of aromatic acids, aldehydes, and phenols [10,11], polyaromatic hydrocarbons [12], nucleotides [13], and anthrocyanins [14,15]. Additional work in our laboratories has demonstrated Polyclar AT affinity for aromatic nitro compounds, estrogens, aromatic amines, and phenylhydrazones. Aliphatic compounds have shown no complexation with the pyrrolidone ring. Overall, Polyclar AT shows the necessary complexation characteristics for selective separation, but tends to lack the desired physical properties.

To apply 1-vinyl-2-pyrrolidone to HPLC, it was necessary to bond the butyrolactam to an inert, mechanically stable support. One procedure found suitable was adapted from that of Wheals [16]. 1-vinyl-2-pyrrolidone was chemically bonded to 8 μm spherical silica via polymerization to a vinyl modified surface (Fig. 3).

Separations selective for aromatic compounds alone have been obtained in a reverse phase mode. Estrogens have been selectively separated from mixtures of the non-bezenoid progestins and androgens, mixtures typically encountered in pregnancy testing and oral contraception formulations. Similarly, aromatic nitro compounds have been isolated from aliphatic nitro substituted materials, a mixture that is typically encountered in commercial high explosive preparations. In both cases aliphatic materials are unretained and thus eluted in the dead volume. Other separations with immobilized pyrrolidone include phthalic acid isomers, polyaromatic hydrocarbons, and substituted phenols. In addition, initial investigations show that this stationary phase may be used in an adsorption mode as well.

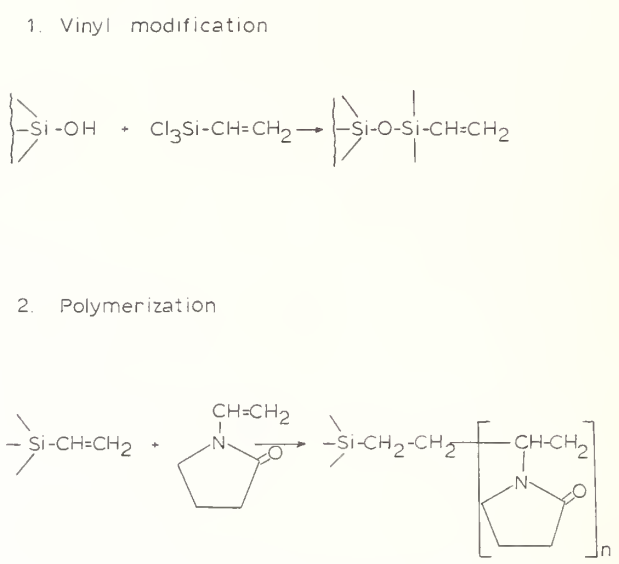

Figure 3. Synthesis of an immobilized 1-vinyl-2-pyrrolidone stationary phase.

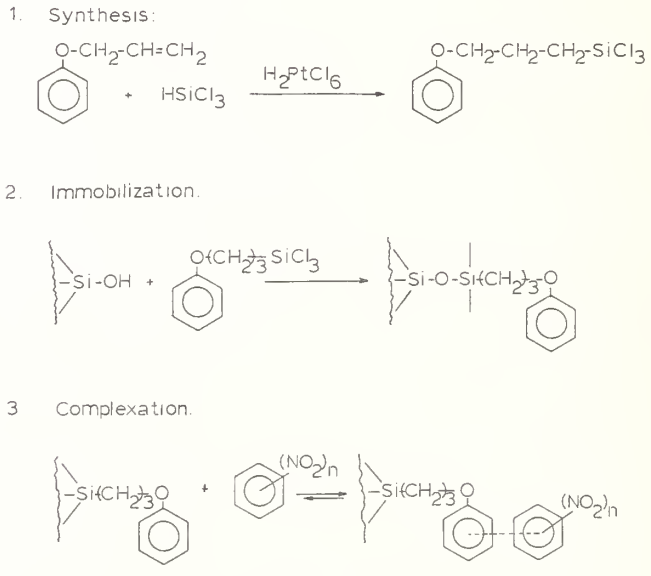

Figure 4. Synthesis and reactions of an immobilized aryl ether.

D. Aromatic Nitro Compounds

Aryl ethers form molecular complexes with nitro substituted aromatic compounds. Picrates are frequently used as solid derivatives of aryl ethers and vice versa for aromatic nitro compounds. A functional group selective stationary phase can be readily obtained by immobilizing the aryl ether onto a solid support. Starting with the allyl phenoxide, immobilization was achieved by generation of an aryl silanizing reagent (Fig. 4). Retention is favored in mixed, nonaqueous solvent systems.

III. Conclusions

All of the functional group selective stationary phases discussed rely on highly specific chemical interactions. Their synthesis is fairly rapid and straightforward, as is their characterization. There is considerable work to be done in optimization of efficiency and dedication to isolated, difficult analytical problems. Work to date is encouraging in both respects. It has also opened up a number of possibilities in the immobilization of yet other reagents capable of providing selective separation of functional groups in multicomponent samples.

IV. Acknowledgment

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A PROPOSAL FOR IMPLEMENTING A REFERENCE COLLECTION OF HUMIC AND FULVIC ACIDS

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Four humic and fulvic acids from a mineral soil, an organic soil, a geologic deposit, and from a stream water are proposed as international reference samples of natural organic matter. The acids will be extracted and isolated by the best present technology to insure well-humified, representative, ash-free preparations for geochemical research. A proposed isolation procedure is outlined using XAD-8, an uncharged acrylic resin. An international committee of scientists will oversee all aspects of the establishment of the reference collection.

Key words: Aquatic humus; fulvic acid; humic acid; humic substances; reference collection of humic substances; reference samples; soil organic matter.

I. Introduction

The need for the establishment of an international reference collection of humic and fulvic acids has been presented in a paper by MacCarthy and Malcolm [2] in these proceedings. The desirability and benefits of such a collection has been voiced by MacCarthy [1] and many others in the past, but a definite plan to accomplish this objective has never been formalized. Informally, isolates of natural organic matter have been exchanged among researchers on a limited basis, but due to the time and effort required to obtain even small quantities of humic and fulvic fractions, many requests for such materials must be refused in order to avoid the exhaustion of the researchers' limited supply or to prevent the necessity of additional extractive efforts. The authors have recognized the ever-increasing need and demand for reference samples and for the past 3 years have solicited ideas and support for establishing a reference collection of humic and fulvic acids [1]. Considerable international interest and support has been generated, and the following organizational scheme and isolation techniques are proposed to accomplish this goal.

II. General Plan and Organizational Scheme

The proposal to establish such a collection is divided into two phases. Phase I incorporates the planning stage, and Phase II involves the actual sample preparation for the reference collection. Phase I includes:

(i) the development of a detailed plan by the authors for selection, collection, extraction, and isolation of reference samples,

(ii) the endorsement of this plan by scientific societies and organizations,

(iii) the procurement of limited financial support for Phase I, and

(iv) the formation of an international committee to evaluate, modify, and finalize the authors' plan. This plan will then become the committee's comprehensive scheme to establish the reference collection incorporating the expertise and present technology of the world community in humic and fulvic acid research.

Oral presentations by the authors of their plan at several national meetings, and this paper, document the completion of Phase I, part 1, i.e., the planning stage. The second part of Phase I has largely been achieved by the endorsement of the proposal by several national societies and hopefully will culminate in an endorsement from the Congress of The International Society of Soil Science in June 1978 at Edmonton, Canada. A proposal for partial funding of Phase 1 has been submitted to the National Science Foundation. This organization has shown considerable interest in funding the proposal, but precise details have not yet been finalized. Once funding is assured, the 12–15 member international committee will be assembled. The majority of the committee membership will be composed of representatives of national and international societies interested in establishment of the reference collection. Each society will select its own representative to the committee.

Phase II, the actual establishment of the reference collection, includes the following:

(i) Collection of bulk reference samples of solid and aquatic materials.

(ii) Homogenization and storage of bulk earth material samples.

(iii) Extraction, purification, and concentration of reference humic and fulvic acid fractions from bulk samples.

(iv) Storage and distribution of reference humic acids, reference fulvic acids, and bulk soil or geologic samples.

(v) Maintenance and dissemination of data collected on the reference samples.

(vi) Procurement of manpower and monetary support to effect phase II.

All of the Phase II activities would be under the direction of the international committee. Hopefully, Phase II funding will reflect international interest in the establishment and objectives of the reference collection.

III. Proposed Sources of Reference Samples

Three solid earth materials (a mineral soil, an organic peat or muck soil, and a geologic deposit) are suggested as bulk reference samples. The well-characterized Armendale Podzol used in the studies of Dr. M. Schnitzer of Canada would be an excellent choice for the mineral soil. Eastern Europe would be a logical place for the choice of a peat or muck soil. For the geologic community, possible geologic samples would be a Leonardite (an oxidized lignite coal deposit) from Wyoming, or a Dopplerite (a gel-like peat deposit) from Germany. A fourth reference sample representing aquatic humic substances would be very desirable because of the recent increased interest and research in organic hydrology. The Suwannee River flowing from the Okefenokee State Park in south Georgia as studied by several researchers, including the authors, would be a very practical source of aquatic humus. Thus river maintains a dissolved organic carbon (DOC) level of 25–50 mg/L for most of the year with approximately 75 percent of the organic load as fulvic and humic acids.

Bulk samples of the three soil and geologic materials will be collected in the amount of 2–3,000 kg. A portion of this bulk sample will be used for the extraction of humic and fulvic acids; the remainder will be available for dispensing as bulk samples and to serve as a common source material for more advanced extraction techniques which may be developed in the future. It is anticipated that humic and fulvic acids will be prepared in excess of 1 kg from each bulk sample with the exception of the aqueous sample. By processing an enormous quantity of river water (10,000 L), approximately 0.5 kg of humic and fulvic acids can be isolated. Processing of larger quantities of water seems unrealistic.

IV. Proposed Extraction and Purification Scheme

Almost universally, humic and fulvic acids are extracted from soils and sediments with aqueous sodium hydroxide. The normality of the base and the procedures to rid the humic substances of clay mineral, trace metal, and inorganic salt impurities vary tremendously. The need for obtaining humic and fulvic acids of high purity for geochemical studies, and a method for obtaining them has been presented by Malcolm [3]. A recent modification of this method in obtaining humic and fulvic acids from natural waters using XAD-8, a nonionic macroreticulate acrylic resin, is proposed for the isolation and purification of humic substances for the reference sample collection [4]. Protonated humic and fulvic acids in water at pH 2 are quantitatively adsorbed onto the resin, then quantitatively eluted from the resin with dilute base. The fraction eluted with base has been designated as the hydrophobic acid fraction. In addition to the high-molecular-height humic and fulvic acids, this fraction may contain many low-molecular-weight constituents if they are initially present in the aqueous sample. These low-molecular constituents, which include aliphatic acids (C_5–C_{10}), aromatic acids, and phenols, are removed from the high-molecular-weight humic and fulvic acids by molecular exclusion gel chromatography using Enzacryl gel (a polyacryloylmorpholine gel).

After the Enzacryl fractionation, the classical humic-fulvic separation can be accomplished by hydrochloric acid addition to reduce the pH to 2. Following centrifugation the soluble fulvic acids are readsorbed onto XAD-8, eluted in weak base, passed through a H-saturated exchange column, then freeze-dried. The precipitated humic acids are solubilized in dilute base, adsorbed onto XAD-8 resin, and treated as the fulvic acid fraction.

Dilute base (0.1 mol/L NaOH) under N_2 gas will be used to extract the humic and fulvic acids from the three soil and geologic samples. The basic extract will be centrifuged and then filtered through a 0.45 μm filter using nitrogen pressure to remove particulate organic materials and clay minerals. Filtered aqueous extracts of each soil or geologic sample will then be treated as an aquatic humus sample and purified accordingly.

This procedure overcomes many of the objections voiced in the past concerning sodium hydroxide extraction of soil samples. The advantages are:

(i) Only a short period of the extraction is conducted in basic solution to prevent O_2 degradation. The extraction is carried out under a nitrogen atmosphere; therefore, possible degradation is minimal.

(ii) Desalting is accomplished rapidly and effectively by adsorption on XAD-8 without possible microbial alteration during the long dialysis period, frequently employed.

(iii) Low-molecular-weight specific organic acids are not included in the humic and fulvic acids because C_1-C_5 acids are not retained on XAD-8, C_{10} or greater acids are retained but not eluted with dilute base, and the C_5-C_{10} acids are fractionated from the humic and fulvic acids by Enzacryl gel fractionation.

(iv) Low-molecular-weight neutral specific compounds are not included in the humic and fulvic acid fractions because some are not retained on XAD-8, or if retained, they are not desorbed during base elution of fulvic and humic acids.

(v) Organic carbon balance is monitored by DOC analysis to assure no specific fractionation losses during resin treatments.

The detailed isolation procedure and organizational scheme as presented in this paper should serve as a workable basis for the establishment of an international reference collection of humic and fulvic acids.

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THE NEED TO ESTABLISH A REFERENCE COLLECTION OF HUMIC SUBSTANCES

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Humic substances constitute an ubiquitous class of natural organic matter occurring in soils, sediments and natural waters. These materials are found in concentrations ranging from trace to macroscopic levels, and they play an important role in many agricultural, environmental and geochemical processes. Fundamental and applied research on humic substances is hampered by the fact that presently there is no objective mechanism for the interlaboratory comparison of experimental data on humic substances. A proposal to remedy this situation through the establishment of an international stockpile of standard humic materials is discussed; the potential benefits of this program to research on humic substances are outlined.

Key words: Fulvic acid; humic acid; humic substances; reference collection of humic substances; soil organic matter; standard humic materials.

I. Introduction to the Humic Acid Problem

Humic substances constitute a very important class of natural products: they participate in many significant agricultural, geochemical and environmental processes [1–6]. It is evident from an examination of the recent literature that there is an increasing interest in these materials by chemists, soil scientists, hydrologists, organic geochemists and others in environmental sciences. Although these substances have occupied the attention of scientists for almost 200 years [7], and have been studied by many of the well-known classical scientists such as Liebig [8], Berzelius [9] and Waksman [10], *relatively* little progress has been made in the elucidation of their chemical nature compared to that of many other natural products such as proteins, polysaccharides, polynucleotides, etc. However, this is not to imply that no progress has been made in the study of these materials. A vast amount of information has been accumulated on the chemical, physical, biological, geochemical and agricultural properties of humic substances, but it has not been possible to integrate this knowledge within a satisfactory conceptual framework due to a lack of understanding of the nature of these materials at the molecular level.

The objectives of this paper are to critically examine the problems resulting from the lack of humic acid (or fulvic acid, etc.) reference materials and to consider the potential benefits which would ensue from the establishment of such a collection. In the following paper [11] a proposal outlining tentative experimental procedures for the establishment of a reference bank of humic substances is discussed.

II. Basic Causes of the Problem

It is rather easy to establish the basic reasons for the lack of progress in our understanding of humic substances.

(i) These substances are complicated mixtures of macromolecular material which are extracted from soils, marine or fresh-water sediments, natural waters or similar environs. Humic substances are defined in operational terms on the basis of their extractability by a variety of extractive procedures, or on the basis of their solubility under varying conditions.[1] As a result, the term humic acid (or fulvic acid, etc.) does not refer to a specific substance, or even to a unique mixture of substances. Rather, the term embraces non-identical mixtures of substances which are extracted by common or related methods and whose average gross chemical and physical properties are similar.

(ii) For all practical purposes there has been virtually no standardization of extractive procedures for humic substances. Although proposals to adopt standard methods of extraction have been made in the past (see, for example, ref. 4), these have so far not been implemented on a broad scale. Similar comments apply to the lack of standard "purification," or other methods of pretreatment, for humic substances. All of these considerations, in addition to the wide diversity of substrate materials from which the humic substances can be extracted, lead to a considerable variability in the final products.

(iii) As humic substances are complex mixtures, the application of all chemical and physical methods to their study is very limited and the detailed interpretation of the data is rendered very difficult and virtually impossible at present.

(iv) Despite numerous attempts by many workers, no one has yet succeeded in achieving a satisfactory fractionation of humic substances. No pure substance which could be called a humic acid has yet been isolated. Each fraction obtained by a separation process still consists of a complicated mixture. The reasons for these results have not yet been unambiguously established.

III. The Heart of the Problem

As a result of the considerations enumerated above, workers in various laboratories who are carrying out research on humic substances are generally working with basically different materials. Of course, there is *some* exchange of samples between workers in different laboratories. "Humic acids" are also available commercially from a number of chemical suppliers.[2] However, an examination of the literature does show that the great majority of workers extract their own humic samples from soil, sediments, natural waters, etc. This is totally understandable, and in many cases one may be interested only in the behavior of a humic acid obtained from a specific locale. However, this type of situation does create a particularly difficult problem in the area of interlaboratory calibration of analytical methods and critical comparison of experimental results. Interlaboratory comparison of results can often present serious problems even when different workers are using identical samples of a pure substance.

The problem becomes vastly more complicated when the substrate varies from worker to worker as in the case of humic acids, and where there has been no standardization of extractive, fractionation or purification procedures. It is thus virtually impossible to objectively and critically compare the laboratory results of different workers in this interdisciplinary area of research.

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[1] Humic acid is defined as the alkali-soluble, acid-insoluble fraction of natural organic matter; fulvic acid is, by definition, soluble in both acid and basic media. Humin refers to the fraction of the organic matter which is insoluble in both acid and base.

[2] There are at least five chemical distributors in the U.S. which supply so-called "humic acids." Use of these commercial humic acids would not solve, but would likely aggravate the various problems, because these materials are non-homogeneous and vary from batch to batch. They are exceptionally high in clay mineral and metal impurities, some containing about 10% oxides of iron. There is little or no information available concerning the origin of the commercial humic acids or their mode of extraction. Virtually no elemental or other analytical data are available for these materials and it appears that there is little quality control involved in their procurement.

IV. A Rational Proposal to Minimize the Problem

These complications could be minimized to a considerable degree if many researchers were to work with identical samples of humic materials obtained from a single distributing source. It has been proposed that a reference collection of humic materials be established, and that samples from this collection be made available to researchers on a worldwide basis [12]. The implementation of such a proposal would result in a number of important benefits.

V. Potential Benefits Resulting from Implementation of the Proposal

(i) It would lead to the establishment of a large homogeneous stockpile of organic matter from which workers throughout the world could withdraw humic acid and fulvic acid samples for which extensive analytical data would be available. The establishment of a reference material or materials is a basic requirement in all areas of quantitative measurement.

(ii) By the use of such a common-source material, the analytical methods of various workers could be compared more objectively, and hopefully the optimum methods of analysis, including reliable levels of accuracy and precision, established. This would provide a more valid framework for the evaluation of analytical methods for characterizing humic substances.

(iii) In addition to serving the more conventional use of standards for elemental analysis, it is intended that the reference humic materials will also serve as standards for chemical reactivity, fractionation, structure-determination, etc. Thus, experiments such as fractionation, chemical derivatization and degradation, complexation with metal ions, spectroscopic studies, investigation of effects on plant growth, etc., could then be carried out using a common material. This would allow a more critical comparison of the results of different workers in these areas.

(iv) An intensive study on the chemical structure of a limited number of reference samples by many workers could possibly help to accelerate efforts to determine the chemical structure of these materials.

(v) General acceptance of these reference materials should considerably curtail the use of, and problems caused by ill-defined, non-representative commercial[2] and personal humic preparations.

(vi) Finally, once common methodologies and reliable standards have been developed using "standard" humic substances, these methods could then be applied to different humic acid samples. This would allow true differences between various humic acids to be more readily and quantitatively established as distinct from discrepancies resulting from differences in methodology. In other words, the "standard" samples would establish a reference point of chemical composition, reactivity, etc., from which other humic substances could be objectively compared.

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ANALYSIS OF 4.5 MOL/L SULFURIC ACID FOR ORGANIC COMPOUNDS LEACHED FROM BATTERY SEPARATORS

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Organic compounds present at $\geqslant$10 μg/mL in 4.5 mol/L H_2SO_4 were separated, identified, and quantitated. These compounds were solubilized from lead-acid battery separators, made of phenol-formaldehyde resin-impregnated cellulose, by the action of 4.5 mol/L H_2SO_4 at 75 °C for 20 h. Separation techniques include: steam distillation, ion-exchange, TLC, GC, centrifugation, chemical precipitation, paper chromatography and reverse phase HPLC. Identification and quantitation involved the use of GC, IR, NMR, UV-visible and "total carbon" analysis. Glucose, formaldehyde, acetic acid, and formic acid are among the many products found in the leach acid.

Key words: Battery; chromatography; sulfuric acid; trace organics.

This paper reports our approach to determine organic compounds in 4.5 mol/L H_2SO_4. Lead-acid battery separators made from cellulose fibers impregnated with a phenol-formaldehyde resin were leached in 4.5 mol/L H_2SO_4 at 75 °C for 20 h; our objective was to determine the soluble organic compounds present at $\geqslant$10 μg/mL in the leach acid.

Battery cellulose separators have four functional components: cellulose fibers, phenol-formaldehyde resin, wetting agent, and cellulose binder. Compounds expected to be obtained from each of the separator components are listed in Table 1.

Since acetic acid (HOAc) is know to be harmful to the battery [1], the first stage of our work was directed toward the separation, identification, and determination of the C_1-C_6 volatile fatty acids and the C_2-C_6 dicarboxylic acids. The separation was accomplished by the following schemes:

a) Solvent extraction by a batch process with chloroform, ether, or petroleum ether, and by a continuous process with ether. The organic solvent was then removed with the aid of a rotary evaporator. The protonating effect of H_2SO_4, which would tend to hinder volatility, was reduced by diluting the leach acid (1:1 and 1:4), or, more effectively, by adjusting to pH 2 with 9 mol/L NaOH in an ice bath.

b) Steam distillation at pH 2. The distillate was neutralized and evaporated to dryness. The salts were converted back to their corresponding acids by percolating their solutions through a column of strong cation exchange resin, Fisher Scientific Rexyn 101 (H^+). Steam distillation had the advantage over the solvent extraction of not adding additional organic compounds to the aqueous solution. This allowed us to use "total organic carbon" measurements, with a Dohrmann DC-50 carbon analyzer, to obtain a carbon balance between fractions.

Through the use of thin layer chromatography (TLC) [2] and infrared spectrometery (IR), we detected formic and acetic acids in the steam distillate. We detected no other volatile fatty acids or

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TABLE 1. *Suspected compounds leached from the separator components*

Cellulose	Phenol-formaldehyde	Wetting agent	Cellulose binder
D-glucose	o-hydroxybenzyl alcohol	dioctyl sulfo-succinic acid	polyamide-epichlorohydrin resin
C_1–C_6 carboxylic acids	p-hydroxybenzoic acid		
C_2–C_6 dicarboxylic acids	p-phenolsulfonic acid		
gluconic acid	1,4-benzoquinone		
glucuronic acid			
glutaric acid			
D-cellobiose			
levulinic acid			
5-(hydroxymethyl)-2-furfural			
2-furfural			
aliphatic aldehydes			
aliphatic ketones			

dicarboxylic acids. Solvent extraction provided no additional information on the carboxylic acids, but did show evidence of aromatic compounds. These compounds will be discussed later.

Acetic acid resists oxidation to CO_2 at the PbO_2 electrode [3], whereas the other organics in the leach acid are oxidized readily to CO_2. This reduced interference from the other compounds and allowed us to determine HOAc quantitatively by gas chromatography by direct injection [4] of a 0.5 μL sample of 2.5 mol/L H_2SO_4 solution. Direct injection a small sample and eliminating the errors of an additional separation step. We used 45 cm of Carbowax 20M/0.5% H_3PO_4 on 60/80 mesh Carbopack B (supplied by and conditioned as described by Supelco, Inc.). A sample chromatogram and conditions are shown in Figure 1.

Acetic acid may be measured at $\geqslant 5$ μg/mL over the life of the column; 15–20 injections of 2.5 mol/L H_2SO_4 were possible. The control chart, Figure 2, shows the empirical precision of the method at 80 μg/mL in 2.5 mol/L H_2SO_4. The positive bias of the mean (87 μg/mL found, x for $n=24$) reflects the data system's imperfect integration of the peak area with changing peak width and sloping baseline—caused by the degradation of the column by H_2SO_4; calibration solutions were prepared from glacial acetic acid in H_2O.

Although it is possible to detect the presence of formic acid, and to estimate its concentration from IR and TLC measurements, we did not pursue this analysis because formic acid is easily oxidized to CO_2 at the PbO_2 electrode in the lead-acid battery.

Preliminary TLC analysis of solvent-extracted leach solutions indicated that aromatic compounds were present. These were separated by reverse phase adsorption chromatography using SM-2 Bio-Beads (Bio-Rad Inc.). A 20×2.0 cm column of Bio-Beads was conditioned with 50 mL each of $CHCl_3$, $(CH_3)_2CO$, *n*-PrOH, MeOH, and 2 L of deionized H_2O. A glass filter was placed on the bed to prevent the bed from floating when the acid was added. The leach solution was poured through the bed at 5–10 mL/min and the H_2SO_4 washed out with H_2O. The absorbed organics were eluted with *n*-PrOH.

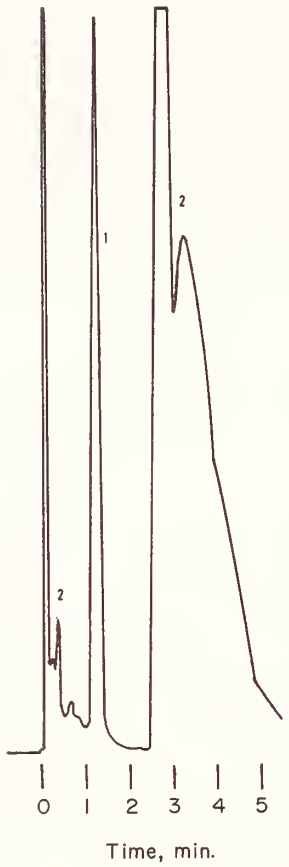

Figure 1. Gas chromatogram of 100 μg/mL acetic acid in 2.5 mol/L sulfuric acid—(1) acetic acid and (2) column degradation products caused by H_2SO_4. Conditions: sample size, 0.5 μL; glass column, 45 cm×2 mm i.d., 60/80 Carbopack B/Carbowax 20M/0.5% H_3PO_4 (Supelco); carrier, 30 mL/min He; flame ionization detector, 32×10^{-12} A/s; injector temperature, 180 °C; column temperature, 120 °C; detector temperature, 210 °C; instrument, Varian 3760; data system, Varian CDS 101.

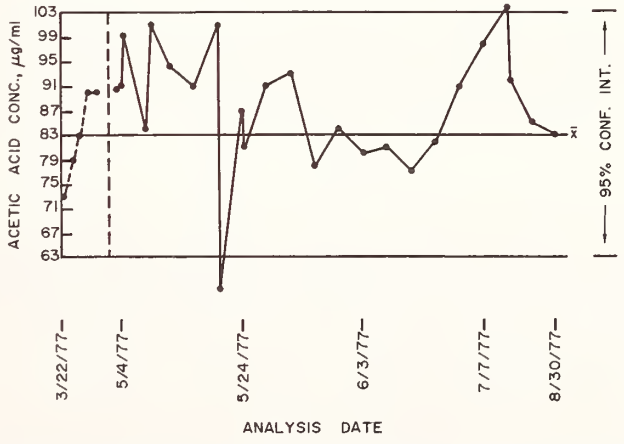

Figure 2. Control chart for 80 μg/mL acetic acid in 2.5 mol/L sulfuric acid—direct injection into gas chromatograph; individual daily results. n=5, x=83 μg/mL, s=7.3 μg/mL, 95% confidence interval=83±20 μg/mL.

Waters Assoc., Inc., also used reverse phase absorption chromatography to determine the feasibility of separating the major phenol-formaldehyde decomposition product by HPLC. A $C_{18}\mu$-Bondapack column was used with a H_2O to MeOH solvent gradient and 254 nm UV detector with these samples: (a) the leach acid, (b) the *n*-PrOH effluent of the Bio-Beads, and (c) a solution of 5.0 g/L glucose in 4.5 mol/L H_2SO_4 that was heated to 75 °C for 20 h. The chromatograms indicate that most of the peaks are from the decomposition of glucose. Peaks 12, 13, and 14 in Figure 3a are presumed, from comparison with Figure 3c, to be products of the resin. Figure 3b is included to show that the peaks are not caused by reaction of H_2SO_4 with the column.

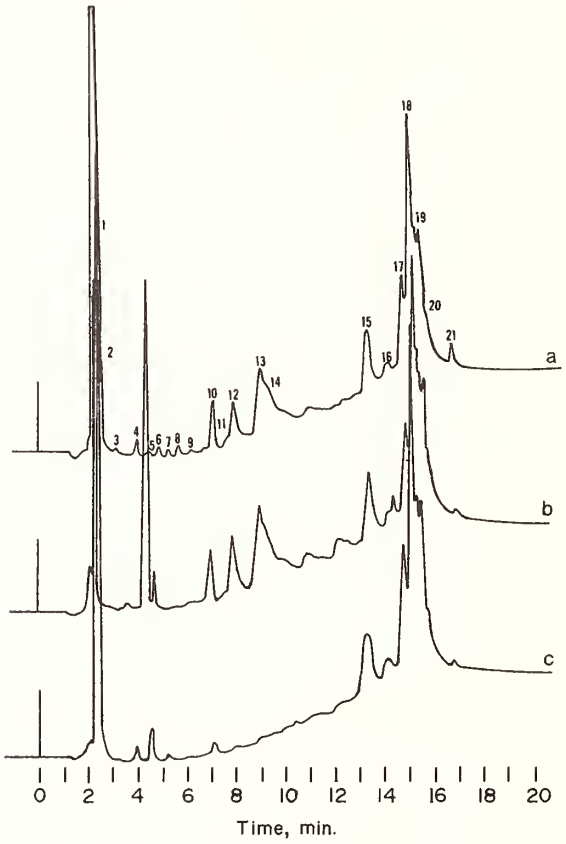

Figure 3. High pressure liquid chromatograms of (a) separator leach acid, (b) *n*-propanol effluent of Bio-Beads after absorption of more hydophobic organics from separator leach acid, and (c) 5.0 g/L glucose in 4.5 mol/L sulfuric acid that was heated to 75 °C for 20 h. Conditions: sample size, 5 μL; column, $C_{18}\mu$-Bondapak; solvent, water to methanol gradient; instrument, Waters Associates model 204 with 440 detector at 254 nm, 6000A pumps, and 660 solvent programmer.

We separated an organic material from the separator leach acid by neutralization with $Ba(OH)_2$, centrifugation, reverse phase absorption chromatography with Bio-Beads, and evaporation. The organic material thus obtained was identified primarily as glucose by comparison of IR spectra, Figure 4. Glucose showed a close match except for a peak at 1600 cm^{-1} in the residue from the leach. But when a glucose solution in 4.5 mol/L H_2SO_4 was heated to 75 °C for 20 h, the separated organic material showed a strong peak at 1600 cm^{-1} (Fig. 4c). Concurrent NMR, paper, and thin layer chromatography tests also indicated glucose.

Quantitative determination of glucose was based on Dische's method [5]. L-Cysteine was reacted at room temperature with the product formed by glucose in hot 86% H_2SO_4. The absorbance of the resultant yellow solution was measured at 406 nm for 10–100 μg/mL glucose.

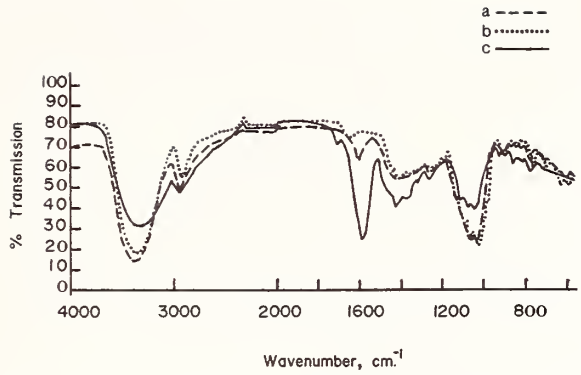

Figure 4. Infrared spectra of evaporated film on potassium bromide from methanol. (a) Organic material separated from leach acid, (b) glucose, and (c) glucose and its reaction products separated from 4.5 mol/L H_2SO_4 after reaction at 75 °C for 20 h.

There was an interfering absorption in the leach acid, but the concentration of glucose was so high that a sample diluted 1/100 eliminated the need for corrective measure.

Formaldehyde was separated from the leach acid as an insoluble derivative of 2,4-dinitrophenylhydrazine. The IR spectrum in Nujol matched that of an authentic sample.

Summary of Results

Table 2 gives a summary of the results. Glucose was present in the greatest concentration: 5500±300 μg/mL (95% conf., 6 runs). HOAc was present at a concentration of 150±40 μg/mL. Formic acid appeared from the TLC data to be present at a slightly higher concentration than HOAc. Formaldehyde is present in the leach acid and is probably a product of cellulose and phenol-formaldehyde decomposition. We observed no other ketones or aldehydes. The results of

TABLE 2. *Summary of analysis of 4.5 mol/L H_2SO_4 separator leach acid*

Separation Technique	Fraction	Total carbon (μg/mL)	Total carbon (%)	Approx. No. of compds.[a]	Compd.	Conc. (μg/mL)
	hydrophobic	50±100	2±4	22	b	--
reverse phase ads. chromatog. SM-2 Bio-Beads						
	hydrophilic non-volatile	2400±500	90±20	4	glucose[c]	5500±300
steam distillation						
	hydrophilic volatile	200±80	7±3	3	acetic acid[d]	150±40
					formic acid	200 (est.)
					formaldehyde[e]	---

[a] Estimated primarily from HPLC and TLC results.
[b] Benzene sulfonic acid class compound present. Most of the compounds are reaction products of glucose.
[c] Di- and trisaccarides, and anhydroglucose tentatively identified.
[d] Electrochemically treated solution.
[e] No other adehydes or ketones detected.

the HPLC data indicate that the major aromatics may be separated by this method and could presumably be identified. It appears that most of these compounds are decomposition products of glucose.

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ANALYSIS OF TRACE PLANT CONSTITUENTS

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The isolation and identification of trace plant constituents such as N,N-dimethyltryptophan, *m*-hydroxyphenylacetic acid, and 5β-cholanic acid were achieved by microanalytical methods. Two methods for analysis of abscisic acid in the plant tissue are described.

Key words: Abscisic acid; gas chromatography; liquid chromatography; plant constituents.

In medicine, food and pollution, there is an ever increasing necessity to detect and analyze trace plant constituents at subnanogram levels. Many of the analytical and chemical methods fail to detect these plant constituents at such low levels unless suitable modifications of existing methods or new techniques are introduced. Since the need for such analyses is so great in many areas of chemistry of natural products, we have undertaken work on analytical methods in the following areas:

(1) Plant growth regulating substances;
(2) toxins, compounds produced by microorganisms acting as plant parasites;
(3) biogenetic markers which are trace compounds obtained from plants and useful for elucidating biogenetic pathways and plant taxnomic classifications.

Some examples in the above areas are shown in Figure 1.

Many naturally occurring plant growth substances have been detected, isolated and identified in the last decade after new analytical methods became available. It may not be an exaggeration to say that without the new methods and instrumentation (particularly recent advances in chromatography and mass spectrometry), many newly identified compounds would not be known to us today. We have isolated a growth inhibitor from *Abrus precatorius* seeds and unequivocally identified the growth inhibitor [1] as N,N-dimethyltryptophan (Ia). This compound inhibits the growth of lettuce, tomato, and other seedlings. It also affects auxin and kinetin-simulated ethylene production and protein and RNA synthesis. Since it is present in a few ppm, it was not detected earlier in *Abrus* seeds, although this seed was subjected to extensive investigation for more than 50 years, because of a) the presence of the toxic substance, abrin and b) its ornamental value. Using GC-MS, we have successfully analyzed the compound Ia. We have also identified N-methyltryptophan (Ib) which appeared to be a biosynthetic intermediate of Ia from the precursor, tryptophan (Ic).

The pathogenic fungus, *Rhizoctonia solani,* is a plant parasite that produces toxic substances which are responsible for its pathogenicity. The literature documents numerous reports on the isolation of fungal toxins from various plants affected by *R. solani.* We became interested in this problem because this fungus reduces the nodule nitrogen fixation of soybeans and thereby affects the yield. In cooperation with Dr. R. Orellana, we have successfully isolated a toxic substance from a culture filtrate and identified it as *m*-hydroxyphenylacetic acid (IIa). We have also found *m*-methoxyphenylacetic acid (IIb) in the same culture filtrate. It is interesting to note that the

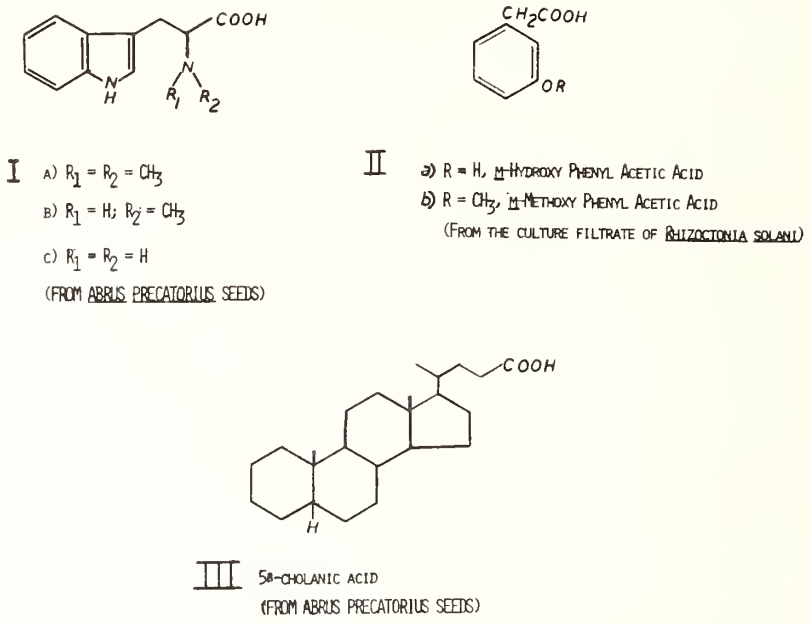

Figure 1. Chemical structures of some biologically important compounds isolated from plant sources.

fungus produces different isomers of hydroxyphenylacetic acid, depending on the plant source. If the fungal strain is isolated from sugar beets, one obtains phenylacetic and *m*-hydroxyphenylacetic acids [2]. On the other hand, if it is from potato, both *m*- and *p*-isomers of hydroxyphenylacetic acid [3] are present in the culture filtrate. From the fungus-affected soybeans, we obtained *m*-hydroxy and *m*-methoxyphenylacetic acids. We have developed trace analytical methods to detect the hydroxyphenylacetic acids. It appears that on the basis of isolated hydroxyphenylacetic acids one could develop a screening method to find plants which are affected by the fungus. Incidentally, phenylacetic acid and its hydroxylated derivatives obtained from such plants as tobacco have been reported to possess growth-regulating properties.

Our analytical methods for the analysis of phenylacetic acids include high performance liquid chromatography (HPLC) (see Fig. 2) [4] and gas chromatography (GC) using TMS derivatives. These methods are more sensitive than the previously described TLC and paper chromatographic systems. For a complete structural analysis, carbon-13 NMR appears to be very promising (Table 1), although one could use the proton NMR, IR, UV and mass spectrometry as well.

It has long been thought that certain chemicals are only derived from animal origin and not from plant sources. One such chemical is a steroidal acid, 5β-cholanic acid (III) which was found previously only from an animal source. On the basis of its presence, petroleum containing it was thought to be of animal origin. We have identified the acid (III) in trace amounts from Jequirity bean seeds by GC-MS after purification by various chromatographic methods [5]. This is the first example demonstrating that plants are capable of producing both isomers (5α and 5β) of cholanic acid. Further, the presence of acid III along with other chemicals may be useful in classifying plants on the basis of chemical compounds, a newly emerging field of chemical taxonomy which will complement plant taxonomy based on morphological attributes.

In connection with our physiological, biochemical and metabolic studies on plant hormones, we required methods to measure them at submicrogram levels. Bioassays were often used for this purpose but quantitation is lacking in this approach. To develop new methods we chose abscisic acid (ABA) because it is present in many plant tissues. We developed two methods for analysis. One method involves the isolation of ABA by a standard procedure and the active fraction was

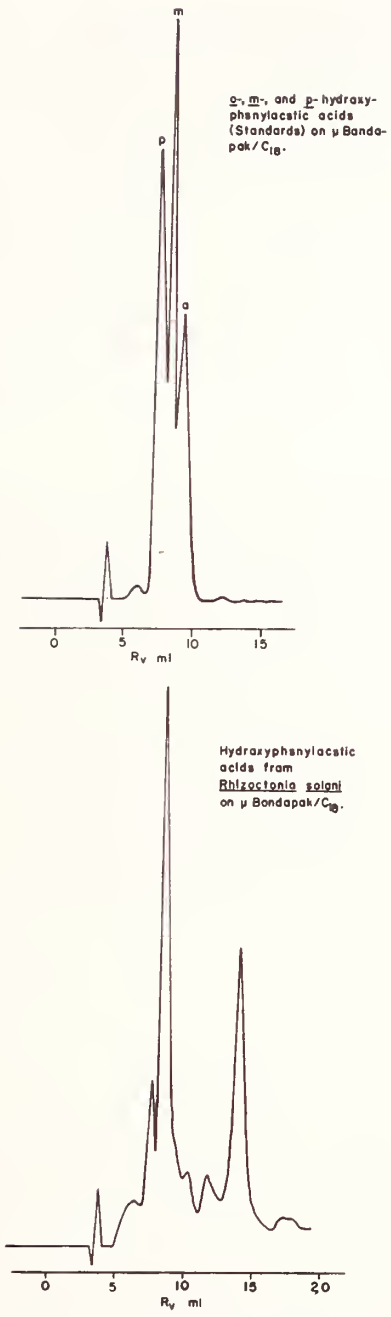

Figure 2. HPLC comparison of hydroxyphenylacetic acids (standards) and the isolated *m*-hydroxyphenylacetic acid from the culture filtrate of *R. solani*.

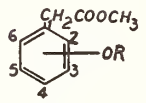

Table 1. Carbon-13 NMR Data on Phenylacetic Acid Derivatives

Methyl Ester Of	Carbon - 13 Chemical Shifts (δ_C ppm from TMS)									
	C=O	Ester CH_3	Methoxy CH_3	CH_2	C-1	C-2	C-3	C-4	C-5	C-6
1. o-Hydroxyphenyl-acetic acid	174.4	37.3	--	52.6	120.7	155.2	117.4	131.1	120.9	129.3
2. m-Hydroxyphenyl-acetic acid	173.0	41.0	--	52.2	129.9	114.5	156.3	116.4	135.4	121.5
3. p-Hydroxyphenyl-acetic acid	173.5	40.2	--	52.1	125.6	130.5	115.7	155.3	115.7	130.5
4. o-Methoxyphenyl-acetic acid	172.4	35.6	55.4	51.7	120.6	157.7	110.6	130.0	123.2	128.6
5. m-Methoxyphenyl-acetic acid	172.1	41.1	55.1	51.9	129.7	115.0	159.9	121.7	135.5	121.7
6. p-Methoxyphenyl-acetic acid	172.5	40.2	55.2	51.9	126.2	130.4	114.1	159.0	114.1	130.4

analyzed by GC and HPLC methods. We prepared methyl and p-nitrobenzyl (PNB) esters. The PNB ester has higher (60%) UV detection limits in HPLC although in GC analysis, the methyl ester appears to be superior with electron capture detection [6]. Application of GC and HPLC methods (Fig. 3) resulted in detecting ABA at submicrogram levels.

In another method, the fragment ions resulting from ABA-methyl ester [7,8] were selectively monitored. The intensities of these fragments were compared with the fragment ions of the hexadeutero-ABA methyl ester which served as an internal standard. In this mass fragmentometric method, we have chosen to simultaneously measure the m/e 190 and 162 from ABA samples. These fragment ions were compared with the fragment ions arising from the standard ABA-d_6-methyl ester which gives corresponding fragments at m/e 194 and 166. A calibration curve (Fig. 4) was constructed with different concentrations of ABA in which 100 ng of internal standard was used. This curve can be used to measure the amount of ABA per gram of tissue. From our initial work it appears that this method is very sensitive for ABA detection in subnanogram quantities.

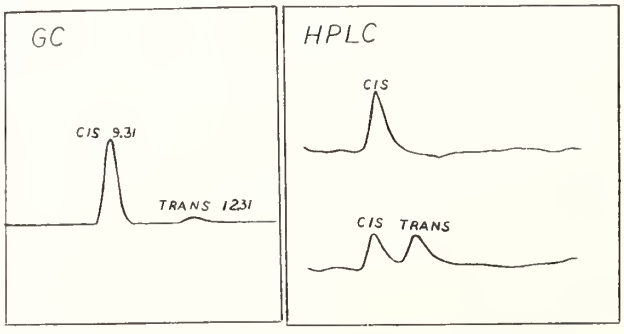

Figure 3. Analysis of ABA-PNB ester by GC and HPLC.

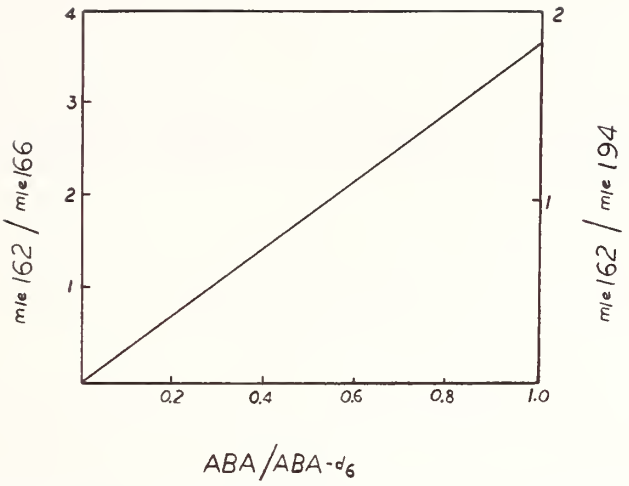

Figure 4. Calibration curve for ABA quantification by MF suing 100 ng ABA-d_6 as an internal standard.

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GC-ECD ASSAY AFTER SAMPLE WORK-UP: NOMENCLATURE FOR STANDARDS, AND SOME POINTS OF PRACTICE

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For trace-organic assays where spiked-in standards are put through the whole procedure, a nomenclature is advocated to reduce present-day diversity and ambiguity, especially where the end-step is GC, HPLC or TLC. The reliability of such assays, e.g., for basic drugs in blood, can be improved by measures to deal with problems such as adsorptivity.

Key words: Chromatography/column packings (GC); electron-capture detection; evaporation; nomenclature; sample work-up; standards.

I. Standards

The following terms are suggested (with alternatives in parentheses) as the *adjective* to distinguish the type of standard, in the context of multi-step assays with a final separatory procedure which can allow the use of an internal standard, normally akin to the test compound "X."

(1a) "X" itself, spiked initially into realistic raw samples, to furnish the calibration curve: *Determinate* (a term suggested by J. A. F. de Silva) (or *Processed*).

(1b) "X" introduced near the end of the procedure, merely for recovery assessment or for reassurance about the late part of the assay: *Unprocessed* (or *Chromatographic* or *Instrument*), except where "X" has to be chemically modified and an appropriate adjective such as *Derivatized* or *Hydrolyzed* is preferable, signifying semi-processed starting midway.

For a "wobble corrector," different from "X" itself and put into each test sample to improve precision, the term "internal" is equivocal. Hence

(2a) Introduction at the outset of a compound which behaves similarly to "X" and is finally measured: *Processed-analog* (or *Processed-internal*), with the *prefix Isotopic* if applicable (can be Radioisotopic, Stable-isotopic).

(2b) Where radiolabelled "X" is introduced initially but has to be counted prior to the final step, serving to assess sample-preparation losses: *Isotopic work-up.*

(2c) Inclusion in the final step of a compound which need not resemble "X" and is a check on load applied: *Load* (or *Injection* (inapplicable to TLC) or *Unprocessed-internal* or *Non-extracted internal*).

The following terms are *not* favoured, because of ambiguity: Assay, Calibration, External, Recovery, and—although advocated by J. A. F. de Silva for (2a)—Reference.

II. Points of GC Practice

It was through collected lore rather than literature that we eventually solved problems in the assay of haloaklylated basic drugs, including metoclopramide [1], by GC with a ^{63}Ni EC detector of fixed-pulse type. For two reasons, we were merely glimpsing the drug peak: there was tenacious adsorption onto the column packing, and swamping of the detector attributable to insidious

chronic bleed of stationary phase (or, at one stage, to traces of flux from a new N_2 pipeline). The column bleed, which did not impair detector cleanliness, was evidently particularly prone to occur if the support had been unevenly coated (also a cause of adsorptiveness), as when support particles were tipped into a chloroform dispersion of the stationary phase (e.g., SP-2100) in the first stage of the "evaporative" procedure. We rediscovered an old, undocumented observation [2]—that the stationary phase can best be *added to* the slurried support: otherwise the first lot of support particles presumably acquires irreversibly all the dispersed stationary phase. This is less likely to happen if thorough pre-dispersal of the stationary phase is ensured by refluxing or aging. On-column silylation offers no cure, but has proved to be of prophylactic use where there is incipient adsorptiveness towards a troublesome compound [1].

III. Points of Sample-Preparation Practice

(A) When only μL volumes of standard are spiked in (by syringe), to avoid subsequent solvent extraction by solvent from the spike medium, resulting in erratic losses, the order of addition to the dry tube should be blood *followed by* spike. (B) Later, however, an alcohol supplement can be helpful, not only as a means of minimizing adsorption when a solvent extract is to be stored overnight [3] but also, suitably with methanol in small volume, to rinse down the tube wall when an extract is being dried down under a nitrogen stream. (C) In the latter operation it should not be taken on trust that both drug and analog (internal standard) are involatile, especially after derivatization for GC-ECD. If radiolabelled drug was added at the outset, counts should be checked *after* derivatization. (D) As can likewise be revealed by counts, a low-polarity solvent such as haptane may be inefficient in dissolving a derivatized residue for GC. (E) When it is necessary to cold-store glass vessels such as GC vials, one should not take it on trust that a press-in as distinct from press-on plastic cap will remain tight-fitting.

IV. Acknowledgments

Suggestions and help came from colleagues including Dr. J. P. Leppard and Dr. A. D. R. Harrison, and from many outside experts including Dr. B. S. Thomas (Imperial Cancer Research Fund Labs.), Mr. L. Zappe and Mr. P. J. Joynes (Pye Unicam), and Dr. B. Scales and colleagues (ICI Pharmaceuticals).

V. References

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16. ABSTRACT (*A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here.*)

Researchers in diverse areas must currently perform critical analyses on minute quantities of organic compounds in various matrices. It was the aim of this Symposium to bring together these scientists to discuss their common problems and to explore current and impending technology for organic analyses. Emphasis was placed on the total analysis, from collecting the sample through interpreting the results, rather than upon the measurement only.

The Proceedings consist of a series of invited papers by experts as well as particularly appropriate contributed papers. Topics covered in the Proceedings are as follows: Sampling and Sampling Handling for Trace Organic Analysis, State-of-the-Art Analytical Systems, Analytical Techniques on the Horizon, Analysis of Nutrients, Analysis of Organic Pollutants and Their Metabolites in the Ecosystem, Analysis of Drugs in Body Fluid, Analysis of Food Toxicants, and Analysis of Hormones and Neurotransmitters.

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