NBS SPECIAL PUBLICATION 635

U.S. DEPARTMENT OF COMMERCE/National Bureau of Standards

Reference Materials for Organic Nutrient Measurement

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Reference Materials for Organic Nutrient Measurement

Proceedings of a Workshop held at
the National Bureau of Standards,
Gaithersburg, Maryland, October 23, 1980

Edited by Sam A. Margolis

National Measurement Laboratory
Center for Analytical Chemistry
National Bureau of Standards
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PREFACE

In 1980, President Carter signed into law a bill (Public Law 96-359) regulating the minimal nutritional content of infant formula. Furthermore, the FDA regulation promulgated in 1973 mandated full nutritional labeling on products whose merchandising includes any nutritional claims or information. The accuracy and precision of some of the methods used in measuring nutrient content are inadequate and high-quality standard materials in representative food matrices, are not always available for use in standardizing analytical procedures.

The objectives of this Workshop were to assess the state-of-the-art of the measurement of organic nutrients in food matrices for the purpose of developing a sound rationale for preparing suitable Standard Reference Materials (SRM's). These proposed SRM's will complement the SRM's which are already certified for inorganic nutrients in food matrices. The aim of this meeting was to bring together scientists from the industrial, research, and regulatory communities to discuss the problems and propose strategies for the development of SRM's including accurate and precise methods of measurement. A priority list of organic nutrients and suitable matrices to be certified as SRM's, was developed by the workshop participants, who made a very important contribution to the knowledge and understanding of the stability and measurement of organic nutrients in foods.

ABSTRACT

This publication is the formal report of the Workshop on Reference Materials for Organic Nutrient Measurement held at the National Bureau of Standards on October 23, 1980. There were seven formal presentations which provided the framework for three workshop sessions. Each workshop session focused on one of three groups of nutrients: (1) cholesterol, fat, and fat-soluble vitamins; (2) water-soluble vitamins; or (3) sugars. Each workshop session reported on the state-of-the-art in measurement techniques, suggested matrices which were appropriate for Standard Reference Materials (SRM's), and indicated areas where there were problems in measurement methodology. These recommendations are included in this publication.

Key Words: food matrices; methods of measurement; nutrients; SRM's; stability; vitamins.

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WELCOMING REMARKS

I welcome you on behalf of the National Bureau of Standards to this Workshop on Reference Materials for Organic Nutrient Measurements. This workshop is a cooperative effort between NBS and the Food and Drug Administration, Department of Agriculture, and the National Food Processors Association. We are very pleased to see the turnout this morning, which I think is a bit more than we anticipated. I would like to call your attention to the brochure which states rather explicitly the purpose for this meeting. Let me articulate that purpose in my own words. This meeting has been called to develop rationalizations for a series of Standard Reference Materials for the measurement of nutrients. I would like to point out to you that NBS has been involved for many years in problems of measurement standardization in various areas of the health care industry. Our work in clinical chemistry, dental materials, and certain surgical implant materials is well known. We also play the leadership role in developing reference materials in many of these areas and, in fact, we now offer Standard Reference Materials for trace elements in food matrices, such as spinach and flour. We recognize that there is a growing interest in standardization and measurement of nutrients in other matrices and the exploration of this area of interest is the purpose of this meeting today. According to your organizers during the next few hours you will assess the state-of-the-art in the measurement of organic nutrients in various matrices, examine nutrient stability in the matrices, develop priority lists of nutrients for standardization (That is something that is exceedingly important to us, particularly for the people operating our Standard Reference Materials program.) and develop a list of most appropriate matrices for each group of nutrients. This is a big job and is going to require a lot of deliberation in your working groups this afternoon. I think your steering committee has done an excellent job in arranging speakers for you this morning. I wish you the best of success in the deliberations over the next day and a half and I hope that you will provide a very useful list of priorities for us. Thank you.

D. R. Johnson
Deputy Director
National Measurement Laboratory

A GENERAL OUTLINE OF THE PROGRAM FOR THE NBS WORKSHOP ON MEASURING NUTRIENTS IN FOOD

The major stimuli for this workshop came from three directions: the recent legal requirement to measure accurately the nutritional content of infant formula, the lack of availability of suitable standard materials for the measurement of organic nutrients in food matrices, and the wide industrial and governmental acceptance of NBS Standard Reference Materials (SRM's) for the measurement of trace elements in foods and the measurement of alcohol in wine. With these points in mind the first paper of the first session of the workshop examined the industrial need for SRM's in the development of an accurate data base for assessing human nutrition with respect to the levels of organic nutrients and trace elements. SRM's are available from NBS for the measurement of trace elements in foods. However there is no single source of Certified Reference Materials for the measurement of organic nutrients.

Some reference materials for specific development or evaluation of methodologies are intermittently provided by the National Food Processors Association and the Association of Official Analytical Chemists. There is no traceable, continuous source of SRM's for the measurement of organic nutrients in food matrices which are comparable to the NBS SRM's for the measurement of trace elements. The second paper outlined the process of development of the SRM's proposed for organic nutrients and the possible cooperative role of NBS and industry in the development of such standards.

The remaining formal presentations examined the current state of the accuracy and precision of the measurements of organic nutrients in foods and identified the major problem areas in the measurement of these nutrients. We will try also to delineate some of the conditions required for long-term stability of organic nutrients in foods.

The afternoon workshop session composed of three parallel meetings dealing with separate classes of nutrients was charged with examining the current methods of measurement and recommending the most accurate ones for use in the analysis of potential SRM's. Each workshop group was also instructed to recommend a priority list of organic nutrients and appropriate matrices for development as SRM's. These efforts will provide the guidelines for the NBS effort to prepare organic nutrient SRM's to meet the need of the industrial community.

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National Bureau of Standards Special Publication 635. Proceedings of the Workshop on Reference Materials for Organic Nutrient Measurement held at NBS, Gaithersburg, Maryland, October 23, 1980.

THE ROLE OF STANDARD REFERENCE MATERIALS IN THE DEVELOPMENT OF A SOUND DATA BASE FOR THE ASSESSMENT OF HUMAN NUTRITION

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1. Introduction

Food composition data bases are basic tools for nutrition researchers, clinical nutritionists, dieticians, and food scientists. Such data bases are essential for developing menus for groups of people and formulating specific therapeutic diets for individuals. They are necessary for assessing the nutrient adequacy of the dietary intake records of groups or individuals. Establishment of the relationship of foods to health requires use of data bases listing the various organic and inorganic nutrients and contaminants in specific foods. Likewise, the challenge of improving the nutritional quality of foods, meeting standards of food identity and nutrient fortification, and formulating new food products also requires extensive data on food composition.

Food composition data are of great importance to the Food and Drug Administration in carrying out its responsibilities of safeguarding the public health by insuring the safety and nutritional quality of the food supply. Such a task requires surveillance of individual food comsumption; conversion of food consumption data to nutrient consumption data (with food composition data bases); and research activities on the use of nutrient consumption as a predictor of health status. Surveillance of food consumption allows FDA to continue assessing the nutritional effects of the food supply on population groups.

The development of food composition data bases is a highly complex task. Data, old and new, are available from many sources – private laboratories, food industry, academic institutions, and government agencies. The means, ranges, and standard deviations of nutrient values obtained in various laboratories by various methodologies need to be evaluated and integrated. Standard reference materials (SRM's) are going to be extremely valuable in the integration of nutrient values from various laboratories so that the quality of nutrient composition data bases can be improved. These reference materials of certified quality serve as points of common reference and allow data from different laboratories determined by different methods to be compared, evaluated and integrated. SRM's will promote the development of new methods of nutrient analysis which will be comparable with current methods. SRM's will play a valuable role in nutrition labeling of foods. It is anticipated that future additional nutrition labeling will be based on nutrient data banks. Use of data banks for nutrition labeling of foods is desirable to prevent severe economic burdens on small food companies who do not have the resources for extensive analytical work. It is essential that the data from the nutrient banks be accurate to fulfill the purposes of nutrition labeling.

2. Food Labeling

The concepts of nutrition labeling were initiated at the White House Conference on Food Nutrition and Health in 1969. These concepts were later incorporated in an FDA regulation proposed in March 1972, and promulgated as a final regulation in 1973, entitled "Food, Nutrition Labeling". This regulation provides for voluntary declaration of nutrition information, but regulates the substance of the information, and specifies a mandatory format for presenting that information. For food products which contain added nutrients or for products whose merchandising includes any nutrition claims or information, full nutrition labeling is mandatory. Fresh fruits and vegetables have been exempted from the provisions of these nutrition labeling regulations.

The nutrition information on the label includes: serving size; servings per container; calories; protein, carbohydrate and fat in grams; and eight mandatory nutrients expressed as a percentage of the U.S. Recommended Daily Allowance (U.S. RDA). These eight mandatory nutrients are:

protein	riboflavin
vitamin A	niacin
vitamin C	calcium
thiamin	iron

Optional nutrients (vitamin D, vitamin E, vitamin B-6, folic acid, vitamin B-12, phosphorus, iodine, magnesium, zinc, copper, biotin, and pantothenic acid) may be listed on food labels and must be listed when they are added to a food. Currently the amount of sodium may be declared without requiring full nutrition information. Nutrient quantities are expressed in terms of an average serving. If the nutrients occur in amounts less than 2 percent of the U.S. RDA per serving, this is indicated by a zero or the statement "contains less than 2 percent of the U.S. RDA for this nutrient".

Since the 1973 regulation, many consumers have advocated more label information, whereas industry spokespeople have questioned whether labeling has reached the saturation point both for label space and for the consumer's capacity to utilize the information. In 1978, FDA joined with USDA and the Federal Trade Commission (FTC) to review the total food label and consider updating the existing food labeling laws and implementing regulations. USDA is responsible for the laws and labeling of meat and poultry products, FDA is responsible for all other foods, and FTC is responsible for the regulation of food advertising.

This was the first joint effort of these three agencies to review the entire field of food labeling laws and regulations. They developed issue papers on various labeling topics and published these papers along with an announcement of public hearings which were held in five cities during the fall of 1978. Consumers were also invited to submit written comments regarding these papers. The topics included: ingredient labeling; nutrition labeling and other dietary information; open date labeling; food fortification; imitation and other substitute foods; safe and suitable ingredients; and the total food label as a communications device.

Over 9000 comments were received, more than 2800 people attended the hearings, and 452 individuals testified. After careful review, a task force of FDA, USDA, and FTC members prepared a report entitled "Food Labeling Report on the Analysis of Comments".

This report plus the results of a Consumer Food Labeling Survey of a statistically representative sample of the population were used to formulate tentative positions on changes in food labeling. These tentative positions are summarized in the December 21, 1979 Federal Register which lays out the strategy that FDA and other agencies plan to use in carrying out the first comprehensive revision of food labeling. This revision has a high priority in FDA and is expected to take 3-4 years to accomplish. The major concerns relative to nutrient labeling are: voluntary vs. mandatory requirement for nutrition labeling; information furnished by the label; and format of the information on the label.

The following regulatory initiatives are being pursued:

1) FDA and USDA will seek legislative authority to make nutrition labeling mandatory or to require nutrition labeling at their discretion. Many foods are already labeled nutritionally. An FDA survey based on 1976 sales showed that on a dollar basis, approximately 40 percent of all packaged processed foods and 24 percent of all foods in supermarkets carried nutrition labeling and that 60 percent of foods with nutrition declarations were labeled voluntarily. The three agencies have formed a task group to develop criteria for determining which additional foods should carry nutrition labeling. This group has delayed action pending legislation in Congress. The criteria will primarily reflect the significance of the food in the diet, the potential for misleading the public without labeling, and other matters of public health significance. FDA seeks authority to require nutrition labeling on many food categories which contribute to the diet in a significant way. To help relieve industry of the task of nutrient analysis, the agencies have formalized a policy pertaining to the use of a composite data base for labeling those foods to which no nutrients have been added.

2) The agencies have agreed to conduct research to determine which nutritional labeling format consumers find more useful and convenient. Industry will be encouraged to experiment with formats consistent with the principle of the current quantitative system.

3) FDA will propose regulations to establish uniform serving sizes for foods.

4) The Agencies plan to make sodium and potassium labeling a mandatory part of nutrition labeling format and will ask for legislative authority to require such labeling on appropriate foods.

5) The Agencies will also require that cholesterol and fatty acids be linked in nutrition labeling so that, if a claim is made for either, or if the manufacturer elects to include one on the label then the other would also be required.

6) The Agencies plan to make labeling of total sugars a mandatory part of nutrition labeling format and will ask for legislative authority to require such labeling on appropriate foods.

7) No change in fiber labeling is proposed, but FDA will intensify research efforts relative to methodology for fiber and its value in the diet.

Nutritional labeling is an effective educational tool. Its principal purpose is to provide consumers with meaningful nutrition information and allow for product comparisons. It likewise allows the consumer, measured assurance of nutrient content through government regulations. Any product which carries nutrition information is subject to FDA laboratory analyses for all nutrients claimed to be in the packaged product. To be in comformance with regulations, labeled products must meet three criteria:

1. For essential nutrients which have been added to the product, FDA's analytical values for a composite sample from 12 packages of the product must equal or exceed label claims.

2. For essential nutrients that are indigenous to the product or its food ingredients, or for claimed polyunsaturated fat, the analytical values must equal or exceed 80 percent of the label claims.

3. Analytical values for calories, carbohydrate, fat, saturated fat, cholesterol, and sodium must not exceed 120 percent of the label claims. Products not meeting these criteria may be declared misbranded.

3. Infant Formula

One specific type of food which requires mandatory declaration of nutrients is infant formula. The President recently signed a bill which specifies the nutrient composition for infant formula. These nutrient specifications are based on the recommendations of the Committee on Nutrition of the American Academy of Pediatrics (CON/AAP). In addition to the responsibility of the manufacturing company for the information provided on the nutrition label, a large number of independent, university, and government laboratories are performing additional analyses on infant formula collected from store shelves. For many of these laboratories this is the first time they have analyzed infant formula. As a result, FDA receives many complaints that Infant Formula Brand X is deficient in certain nutrients. When these complaints are received, samples are collected and analyzed, not only for the suspected deficient nutrients but for the other nutrients as well. The result has been that most of the reported deficiencies are not really deficiencies at all but are errors in the analyses of the products. The availability and use of a Nutrient Standard Reference Material would not guarantee accurate analyses but would at least provide the laboratory with a benchmark to gauge the accuracy of their analytical results.

4. Association of Official Analytical Chemists

The Association of Official Analytical Chemists (AOAC) is a unique, non-profit scientific organization whose primary purpose is to serve the needs of government, regulatory and research agencies for analytical methods. The goal of the AOAC is to provide methods which will perform with the necessary accuracy and precision under usual laboratory conditions. Since its founding in 1884 the AOAC has provided a mechanism to select methods of analysis from published literature or develop new methods, collaboratively test them through interlaboratory studies, approve them, and publish the approved methods. Only methods validated by collaborative tests receive approved status and only approved methods are included in the AOAC compendium, "Official Methods of Analysis."

Although "Official Methods of Analysis" contains many analytical methods, numerous methods in widespread use are not currently included. These methods are not included because the collaborative studies necessary for acceptance of the methods have not been completed. Perhaps the primary reason for this situation is that laboratories are unwilling to participate in these studies. Therefore, although the AOAC Book of Methods may be regarded as unique and fulfilling a valuable function by compiling tested, approved methods, it must also be recognized as being incomplete because it does not contain all currently used analytical methods for nutrients.

Perhaps the ideal analytical results would be produced by a capable analyst, using proven AOAC methodology and analyzing National Bureau of Standards (NBS) SRM's along with the samples to provide a quality control check. Problems arise because sometimes all three are not available. There are certainly many capable analysts and the AOAC Book of Methods contains many analytical methods. There are even several SRM's available; however, these are certified only for trace and major elements. There are currently none for organic nutrients. A primary purpose of this workshop is to investigate the development of one or more SRM's certified for organic nutrients.

5. Summary

In summary it can be said that:

1. Nutrition labeling currently is a voluntary regulation, but it is in widespread use. Pending legislation would provide USDA and FDA with authority to make nutrition labeling mandatory.

2. Data banks have been used to provide nutritional information for foods which contain only indigenous nutrients, and use of data banks is expected to increase as mandatory labeling requirements are issued. The information put into the data banks must be of the highest quality to ensure accurate nutritional information.

3. New and/or improved analytical methodology will be developed which is rapid and perhaps automated in order to provide continuous quality control and to further enhance the data banks.

4. Availability of Standard Reference Materials is vital to the integration of nutrient data, laboratory quality control, and analytical methods development. It is hoped that this workshop will provide the basic information necessary to the development of SRM's for organic nutrients and will provide a framework whereby future SRM's may be selected and developed.

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THE PROCESS AND REQUIREMENTS FOR THE DEVELOPMENT OF A STANDARD REFERENCE MATERIAL

(Copy of Notes Used During Presentation)

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1. Reference Materials

Standard Reference Materials are well-characterized stable materials that are produced in quantity and have one or more physical or chemical properties measured and certified by NBS. Today I'd like to talk about the technical and programmatic requirements for the development of an SRM – with particular emphasis on potential SRM's for organic nutrient analysis. I'll also comment briefly on what I perceive is the role of SRM's in helping to assure accurate and compatible measurements on a national scale. NBS now has available for sale nearly 1,000 different SRM's for use in four major areas:

(1) Industrial Quality Control

(2) Environmental Analysis and Monitoring

(3) Clinical Analysis and Health Related Measurements including Botanical/Biological

(4) Basic Metrological Applications.

During fiscal year 1980, NBS distributed over 40,000 SRM units to approximately 10,000 users throughout the world.

2. Guide for Requesting Development of SRM's

Generally the three criteria shown here should be satisfied before NBS will undertake development of a new SRM.

1. A technical need – usually associated with a critical measurement problem – must be identified, i.e., improving the accuracy of an important measurement method.

2. The production of the SRM at an organization other than NBS should not be technically or economically feasible or appropriate.

3. The SRM should address an industry-wide or community wide need rather than one of a particular manufacturer or individual laboratory.

In a few cases, the SRM's are mandated for use by federal regulations – many of these address public health and safety problems such as in Air Pollution monitoring.

NBS would appreciate obtaining information which justifies the technical need for particular SRM's for organic nutrient analysis in terms of economic impact and/or impact on public health and safety. Some consideration should also be given to estimating future demand for the particular SRM. For your information, the average compositional SRM sells about 100 units per year. NBS would also like to be made aware of laboratories that are willing to participate in the preparation or analysis of the materials. Evidence of outside laboratory cooperation considerably strengthens justification for producing the SRM.

3. NBS SRM Certification Philosophy

Three principal modes are used by NBS to certify SRM's.

1. Measurement by a so-called definitive method of known and demonstrated accuracy having essentially no systematic error – an example is the Cholesterol in Serum method, based on isotope dilution mass spectrometry.

2. Measurement by two or more independent – so-called reference methods – having inaccuracies (including systematic errors) that are small relative to certification requirements. Example – the two independent gas chromatography and liquid chromatography techniques used to certify the anti-convulsant drug in serum SRM – the first SRM ever certified by NBS for trace organic constituents.

3. The third certification mode involves interlaboratory testing and is generally used only for renewals of previously issued SRM's or to obtain supplemental data.

The objective of the SRM Program is to achieve interlaboratory compatibility and accuracy on a national scale.

The total uncertainty assigned to the certified value of an SRM generally includes three components:

1. The first is due to method precision and is amenable to standard procedures for statistical analysis.
2. The second is due to systematic error of the method or bias among methods. In many cases the value assigned to this component is based on experience and/or judgment of the analyst.
3. If significant, material variability or inhomogeneity accounts for the third component.

With regard to SRM's certified for chemical composition, three of the most important criteria to be met during certification are:

1. The material must be homogeneous with regard to the constituents of interest. A testing protocol must be developed to quantitatively assess homogeneity within or between bottles and/or samples.
2. Long term stability of the material must be assured usually through the use of special processing procedures or treatment of the material. Special storage requirements may also be required.
3. Another necessary requirement to be met is the availability of one or more certification methods, which have been well characterized with regard to precision and accuracy.

4. Homogeneity Testing

Normally preliminary homogeneity testing is performed on a limited number of key constituents to detect trends or patterns in the variation of the levels of constituents, which could cause problems.

If inhomogeneity is shown to be small, we normally proceed directly to carry out a certification program.

If inhomogeneity is very large – causing the total uncertainty to be large relative to end-use requirements – the material must be either rejected or reprocessed.

If inhomogeneity is of the same magnitude as the estimated errors of certification methods and the total estimated uncertainty is small relative to end-use requirements, then one proceeds to quantify the inhomogeneity for inclusion in the uncertainty. Certain restrictions such as requiring the use of minimum sample size for analysis may be imposed to assure proper level of homogeneity.

5. Stability of Food SRM's

The stability of materials is undoubtedly going to cause more serious problems than inhomogeneity with regard to developing organic nutrient SRM's. Stability of constituents and the matrix itself may be affected by microbial attack, and/or chemical reactions which in turn are sensitive to environmental conditions such as temperature, radiation, or presence of oxygen or moisture.

The following questions are pertinent to this aspect of SRM development.

(1) What materials could be used that would minimize stability problems? What steps might be taken during processing and/or storage to assure adequate stability?

(2) Are there simple and accurate methods for monitoring the long term stability of the material without having to essentially duplicate the certification procedure?

(3) What constraints do you have to impose on the user to minimize stability problems?

NBS has not had much experience with developing SRM's for organic nutrient analysis. We have certified over the last 10 years, a series of agricultural products and plant tissues for a number of elements at the trace, and minor constituent levels. The first in this series was orchard leaves and the last, oyster tissue.

A number of different processes have been used to assure adequate stability of these materials. These include freeze and air drying and Co-60 irradiation. Some of these techniques may not be useful for stabilizing organic nutrient SRM's. For example, the use of Co-60 radiation would probably damage both the matrix and many organic constituents of interest.

We probably will have to develop special processes to stabilize SRM's for organic nutrient analysis. To be a useful SRM, the material should be stable for at least 4 or 5 years and preferably 10 years or more.

Sometimes, it is necessary to take a number of different steps to assure adequate stability in a material. A case in point is the soon-to-be-issued wine SRM[1], which is being certified for its ethanol content. At the recommendation of oenologists at the University of California, Davis and a committee of experts from the wine industry, a number of separate special processing steps were undertaken to stabilize the wine. These included ion exchange of K for Na, increasing the alcohol content, and addition of Cu and Fe. I won't elaborate on why steps were necessary to stabilize the wine. Suffice it to say that to obtain requisite stability – i.e., 5 years or more – some unusual steps may have to be taken during the processing of material intended for use as an SRM.

6. Summary

In summary, certification of organic constituents in a food SRM will depend on (1) assuring the homogeneity and long-term stability of both constituents and matrix, and (2) the availability of certification methods of requisite precision and accuracy. Subsequent speakers will address these questions. Particular attention must be paid to the question of stability.

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[1]Editor's Note: This material is available from NBS as SRM 1590, Stabilized Wine.

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AN ASSESSMENT OF THE ACCURACY AND PRECISION OF THE METHODS USED FOR THE MEASUREMENT OF ORGANIC NUTRIENTS IN CEREAL AND GRAIN PRODUCTS

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A sincere concern for the nutritional quality of the U.S. food supply on the part of industry and government has given rise to nutritional labeling and nutrient fortification of foods. Under the authority of the 1938 FD & C Act, the FDA formulated in 1941 requirements for Minimum Daily Requirement labeling and in 1942 standards for nutrient enriched flour became effective. Since that time, these programs have been credited with the elimination of certain nutrient deficiency diseases in the United States.

In 1973, the FDA published "voluntary" food labeling regulations with nutritional labeling expressed in terms of the percent of the National Academy of Sciences' Recommended Daily Allowance of nutrients on a per serving basis. Also, in January of this year the FDA published a food fortification policy. Clearly, the implication of all this interest and regulatory activity for the food chemist, in the public or private sector, is an increased need for accurate nutritional analysis of foods – the subject of this presentation.

The Code of Federal Regulations states that "... food composites shall be analyzed by AOAC methods where available and when no AOAC method is available, by reliable and appropriate analytical procedures. Alternative methods of analysis may be submitted to the FDA to determine their acceptability". This means that AOAC methods are used to check compliance by the FDA and hence, in a legal sense, are correct or accurate by definition.

It is generally recognized that due to the lengthy, and I might add necessary, collaborative process required for official status, AOAC methods frequently are not the most current methods of choice. Industrial chemists who are concerned with diminishing analytical costs may use any technology at their disposal provided there is sufficient correlation with AOAC methodology to guarantee compliance upon FDA sampling. This striving for efficiency has lead to a proliferation of analytical methods for nutrient analysis in foods. The objective of this workshop is to help the NBS provide reference materials for organic nutrients. These reference materials may then be used to assess the accuracy of analytical methods, as well as laboratory performance. It is worth repeating, however, that for nutritional labeling, comparison with AOAC methods used by the FDA and state agencies is currently the most fundamental criterion. No amount of checking with reference materials will change a non-compliance situation.

In discussing the precision and accuracy of methods used for organic nutrient analysis, I will focus primarily on the five vitamins required when a food product is labeled. Although protein, carbohydrate, and fat analyses are important for labeling and calorie calculation, the methods used are quite standardized for cereals and the CFR allows a 20 percent tolerance in calories, carbohydrate, and fat labeling.

In discussing the precision and accuracy of methods used for riboflavin, thiamin, niacin, vitamin A and vitamin C, I will review some sources of error associated with extraction, method interference, and method execution. Some reproducibility, repeatability, recovery and method comparison will be presented. I will be dealing almost exclusively with instrumental and chemical methods leaving microbiological assay to someone with more experience.

Between-laboratory reproducibility of nutrient methods may be assessed by collaborative study. Within-laboratory repeatability is less of a concern and is usually controlled by individual laboratories to within five percent relative error. Accuracy or "the true value" is most important and more elusive; it is assessed operationally by recovery data, between-method comparative data and of course with reference materials which we anticipate will be forthcoming through the efforts of this workshop.

1. Riboflavin

If protected from light, our laboratory has observed no loss of riboflavin in ground cereal samples (2-8 percent moisture) at room temperature over 90 days. Riboflavin exists in foods as the phosphate ester of the carbohydrate moiety as well as the adenine dinucleotide. Because of differences in fluorescence response and extraction efficiency, acid and/or enzymatic hydrolysis is required when other than added riboflavin is analyzed. In the presence of elemental iron and hot acid, riboflavin is reduced; this is usually not a problem with cereals but is a concern with concentrates. If enzymes are used for hydrolysis, a correction is necessary for riboflavin added with the enzyme.

Potential sources of error in the fluorometric method are as follows: (1) filter paper adsorption of riboflavin (glass filter recommended); (2) high iron; and (3) sulfur formation with dithionite addition.

Any fluorescing material interferes which is not oxidized by permanganate and is reduced by dithionite. These exist in certain foods and adsorption/desorption using zeolite, magnesium silicate, and Fuller's earth is recommended for certain foods. Comparison of the fluorometric method with an High Performance Liquid Chromatograph (HPLC) method in our lab for ready to eat (RTE) cereals has demonstrated a bias of about 15 percent (1.5 mg/100 g) from the fluorometric even though both methods show excellent recoveries. Within laboratory repeatability is usually about 4-6 percent relative error and typical data are shown in Table 1. The recovery is from an automated method which also shows better repeatability. Between laboratory variability is shown in Table 2 under controlled method conditions and conditions using different methods. The reproducibility about doubles when many different methods are used. The standard deviation of 25 percent with outliers removed graphically demonstrates the need for standard reference materials.

Table 1. Recovery and Repeatability of AOAC Fluorometric Riboflavin.

Twelve RTE Cereals (Corn, Oat, Wheat)
Average 3.35 mg/100 g (56% RDA)
Within Laboratory Standard Deviation 6.9% (11 degrees of freedom)
Average Recovery Automated 99 ± 4.6%

Table 2. Reproducibility of Riboflavin.

		Relative Standard Deviation
Sixteen laboratories AOAC fluorometric		
Wheat Flour	Average 0.50 mg/100 g	9.6%
	Average 0.13 mg/100 g	18.3%
Bread	Average 0.43 mg/100 g	11.9%
Oat RTE	Average 1.95 mg/100 g	10.9%
Corn-Oat	Average 2.45 mg/100 g	11.0%
American Association of Cereal Chemists (AACC) flour check sample		
Thirteen laboratories any method		
Hard Wheat Flour	Average 0.43 mg/100 g	19.0%
Association of Vitamin Chemists (AVC) mixture		
Nineteen laboratories any method		
Fabricated	Average 1.05 mg/100 g	25.0%

To summarize, fluorometric and chromatographic methods provide reasonably precise and accurate riboflavin results. The between laboratory reproducibility needs improvement.

2. Thiamin

Over a 90-day period, our laboratory has observed no loss of thiamin in ground cereal samples (2-8 percent moisture) stored at room temperature.

Thiamin exists in foods as free thiamin and the phosphate ester which must be cleaved by enzyme hydrolysis for isobutanol extraction in the standard thiochrome method. Like riboflavin, acid/elemental iron reduction is a source of error in vitamin concentrates and the thiamin in an enzyme used must be subtracted.

Potential sources of error in the fluorometric thiochrome method are as follows: (1) thiamin as disulfide; (2) non-standard Decalso column recovery; (3) overloading Decalso (>10 µg/g).

Liquid chromatographic methods have been reported but with limited comparison data. Typical within laboratory repeatability of the thiochrome method is 4 to 6 percent with good recoveries as shown in Table 3. The AACC flour sample and the AVC check sample show a between laboratory reproducibility of about 15 percent relative (Table 4) with outliers removed.

Table 3. Recovery and Repeatability of AOAC Thiochrome Method.

Ten RTE cereals (Corn, Oat, Wheat)

Average 3.10 mg/100 g (57% RDA)

Within Laboratory Standard Deviation 4-6.5%
(18 degrees of freedom)

Average Recovery
Automated 98 ± 3.8%

Table 4. Reproducibility of Thiamin.

		Relative Standard Deviation
AACC flour check sample		
Fourteen laboratories any method		
Hard Wheat	Average 0.83 mg/100 g	19%
	Average C.30 mg/100 g	13%
AVC mixture		
Eighteen laboratories all thiochrome assay		
Fabricated	Average 1.34 mg/100 g	15%

3. Niacin

Over a 90-day period, our laboratory has observed no loss of niacin in ground cereal samples (2-8 percent at room temperature).

Niacin exists in foods as free acid, amide, and amide derivatives, all of which are converted to nicotinic acid by acid or base hydrolysis prior to analysis. Chemical analyses with cyanogen bromide and microbiological methods are commonly used. Liquid chromatographic methods have been reported but with little comparative data.

For cereal products the AOAC microbiological method using *Lactobacillus plantarum* compares favorably with the AOAC colorimetric method as shown in Table 5 giving some of the data from an AACC collaborative study using ten products and seven laboratories. The excellent reproducibility of 11 percent or less is not typical when different methods are used under routine conditions as evidence by a 17 and 15 percent between laboratory reproducibility in two recent AVC collaborative studies (6.61 and 2.66 mg/100 g, respectively).

Table 5. Reproducibility of Niacin.

		Microbiological	Chemical
AACC collaborative study over seven laboratories			
Soy Flour	Average	2.95 mg/100 g	2.56 mg/100 g
	Standard Deviation	2.4%	11.0%
RTE Cereal	Average	17.8 mg/100 g	19.4 mg/100 g
	Standard Deviation	4.0%	5.2%
Flour	Average	1.4 mg/100 g	1.3 mg/100 g
	Standard Deviation	7.1%	6.9%
Bread	Average	7.03 mg/100 g	7.17 mg/100 g
	Standard Deviation	6.3%	5.4%
Rice	Average	1.23 mg/100 g	1.26 mg/100 g
	Standard Deviation	8.1%	10.0%

4. Vitamin A

Vitamin A activity exists in cereal products as pre-vitamin A carotenoids and as retinol esters added as fortification. Over a 90-day period, our laboratory has observed no loss of retinol palmitate in ground cereal samples (2-8 percent moisture) stored at room temperature.

Liquid chromatographic, fluorometric, and Carr-Price colorimetric methods are commonly used for vitamin A analysis. Potential sources of error in the popular AOAC Carr-Price method are: (1) potential destruction during saponification; (2) incomplete partitioning into petroleum ether; (3) incomplete recovery from alumina column; (4) contamination of the Carr-Price reagent, and (5) improper timing of the antimony trichloride reaction. Most of these problems are eliminated using liquid chromatography, but care must be taken to ensure complete extraction with encapsulated vitamin A and to combine the all-trans and 13-cis peaks if the chromatographic conditions used, separate these isomers.

An HPLC method used in our laboratory has a within relative standard deviation of about 4.0 percent, 95 percent recovery, and compares favorably with Carr-Price. In a recent AVC collaborative study, however, 14 laboratories ranged from 0 to 11,880 vitamin A IU's with a mean of 8,313 IU. Clearly, many of these labs could use a reference material.

Error associated with pre-vitamin A determination is related to destruction during saponification and the inability to separate pre-vitamin A carotenoids from other carotenoids prior to quantitation. To a large extent, these difficulties are eliminated by omitting saponification and using HPLC, but it is still very difficult to separate α from β carotene.

5. Vitamin C

Ascorbic acid exists in cereal products due to fortification. It is a reactive vitamin and care must be exercised in storing the sample and extract. In our laboratory a ground cereal product sample (2 percent moisture) showed no vitamin C destruction after 90 days, yet another product with as little as 8.3 percent moisture showed 53 percent loss after 30 days.

Upon extraction of the vitamin C, rapid analysis within 30 minutes is recommended, especially in the presence of solid material.

The ascorbic acid is reversibly oxidized to dehydroascorbic acid which is hydrolyzed to biologically unavailable diketogulonic acid. Only trace levels of dehydroascorbic acid are present in cereal products. Of the many methods available for ascorbic acid determination, the most common are the indophenol titration, microfluorometric, osazone, and HPLC. The indophenol method is subject to great error due to reducing substances (sulfhydryl, reductones, etc.) other than ascorbic acid and it doesn't measure the dehydroascorbic acid. The osazone technique is subject to interference by the gulonic acid although not a major concern. The HPLC method omits the dehydro form unless it is reduced to ascorbic acid prior to analysis. The fluorometric method is comparatively free of problems except when large amounts of browning products are present. Some typical performance specifications for the fluorometric, indophenol, and HPLC methods used in our laboratory are

shown in Table 6. Even though excellent repeatability and recovery data can be achieved, reproducibility data from recent AVC collaborative study are very poor ranging from 3.8 to 293 mg/100 g (11 laboratories, fabricated food).

Table 6. Vitamin C.

HPLC

30 samples (corn, oat, wheat)
Within Laboratory Standard Deviation 4.5%
Recovery 98.4 ± 2.5%
No bias from fluorometric method

Indophenol

16 samples (corn, oat, wheat)
Within Laboratory Standard Deviation 4.0%
No bias from fluorometric method

Fluorometric

34 samples (corn, oat, wheat)
Within Laboratory Standard Deviation 3.0%
Recovery 100.5 ± 2.0%

To summarize, numerous instrumental, chemical, and microbiological methods exist for the analyses of organic nutrients in foods. Many of these are sufficiently accurate and repeatable if applied properly by competent workers. The major difficulty exists in between laboratory reproducibility and poor method execution. I feel that Standard Reference Materials will help improve the situation.

National Bureau of Standards Special Publication 635. Proceedings of the Workshop on Reference Materials for Organic Nutrient Measurement held at NBS, Gaithersburg, Maryland, October 23, 1980.

ACCURACY AND PRECISION OF NUTRIENT METHODOLOGY

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Since 1964, the National Food Processors Association, formerly the National Canners Association, Committee of Canning Industry Analytical Chemists (CCIAC), has been obtaining data on interlaboratory variability of food composition analytical methods.

This paper will describe the CCIAC and discuss interlaboratory variability in nutrition methodology projects which the committee has cooperatively studied. We term these projects cooperative studies because they are not strictly controlled like an AOAC collaborative study. AOAC methods are mostly used, however.

The members of the CCIAC are analytical chemists from the laboratories of companies belonging to the NFPA. These companies are listed in Table 1.

Table 1. Committee of Canning Industry Analytical Chemists.

National Food Processors Association Members

American Home Foods
Baker/Beech-Nut, Inc.
California Canners & Growers
Campbell Soup Company
Carnation Company
Del Monte Corporation
Environmental Science Associates
General Mills, Inc.
Gerber Products Company
Green Giant Company
H. J. Heinz Company
Hunt-Wesson Foods, Inc.
Libby, McNeill & Libby
National Food Processors Association
Ocean Spray Cranberries, Inc.
Ralston Purina Company
Stokely-Van Camp, Inc.
Van Camp Seafood Company
Westgate-California Foods, Inc.

Recently, participation in projects involving testing for nutrient quality and heavy metals has been opened to interested commercial and government laboratories listed in Table 2.

Table 2. Committee of Canning Industry Analytical Chemists.

Government and Commercial Laboratories

ABS Laboratories, Inc.
Anresco
Columbia Laboratories
Food and Drug Administration
Nutrition International, Inc.
Shankman Laboratories, Inc.
H. V. Shuster, Inc.
Stoner Laboratories
United States Department of Agriculture
Warf Institute
Westreco

The committee was organized in 1964 to discuss problems in the field of pesticide residue analysis. Since that time, its activities have broadened to include cooperative studies on environmental contaminants and nutrient testing. The committee meets twice a year to discuss the results of the cooperative studies. Approximately 100 samples have been studied over the past 16 years.

The advent of nutrition labeling made it necessary for the canned foods industry to carry out studies on currently available nutrition methodology; a typical nutritional labeling format is shown in Table 3. The part of the nutrition labeling regulation which deals with method variability states that no regulatory action will be based on the determination of a nutrient value which falls below 80 percent of the declared value by a factor less than the variability generally recognized for the analytical method used in that food at the level involved.

Table 3. Sweet Peas Nutrition Information, Serving Size One Cup.

Calories	130	Carbohydrate	25 g
Protein	8 g	Fat	1 g

Percent of U.S. Recommended Daily Allowance

Protein	10	Riboflavin	8
Vitamin A	15	Niacin	10
Vitamin C	40	Calcium	2
Thiamin	15	Iron	10

This variability has yet to be established. The processed foods industry through the CCIAC is actively engaged in obtaining data on interlaboratory variability for nutrition methods in a variety of foods.

This workshop is designed to answer questions concerning the current state-of-the-art in organic nutrient methodology. This paper will attempt to answer those questions concerning interlaboratory variability for organic nutrients that must be measured if a manufacturer chooses to nutritionally label their products. Since there are no products available in which exact concentrations of these nutrients are known, we can only guess at the accuracy of the methods. We do feel that once a material has been tested by a large number of laboratories, the resulting mean is the best estimate of the concentration of a particular analyte if the standard deviation is reasonably small at the level being tested.

Many samples have been cooperatively studied by the CCIAC since 1973. Some of these are listed in Table 4. For purposes of this paper, we have elected to discuss interlaboratory variability in puréed peaches, green beans, a vegetable and liver mixture and a dry pet food.

Table 4. Committee of Canning Industry Analytical Chemists.

Nutrition Projects

Project Number	
1	Tomato Juice
2	Peas
3	Corn
4	Spinach
5	Formula II
6	Corn
7	Liver
8	Peaches
9	Formula II
10	Meat, Dairy, Vegetable and Cereal
11	Peaches
12	Bread Crumbs
13	Peaches
14	Sweet Potatoes
15	Tuna Fish in Oil
16	Bran
17	Tomato Catsup
18	Apple Sauce
19	Bran
20	Mixed Vegetables and Meat
21	Corn
22	Vegetables and Liver

Table 5 shows data developed by the CCIAC on protein and fat in these products. The method used for protein was the AOAC method, 1980 Edition, section 2.057-2.058; the factor of 6.25 was used to calculate percent (%) protein from Kjeldahl nitrogen value. For fat, the AOAC method, 1980 Edition, section 18.043 of acid hydrolysis was used by the committee. Interlaboratory variation for protein values at the level tested is considered good. The interlaboratory variation for fat analysis at the highest level is acceptable; variation at low fat levels is high, but we would expect it to be.

Table 6 shows interlaboratory variation for carotene, ascorbic acid and niacin content in green beans, peaches, vegetable and liver mixture and a dry pet food. The dry pet food did not contain carotene or ascorbic acid.

Methods of analysis for carotene in yellow fruits and vegetables are poor and this is reflected in the high degree of variation in Table 6. The presence of xanthophylls makes the analysis difficult. HPLC methods for this determination looks promising.

Two methods are currently being used for ascorbic acid determinations. These are:

1. AOAC, 1980 Edition, section 43.056-2-6 dichlorindophenol titration;

2. AOAC, 1980 Edition, section 43.061-microfluorometric method.

The interlaboratory variability for this nutrient at higher levels would appear to be acceptable. Results for niacin in these products at the levels tested, are also good.

Table 5. Interlaboratory Variability in Nutrient Analysis.

Product	No.	Mean	Standard Deviation	Coefficient of Variation
		Protein Percent		
Vegetables and Liver	14	2.7	0.10	4
Dry Pet Food	19	21.4	0.46	2
Green Beans	18	1.6	0.15	9
Peaches	23	0.4	0.10	25
		Fat Percent		
Vegetables and Liver	11	0.46	0.05	12
Dry Pet Food	12	10.66	0.60	6
Green Beans	15	0.26	0.11	42
Peaches	23	0.22	0.18	83

Table 6. Interlaboratory Variability in Nutrient Analysis.

Product	No.	Mean	Standard Deviation	Coefficient of Variation
		Carotene (u/100 g)		
Green Beans	16	247	62	25
Peaches	17	516	200	39
Vegetables and Liver	12	591	135	23
		Ascorbic Acid (mg/100 g)		
Vegetables and Liver	12	2.7	0.8	30
Green Beans	16	3.5	1.7	49
Peaches	19	22.2	7.8	8
		Niacin (mg/100 g)		
Peaches	18	0.49	0.12	24
Vegetables and Liver	12	1.45	0.11	8
Green Beans	18	0.53	0.15	28
Dry Pet Food	16	11.21	1.18	11

In Table 7 are data on interlaboratory variability for thiamin and riboflavin analyses. Again, at the levels tested, these data are considered satisfactory.

Some observations that can be made from these data are:

1. In many cases, but not all, the precision of the methods depends on the concentration of the nutrient being tested. Interlaboratory variability increases as concentration decreases.

2. Interlaboratory variability also depends on the food matrix. Some foods are more difficult to analyze, for example, carotene in yellow foods vs. green foods.

3. Extractions are easier in cooked foods.

Members of the NFPA-CCIAC have for some time used excess sample from a particular study as an inhouse reference material. The indication is that they will continue to do so. So far, these samples have proved to be an excellent quality assurance program. However, the industry does need Standard Reference Materials that have been certified by the National Bureau of Standards.

Table 7. Interlaboratory Variability in Nutrient Analysis.

Product	No.	Mean	Standard Deviation	Coefficient of Variation
		Thiamin (mg/100 g)		
Peaches	18	0.01	0.003	29
Vegetables and Liver	12	0.02	0.008	42
Green Beans	17	0.033	0.008	24
Dry Pet Food	17	1.17	0.21	18
		Riboflavin (mg/100 g)		
Peaches	19	0.033	0.013	39
Vegetables and Liver	13	0.29	0.05	17
Green Beans	17	0.09	0.01	11
Dry Pet Food	17	0.95	0.22	23

National Bureau of Standards Special Publication 635. Proceedings of the Workshop on Reference Materials for Organic Nutrient Measurement held at NBS, Gaithersburg, Maryland, October 23, 1980.

PROBLEMS IN THE MEASUREMENT OF ORGANIC NUTRIENTS IN FOOD PRODUCTS: AN OVERVIEW

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The accurate and precise measurements of organic nutrients in foods are the underpinning of our understanding of the human requirements for those nutrients and our understanding of the nutrient contents of foods so that we may meet these requirements. We would prefer to utilize methods which contain the elements shown in figure 1. Of particular interest to this workshop are those features of the method which are concerned with quality control. The availability of pure standards and adequately documented standard reference materials are critical to adequate quality control for analytical determinations.

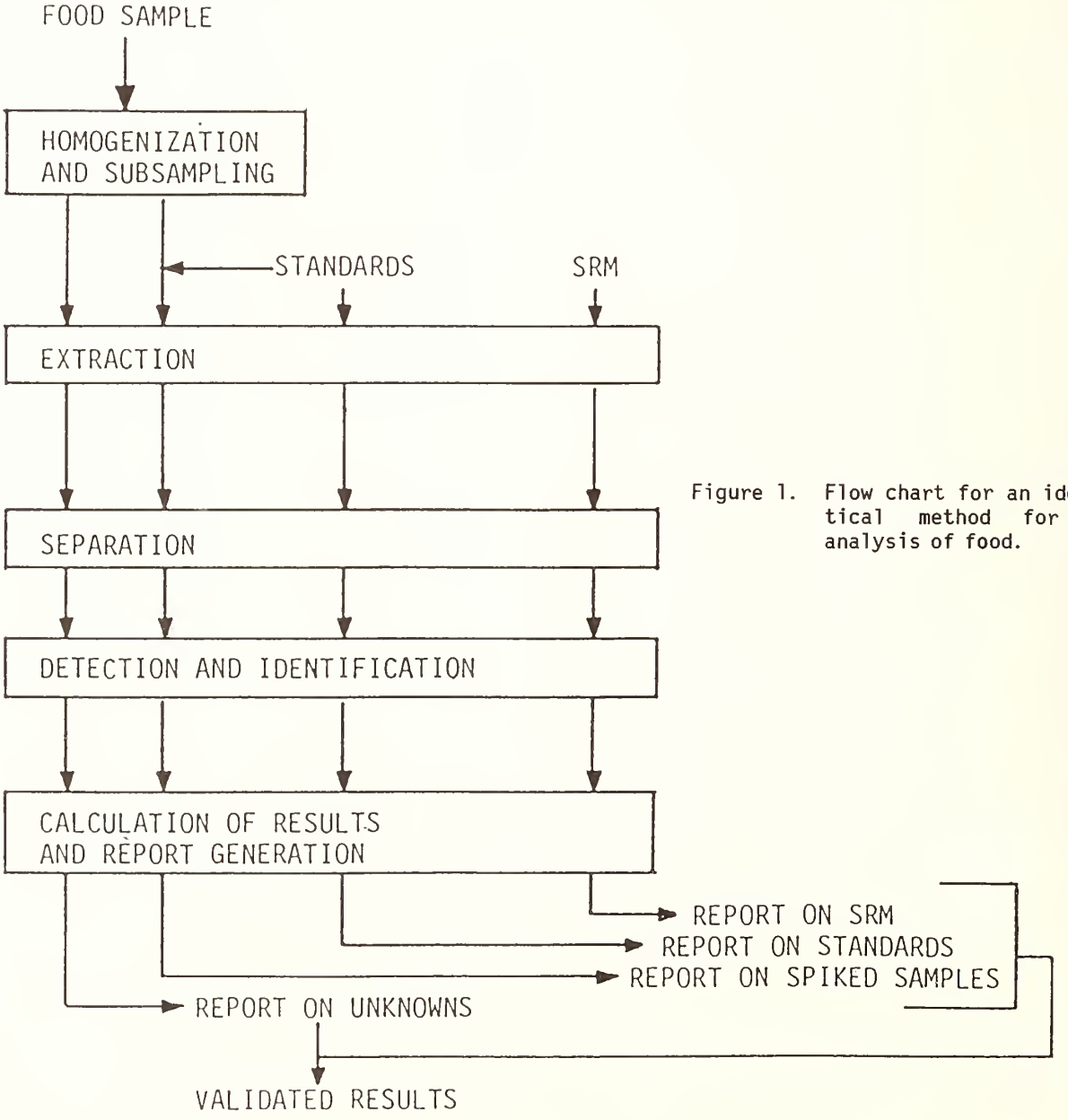

Figure 1. Flow chart for an ideal analytical method for nutrient analysis of food.

Before discussing this topic further, it is useful to briefly evaluate the state-of-the-art for organic nutrient determinations in foods (see Table 1). If asked "If a qualified analyst uses current methods under the appropriate conditions, for which nutrients are the results of the determinations in food matrices likely to have sufficient accuracy and precision?", the answer must be, for most foods, only those nutrients for which the methods are classified as "sufficient" or "substantial". Methods for those labeled "tentative" will probably give acceptable results. Those determinations which are classified "conflicting" yield different results with different methods. No one knows which, if any, of these results are accurate. Those nutrient methods listed as "fragmentory" have had so little study that the accuracy is unknown. As shown in Table 1, there are a large number of organic nutrients for which there are not adequate methods for determination of the nutrient levels in foods.

Table 1. State of Development of Methodology for the Analysis of Organic Nutrients in Foods as of 1980.

Sufficient	Substantial	Tentatively Acceptable	Conflicting	Fragmentary
	Amino Acids (Most)	Amino Acids (Some)	Fiber	Biotin
	Cholesterol	Fat (Total)	Folacin	Choline
	Fatty Acids	Trans-Fatty Acids	Pantothenic Acid	Heme-Iron
	Individual Sugars		Protein (Total)[b]	Vitamin K
	Niacin		Starch	
	Riboflavin		Sterols (Others)	
	Thiamin		Vitamins A	
	Vitamin C		B_6[a]	
			B_{12}[a]	
			D	
			E[a]	

[a] New methods look very promising.

[b] Total nitrogen methods would have a ranking of sufficient.

It is worthwhile to examine some of the background of this state of affairs. Basically "nutrients" are a group of compounds which elicit given biological responses. When too low a level of a "nutrient" is consumed, one set of biological responses – "a deficiency" – is observed in the test animal. When a adequate level is consumed, another set of biological responses – "normal" – is observed. For most nutrients, several isomers of a given compound will elicit qualitatively (though not always quantitatively) similar biological responses. For example, in the case of the vitamins, niacin and vitamin C have two isomeric forms which elicit similar biological responses; riboflavin, thiamin, and vitamin E have three; vitamin D has four; vitamin B_{12} has five; vitamin B_6 has six; and vitamin A and folacin each have greater than 10. Often the relative biological responses of the nutrient isomers is species specific, thus nutrient activities determined using one species (i.e., rats) cannot be directly transposed to another species (i.e., man). Many traditional organic nutrient assay methods depend either upon feeding studies with a test species (usually microorganisms, rats, or chicks) or upon single chemical reactions for which a number of isomers give similar responses. While the originator of the assay usually did the necessary development and quality control for the determination of a given nutrient in a given food matrix, these techniques have often been utilized for the determination of that nutrient in different matrices without revalidating the methods. Often these traditional methods do not provide sufficiently accurate and precise results for all food matrices. It is desirable to have assays which separate the biologically active isomers from each other as well as from the bulk of the food matrix, to quantitate each isomer separately, to assign a biological activity to each isomer and then to sum the biological activities to obtain the total nutrient activity for the food. These desired techniques should have built in quality control systems which include internal standards, pure standards of each nutrient isomer and standard reference materials of similar matrices to the food sample. Many believe that it is desirable to do the quality control operations in each set of determinations. Thus, it will be necessary to have pure standards for each isomer of each nutrient of interest and to have standard reference materials representative of the important food matrices for these nutrients. These pure standards and standard reference materials are needed for within method quality control and for development and validation of new or untested methods. This is a large assignment and some sense of priorities

problems in the United States (see Table 3). From the information presented in Tables 2-3 it is possible to set priorities for research on new methods (see Table 4) and for routine determinations (see Table 5).

Table 2. Organic Label Nutrients.

Biotin	Panthothenic Acid
Carbohydrate	Riboflavin
Cholesterol	Thiamin
Fat	Vitamin A
Fatty Acids	B_6
saturates	B_{12}
polyunsaturates	C
Folacin	D
Niacin	E

Table 3. Inadequate and/or Excessive Organic 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
Biotin	Amino Acids[a]	Cholesterol
Choline	Fatty Acids	Folacin
Glucose	Fructose	Lactose
Maltose	Niacin	Nutrient Fiber
Pantothenic Acid	Other Sterols	Riboflavin
Starch	Total Protein[a]	Sucrose
	Trans-Fatty Acids[a]	Thiamin
	Vitamin E	Total Fat
	Vitamin K	Vitamin A
		B_6
		B_{12}
		C
		D

[a]It is unlikely that increased information on the nutrient composition of foods for these nutrients will significantly help in combating in public health problems associated with these nutrients.

Table 4. Suggested Priorities for Research on New or Improved Analytical Methodologies for Organic Nutrients in Foods.

First Priority		
Folacin	Total Fat	Vitamin A
		Vitamin B_6
Second Priority		
	Nutrient Fiber	Trans-Fatty Acids
	Sugars	Vitamin B_{12}
		Vitamin D
		Vitamin E
Third Priority		
Niacin	Riboflavin	Vitamin K
	Sterols	

These priorities were determined by an assessment of the severity of the U.S. public health problems and the adequacy of the analytical methods.

Table 5. Suggested Organic Nutrient Priorities for Routine Analysis of Foods.

First Priority		
Cholesterol	Neutral Detergent Fiber	Total Fat
Fatty Acids (including Trans)		Vitamin C
Second Priority		
Niacin	Sugars	
Thiamin	Vitamin E	
Riboflavin		

These nutrient priorities were determined by an assessment of the relationship of the nutrient to U.S. public health, state of the data for the nutrients, and the adequacy of the analytical methods available for assay.

Pure standards are needed to develop adequate methods for determination of given nutrients (see figure 2). A great deal of work has been done in this area and often either the U.S. Pharmacopoeia or the National Bureau of Standards or both have developed pure standards of many of the major organic nutrients. This is particularly true of those isomeric forms which are used as supplements. However, there are a number of nutrient isomers for which pure standards are not available. This is especially the case for those forms which are relatively unstable.

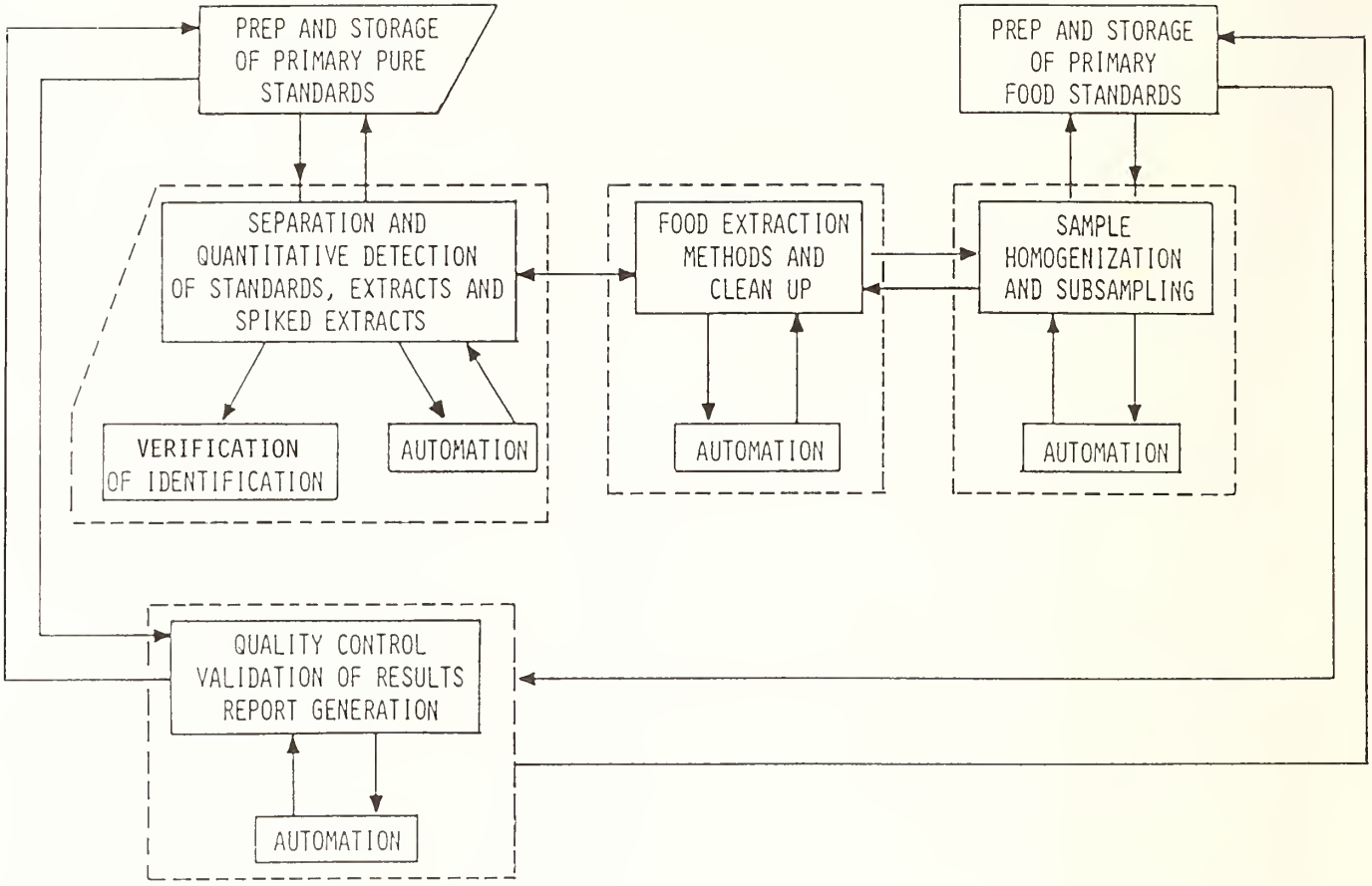

Figure 2. Flow chart for the development of an ideal analytical method.

The Standard Reference Materials for organic nutrients are much less well developed. Table 6 lists the Food Standard Reference Materials available as of 1978 with their certified nutrients. When this is compared with the priorities for routine analysis (Table 5) it is apparent that much remains to be done. The development of food standard reference materials fortified with those forms of the nutrients most commonly added to foods would be an important first step. These SRM's would be useful to the food industry, the regulatory laboratories and the research laboratories. Development of SRM's certified for the other isomeric nutrient contents could then follow.

Table 6. Food Standard Reference Materials Certified for Nutrient Content.

Food Category	NBS Available	Nutrients Certified	SRM Needed
Cereal Grain	Wheat Flour-SRM 1567 Rice Flour-SRM 1568	Some Inorganic Some Inorganic	Wheat Bran
Meat	Bovine Liver-SRM 1577	Some Inorganic	Beef Muscle Pork Muscle
Fish	Tuna-RM 50	Information Values Some Inorganic	Fish Meat
Poultry	---	---	Chicken
Vegetables, Leafy Root, Vine	Spinach-SRM 1570[a] ---	Some Inorganic ---	Lettuce Potato, Tomato
Fruit	---	---	Apple, Orange
Legumes	---	---	Bean, Pea
Eggs and Egg Products	---	---	Egg
Dairy Products	---	---	Cheese
Fats and Oils	---	---	Cooking Oil
Beverages	---	---	Soft Drink, Milk

[a]Editor's Note: SRM 1570 is no longer available. Other plant tissue SRM's are available which may be useful for these analyses.

Nutrient Composition Laboratory; Nutrition Institute; USDA, SEA-FR, NER, BARC; Beltsville, Maryland 20705; 1978.

Summary

The state-of-the-art for determining the organic nutrients in biological materials and particularly foods needs improvement. The development (where necessary) of pure standards for isomeric forms of the nutrients used in food fortification and Standard Reference Materials certified for these nutrients would be an important first step in the development of the needed organic nutrients reference materials.

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LONG TERM STABILITY OF ORGANIC NUTRIENTS IN FOODS

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Today we are going to discuss the long term stability of natural and fortified organic nutrients in food products.

The way to avoid the loss of organic nutrients in food is to pick it fresh from the garden, rinse it, and eat it. However, in modern society this is rarely possible. Most of our fruits and vegetables are transported fresh in refrigerated and/or controlled atmosphere storage or processed into frozen or canned foods which may be stored for long periods.

My talk will be principally about fruits and vegetables since most of our work has been with these foods.

Hurt, in 1979, stated that the primary objective of any form of food preservation is to modify basic raw agricultural commodities into a stable physical form that will ensure a safe and wholesome food supply. In this regard, nutrition becomes only one of the factors in the equation of successful food processing. Processed foods must have the necessary organoleptic qualities of desirable flavor, odor, texture, and appearance, as well as be priced right before nutritional quality has any meaning. People today eat foods not nutrients.

Two major methods of food preservation are canning and freezing.

Table 1 shows the changes in vegetable consumption between 1940 – 1975 and shows a slow increase in the consumption of canned and frozen vegetables.

Table 1. Changes in Vegetable Consumption per Person, 1940 – 1975[a].

Year	Total Consumption (lb)	Percentage Consumed As: Fresh	Canned	Frozen
1940	116	78	21	1
1955	118	71	23	6
1965	115	69	23	8
1975	123	64	24	10

[a]Source: USDA (1977).

When I searched the literature on the stability of vitamins in processed foods to compare it with our experimental data I was surprised to find a great divergency in published data.

Table 2 compares Del Monte experimental data with data published by Lund in 1975. It shows the percent loss of four vitamins in four foods caused by the canning process.

Table 2. Loss of Nutrients in the Canning Process.

Percent Losses of Nutrients

Product	Vitamin A		Thiamin		Riboflavin		Vitamin C	
	Del Monte	Lund[a]	Del Monte	Lund	Del Monte	Lund	Del Monte	Lund
Green Beans	1.9	51.7	20.0	62.5	0	63.6	59.7	78.9
Corn	22.4	32.5	33.3	80.0	0	58.3	20.0	58.3
Green Peas	45.4	29.7	65.0	74.2	41.6	64.3	25.9	66.7
Spinach	0	32.1	66.6	80.0	22.2	50.0	68.2	72.5

[a]1975.

Table 3 lists some of the reasons that there is such a variation in data.

Table 3. Factors Affecting the Apparent Percent of Loss of Vitamins in Processed Food.

1. Variety - growing conditions
2. Handling after harvest
3. Type and temperature of blanch
4. Processing time and temperature
5. Oxygen level in the can
6. Storage after processing
7. Analytical techniques

Causes for Variation in Data

1. Variety - Vitamin levels vary in different varieties of food. Growing conditions may also affect vitamin levels and influence loss of vitamins during processing.

2. Handling after Harvesting - poor handling will reduce levels of vitamin A and C in foods. Under proper refrigerated storage, vitamins are stable until senescence at which time there is rapid loss of vitamins.

3. Type and Temperature of Blanch - Both frozen and canned vegetables are blanched prior to processing. Either hot water or steam blanching can be used. There is a trend to steam blanching because it is more energy efficient. Blanching removes water soluble nutrients (vitamins and minerals) but not fat soluble vitamins. Up to this point the processing of canned and frozen food is similar. Frozen food after freezing is generally held at -18 °C.

Canned Foods

4. Processing Time and Temperatures - The magnitude of the losses in canned foods obviously reflect the sensitivity of the nutrients to the microenvironment in the food and the severity of the thermal process. Canned tomatoes would receive a thermal process no higher than 100 °C while corn would receive a "hot" cook at temperatures near or in excess of 120 °C.

5. Oxygen Level Remaining in the Can - Oxygen levels will affect vitamin A and C levels. In years past, exhaust boxes were used to remove the air from cans. However, they are slow and very energy wasteful. Now most cans are filled with brine or syrup and closed with a steam flow closer where the air is displaced by steam and the can closed in a steam atmosphere. However, more air remains in the can using a steam flow closer.

6. Storage After Processing - Higher storage temperatures may lead to loss of vitamins. This is more pronounced in frozen than in canned food.

7. Analytical Techniques - We will discuss this at some length. Before you can measure the retention or loss of vitamins in foods you must be able to analyze for them accurately.

Analytically, attempts to measure the loss of vitamins leads to a surprising amount of difficulties.

For example, in 1977 J. D. Selman at the University of Technology in Great Britain reviewed the vitamin C losses from peas during blanching in water. He concluded that no conclusions could be drawn due to variations between experiments and the poor control of important parameters.

In our laboratory we found the same problem when we tried to compare the vitamin levels in fresh, frozen, and canned foods. We now analyze the samples for moisture content and express the nutrients on an equi-solids basis and thus levels can be compared.

With water insoluble vitamins the problem is much more complicated. When vegetables and fruits are blanched, water soluble constituents are removed, but not water insoluble vitamins. Then if you analyze the blanched samples you have increased the apparent fat soluble vitamins such as β-carotene. This is very obvious if you compare raw and blanched vegetables on an equal solids basis. After considerable number of experiments we found that the water insoluble solids (WIS) could be used to compare water insoluble vitamins.

Analysis of vitamins on fresh foods is further complicated by the presence of enzymes. Enzymes in plant tissue destroy vitamins, especially vitamins A and C. This is important in two ways. For the analyst it is important to inactivate the enzymes in fresh tissue. If you blend fresh spinach in a Waring blender for 2 minutes, half of the ascorbic acid is lost before blending is completed. If the blendate is allowed to stand for 30 minutes all the ascorbic acid is lost. Therefore, when you analyze fresh vegetables for vitamin C it is necessary to blend the intact tissue with acetic acid/meta phosphoric acid stabilizing solution.

We have found that beta-carotene is rapidly destroyed when fresh plant tissue is blended. We use a procedure developed by Van de Weerdhof where the plant tissue is dropped into boiling methanolic 0.5 mol/L potassium hydroxide solution. This procedure has two advantages:

The enzymes are destroyed and the sample is saponified. After suitable purification the beta-carotene can be quantitated by the AOAC procedure or by high performance liquid chromatography.

We have found that repeated freezing-thawing destroys vitamin C and beta-carotene in frozen blended samples. Commercially both canned and frozen foods are blanched prior to freezing or canning. This operation inactivates the enzymes and softens the food so that it can be put in the can or package.

Stability of Nutrients in Processed Foods

Canned Foods - As previously noted blanching removes water soluble nutrients which includes water soluble vitamins. Nutrients in canned foods are quite stable for as long as the integrity of the can remains intact. In canned foods light and air are excluded and enzymes are destroyed. Some canned vegetables are lower in thiamin; all of this loss being apparently due to the sterilization process.

People frequently ask us how long vitamins are stable in canned foods. Recently we were given a set of canned foods packed in 1939. We believe they were stored in relatively cool storage in a basement. Let me hasten to inform you that the cans used at that time were hot dip tin plate and lasted much longer than would the currently used electrolytic differential plate. We put these canned foods through a complete nutritional analysis and were pleased to find that most of the vitamins were retained and compared favorably with vitamins found in currently marketed foods. Table 4 compares the vitamin levels found in some of these foods compared with the same food available in the markets.

Table 4. Effect of Storage on Canned Food.

(Per 100 Grams)

Food	Storage	Vitamin A (I.U.)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin C (mg)
Crean Style Corn	40 Years	360	0.01	0.05	1.2	8
Cream Style Corn	Current	144	0.04	0.06	0.09	6
Pork and Beans	40 Years	130	0.02	0.04	0.6	---
Pork and Beans	Current	130	0.08	0.03	0.6	---
Fruit Cocktail	40 Years	220	0.02	0.01	0.7	3.1
Fruit Cocktail	Current	233	0.03	0.02	0.5	2.2
Green Peas	40 Years	328	0.07	0.06	0.9	---
Green Peas	Current	442	0.09	0.07	0.7	---

Frozen Foods - Storage temperature has a special significance for frozen foods because they are defined as foods stored at temperatures below -18 °C. Even at such low temperatures certain enzymatic and non-enzymatic changes continue, but at a much slower rate, to limit storage life of frozen foods.

The nutrient that has been most thoroughly researched is ascorbic acid. The destruction of ascorbic acid during storage is influenced by the amount of blanching (to inactivate enzymes) rate of freezing and type of packaging. Generally, we have found that the quality deteriorates and vitamin A and C levels decrease in frozen foods after 18 month's storage. Table 5 demonstrates this loss for frozen green beans, Table 6 for frozen corn, Table 7 for frozen spinach, and Table 8 for frozen green peas.

Table 5. Frozen Green Beans (18 Month's Storage) Nutritional Data on a 7.2 Percent Solids Basis.

(Per 100 Grams)

Storage	Vitamin A (I.U.)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin C (mg)
Initial	503	0.06	0.07	0.46	2.8
6 Months	377	0.06	0.08	0.40	2.1
12 Months	400	0.06	0.07	0.40	1.0
18 Months	396	0.05	0.08	0.45	1.1

Table 6. Frozen Corn (18 Month's Storage) Nutritional Data on a 25 Percent Solids Basis.

(Per 100 Grams)

Storage	Vitamin A (I.U.)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin C (mg)
Initial	728	0.09	0.09	1.16	12.3
6 Months	537	0.11	0.09	1.21	11.4
12 Months	432	0.10	0.08	1.06	11.3
18 Months	340	0.11	0.07	1.15	9.7

Table 7. Frozen Spinach (18 Month's Storage) Nutritional Data on a 7.2 Percent Solids Basis.

(Per 100 Grams)

Storage	Vitamin A (I.U.)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin C (mg)
Initial	6367	0.06	0.14	0.40	24
8 Months	6250	0.07	0.15	0.39	15
18 Months	4752	0.07	0.13	0.43	8

Table 8. Frozen Green Peas (18 Month's Storage) Nutritional Data on a 15 Percent Solids Basis.

(Per 100 Grams)

Storage	Vitamin A (I.U.)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin C (mg)
Initial	678	0.17	0.08	1.03	17
6 Months	898	0.15	0.10	1.11	13
12 Months	611	0.16	0.08	1.15	11
18 Months	566	0.17	0.07	1.00	10

Naturally Occurring vs. Added Nutrients

The question has been raised whether naturally-occurring nutrients are inherently more stable than added nutrients. Some literature seems to indicate that under some conditions added nutrients are inherently less stable than those presumably incorporated in a different way in the natural state.

In summary we can conclude:

1. Vitamins are stable in cool stored fresh vegetables until senescence begins and the food yellows when vitamin loss becomes rapid.

2. If fresh green foods are blended there is a rapid loss of vitamins C and A due to enzyme action and probably oxidation.

3. Both frozen and canned foods are blanched either in steam or hot water to destroy enzymes. Water soluble nutrients (minerals and vitamins) are lost by this operation.

4. Frozen foods begin to lose vitamins after about 18 month's storage.

5. Thiamin is lost in canned food probably due to the high temperatures used to sterilize non acid foods.

6. There is good reason to believe that damage to nutrient levels in both canned and frozen foods occurs to the greatest degree during handling and preparation of the foods in the home.

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THE LONG-TERM STABILITY OF ORGANIC NUTRIENTS IN INFANT AND ADULT DIETARY SUPPLEMENTS

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Many factors affect the long-term stability of organic nutrients. These include raw material composition, processing losses, nutrient-matrix interactions, product-container interactions, product age, and storage temperature. The ability to measure the fat-soluble vitamins accurately is a difficult task. This is due to the inaccuracies in the methods, the occurrence of multiple forms of the vitamins and their natural precursors, and the lack of purity of the reference materials which are used as standards.

In April of 1980, we published a High-Performance Liquid Chromatographic procedure for the determination of vitamins A, D, E, and K from a single sample preparation [1][1]. This method is unique in that an enzyme, Lipase, is used to hydrolyze the lipid material. This more controlled hydrolysis minimizes vitamin degradation and facilitates vitamin extraction.

In using this method, we have observed that vitamin A reference materials contain variable amounts of cis and trans isomers and vitamin K standards contain from 3 to 25 percent of the biological inactive cis isomer. Materials of similar purity are used to fortify foods; therefore, the analytical procedure must quantify all the nutritionally added forms in addition to the naturally occurring vitamin forms. This is evident in the variation of vitamin E isomers, contributed by nutritional seed oils, which are added to fortify infant and adult dietary food products. The distribution of these isomers is so characteristic that identification of a particular oil, in a product, is possible.

Determination of the fat-soluble vitamins in our products, after 2 1/2 years of storage, indicates that vitamin A undergoes facile isomerization to the 13 cis form and other isomers. All have lesser degrees of biological activity and in addition, these isomers are not resolved from the 13 cis form. Extraneous peaks are observed in the E alcohol region and there is evidence of some reduction in the amount of E acetate. The total vitamin K content does not appear to change; however, current procedures do not separate cis and trans isomers, consequently, biological activity is not unequivocably defined. Vitamin D appears to be structurally quite stable in these near-neutral products, in the absence of light, in an atmosphere containing an anti-oxidant, such as α-tocopherol.

The results of these studies demonstrate the critical need for suitable reference materials for the multiple forms of the fat-soluble vitamins. The analytical methodology is improving to the point where more suitable standards of matrices are required to carry out accurate measurements.

REFERENCE

[1] Barnett, S. A.; Frick, L. W.; Baine, H. M. Anal. Chem. 52: 610-614; 1980.

[1]Figure in brackets indicate the literature reference at the end of this paper.

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GENERAL OUTLINE FOR DISCUSSION DURING WORKSHOP SESSIONS

Collaborative studies, summarized by Stewart[1], indicate that the measurement of many of the vitamins are neither accurate nor precise. With this in mind as a starting point, we would like to examine the following questions during each of the three workshops.

Are there new methods which are more accurate than the accepted methods?

Which are the two most accurate and precise chemical methods for measurement of the nutrients listed below in (a) formulated foods? (b) commodity foods?

What are the problems in extracting these nutrients from (a) commodity foods? (b) formulated foods?

Do extraction methods need further study? What is the nature of the needed studies?

What are the five most useful matrices for standards for formulated foods, e.g., infant formula, powdered milk, margarine?

What are the five most useful matrices for standards for commodity foods, e.g., puree peas, wheat flour, frozen dried beef?

What are the conditions under which the analytes cited are stable for 1, 3, or 5 years in these matrices?

Priority List for: (1) Analytes to be standardized
(2) Matrices to be evaluated

PROPOSED LIST OF NUTRIENTS FOR DISCUSSION

The following list of analytes which have special nutritional significance with respect to industrial measurements might serve as a guide for discussion.

Fat Soluble	Water Soluble	Sugars
Group I		
Vitamin A	Niacin	Monosaccharides
Cholesterol	Vitamin C	
Group II		
D	Thiamin	Oligosaccharides
E	Riboflavin	
	Pyridoxine and Analogs	

[1]Stewart, K. K., Nutrient Analysis of Foods: A Reexamination, in NBS Special Publication 519, Trace Organic Analysis: A New Frontier in Analytical Chemistry, U.S. Govt. Printing Office, Washington, D. C. (1979).

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EXECUTIVE SUMMARY OF WORKSHOP SESSIONS

S.A. Margolis, NBS

1. Introduction

The development of standards for the vitamins fall into three categories: those for vitamins which are believed to present few or no analytical problems, those for vitamins which present some analytical problems but the preparation of standards appears feasible, and those for vitamins for which current methods of analysis are neither accurate nor precise.

A major problem for the workshop was the alternative development of standards based on fortified vitamin levels as opposed to standards in which the vitamins occur naturally. The analysis of fortified standards presents fewer problems in extraction and measurement since the fortified vitamin is often added to the matrix as a single species.

2. Workshop on Water-Soluble Vitamins

Most of the naturally occurring vitamins in the water-soluble class, with the exception of ascorbic acid, occur in forms other than those which would be used for fortifying standards. For instance, niacin occurs naturally as NAD, NADH, and related compounds; riboflavin occurs naturally as FAD, FMN, and related compounds; and thiamin is naturally bound to proteins. Similarly B_6, biotin, and pantothenic acid occur naturally bound to macromolecules. Furthermore, the extraction procedures for some of these vitamins or the procedures used to release the vitamins from the bound form may cause extreme rearrangement of the vitamin moiety. This occurs, for example, when niacin or nicotinamide is released from NAD and NADH or NADP and NADPH. The alkaline hydrolysis results in the conversion of the nicotinamide ring of NAD and NADP to 2-hydroxynicotinaldehyde, and other products as well. On the other hand, acid hydrolysis of NADH and NADPH results in the formation of products in which the carbon at C-6 of the nicotinamide ring is hydroxylated and then condenses with the ,2' carbon of the ribose ring to yield $O^{2'}$, 6-B-cyclotetrahydronicotinamide adenine dinucleotide. This acid instability of one form and basic instability of another form may lead to the inability to adequately recover bound vitamins from natural materials. Because of this type of problem, the workshop discussion centered primarily on the development of standards of the class of fortified materials. High priorities were given to the most stable vitamins for which current FDA labeling regulations require that measurements be made to support labeling statements.

Participants in the water-soluble vitamins workshop quickly established a general priority listing. The high-priority group included thiamin, niacin, ascorbic acid, and riboflavin followed by B_6, pantothenic acid, folacin, biotin, and B_{12}. Of the B vitamins, niacin appears to present the fewest problems. It is extremely stable as nicotinamide, easy to extract, and easy to measure. Workshop participants believed that it can be measured quite accurately by the cyanogen bromide method using an autoanalyzer as well as by microbiological techniques.

Thiamin was listed as the most stable compound. It exists in nature as enzyme-bound thiamin pyrophosphate. It is heat sensitive, but can be released by hydrolysis with appropriate enzyme preparations. The binding of thiamin pyrophosphate to the enzyme is through a phospho-ester linkage possibly to serine, and the release of thiamin from the enzyme requires hydrolysis of this bond. Another problem with the assay of thiamin is the occasional unavailability of the ion-exchange material used for the final purification step. An alternate material, Bio-Rex 70, appears to be satisfactory for this separation. The use of thiamin-fortified material would eliminate the need for the hydrolysis and the ion-exchange chromatography. Without these steps the measurement of thiamin is believed to be relatively problem free.

The measurement of riboflavin still represents a significant problem. Riboflavin is unstable above pH 6 and is light sensitive. The microbiological method has not yet been approved as an AOAC official method, and the official method which utilizes permanganate oxidation is of limited accuracy and precision.

In the case of unfortified materials, the major problem is in the extraction procedure. Presumably, the extraction procedures for riboflavin present the same problems as those for bound niacin; and, in general, it is difficult to assess the completeness of the release of riboflavin from the nucleotide forms. Because of the difficulties in measurement and extraction, riboflavin is given

a relatively low priority in the first category of compounds for which standards need to be made. Ascorbic acid, on the other hand, which occurs as the free vitamin, is relatively easy to extract. However, it is somewhat unstable; and in the presence of oxygen, is oxidized to dehydroascorbic acid, a reaction which is speeded up by the presence of moisture and trace amounts of metals. However, vitamin C can be stabilized at pH's in the range of 2-4 and by the use of matrices which have a low water activity. It was pointed out in this workshop that the water content of the matrix is not as crucial as the water activity.

The next group of vitamins which have significant public health impact, but which have problems either in the methods of analysis or isolation, include folacin, B_6, pantothenic acid, and B_{12}. The official methods for the analysis of these vitamins are microbiological techniques, and suitable assays need to be developed for the chemical assay of these vitamins. Folacin was only briefly considered in the workshop because it exists in multiple forms which are not easy to interconvert to a single form. B_{12} also exists in several forms, both adenylo- and cyano-forms. It is measured by radioimmunoassay techniques. At this time, both the researchers and the nutritionists need a well-characterized pure standard to use in the development of better assay techniques. In the case of pantothenic acid, the major problem is the ability to hydrolyze the phosphoserine bond or phosphodiester bond with the AMP. At the moment, pantothenic acid is released by enzymatic digestion; and the resulting material is measured by microbiological assay.

Of the four vitamins in this class which have significant public health impact, B_6 has the highest priority. There is currently an urgent need for reference materials containing the six vitamers of B_6: the free and phosphorylated forms of pyridoxine, of pyridoxal, and of pyridoxamine. The availability of these vitamers in pure form and also in a simple matrix would facilitate the development of methods of analysis for this group of substances. In the case of plant food materials, the incomplete extraction of vitamin B_6, particularly that which is covalently bound to carbohydrate, still requires further evaluation. The pure vitamers would aid in the evaluation of the completeness of the hydrolysis of the vitamers from food substances and in the analysis of the multiple peaks found by HPLC analysis of food extracts.

At the moment, the analysis and measurement of biotin is not considered important for public health reasons; therefore, it was recommended that a biotin SRM would be less urgent than others and would be necessary only to check the process of extraction.

3. Workshop on Analysis of Carbohydrates

Carbohydrate analysis was divided into two parts: a formal discussion of methods of analysis of carbohydrate content and then a discussion of the types of materials needed as Standard Reference Materials for carbohydrate analysis. The first formal presentation by Dr. Kent Stewart consisted of a discussion of the automated analysis of carbohydrate content in food samples. Dr. Stewart pointed out that this method of analysis is not suitable as a primary method but is desirable when a large number of samples must be analyzed. The method involves the use of an autoanalyzer and consists of hydrolyzing the carbohydrate samples with sulfuric acid, and then reacting the hydrolysate with dinitrophenylhydrazine to form a colored derivative that is measured spectrophotometrically. The problem with this method is that different sugars have different molar response factors. Glucose, fructose, and invert sugar have similar response factors, but the factor for maltose is 30 percent and for lactose is 23 percent of the others. For sucrose, the response factor is double the others since it is hydrolyzed into fructose and glucose before the hydrazone is formed.

The second presentation by Dr. Betty Li was a consideration of methods of analysis of carbohydrates by gas chromatography which requires the conversion of carbohydrates into derivatives that are volatile. Four types of derivatives were discussed: trimethylsilyl ethers, trimethylsilylated oximes, products of the reaction with N-methylimidazole followed by reaction with nitroacetate, and products of reaction with sodium borohydride and acetic anhydride. The trimethylsilylated oxime method is preferred since it avoids anomerization and permits distinction between glucose and fructose. It has a high degree of sensitivity, gives good resolution, and can detect as little as 1 percent of any sugar in the presence of other sugars. It permits both identification and quantitation of the sugars, and requires a relatively short period of time for analysis. It cannot be used for sugars more complex than tetrasaccharides. The solvent for the derivatization is 80 percent methanol which prevents enzymatic hydrolysis of the polysaccharides. With automated samplers, the standard deviation of the analysis for glucose was 2 percent. In the case of maltose and lactose, the standard deviation was 5 percent. For food extracts, the precision was approximately 7 percent. Furthermore, this method can be used to analyze sugar alcohols, sugar amines, or sugar phosphates; and it is appropriate for analysis of sugar syrups.

Two presentations dealt with the high-performance liquid chromatographic analysis (HPLC) of sugars. The HPLC methods do not require the derivatization of the sugars. Thus, samples can be analyzed directly. However, HPLC methods are not as sensitive as the gas chromatographic methods.

The first of these methods (presented by M. J. Gray) dealt with the use of ion-exchange resins for carbohydrate separation. The resins were polystyrene-divinylbenzene copolymers which were either polysulfonated or aminated. Calcium, lead, or silver ions are added to the solvent in the use of the polysulfonated resins and are required for the separations. The basis for the separation is not clearly understood. It may be ion exclusion, molecular size exclusion, or ion exchange, or some combination of the three. The method requires the use of a precolumn to remove strong anions or cations depending on the choice of resin and mobile phase. These resins are durable under aqueous conditions and stable up to a temperature of 80 °C. The major disadvantages of this method are that disaccharides cannot be resolved and the resolution is dependent upon the degree of cross-linking and upon the counter ion. Thus, a resin with 6 percent cross-linking in the presence of silver will resolve oligosaccharides up to hexasaccharides; whereas with 4 percent cross-linking in the presence of silver, oligomers as large as nonasaccharides can be resolved. Sugar alcohols can be resolved, by increasing the concentration of acetonitrile.

For the resolution of disaccharides such as maltose and cellobiose, lead is used as the cation. Solutions containing 1 percent saccharides can easily be analyzed. Anion and cation exchange resins bonded to silica can also be used; and for these, the use of metal ions are not required. The solvent commonly used is a mixture of water and acetonitrile, and efficient separations can be achieved through the control of solvent composition and flow rate.

The second presentation by K. Ivie dealt with separations of carbohydrates on aminosilane columns. In this case, the solvent is again water and acetonitrile. The separation can be achieved at ambient temperature, and for the simple sugars appears to give better resolution than the other liquid chromatographic methods. This column has been used to quantify sugars in foods; the example cited was corn syrup. A Corasil™ guard column was used to protect the analytical column. This system resolved sucrose, glucose, and maltose; analyses with a CV of 1 to 2 percent and a relative standard deviation of 1/10 of 1 percent were obtained using an automatic injection mode for more than 100 analyses. Maltose and maltotriose were also resolved by this system.

An alternate method of analysis uses a silica column and a solvent which contains water, acetonitrile, and a small amount of an amine. By controlling the concentration of the amine, one can control the resolution of the column. However, this method does not resolve glucose and galactose. Alpha- and beta-anomers can be resolved by reverse-phase chromatography. The amine modified silicas are adequate for analyzing sugar acids, sugar amines, sugar alcohols, as well as the sugars themselves.

The discussion which followed focused on the sugars most important to both the nutritionist and the industrialist. These included sucrose, lactose, maltose, glucose, fructose, galactose, and malotriose. The participants noted that corn syrup is widely used as a sweetening agent. A reference material for use in determining the degree of adulteration of various corn syrup products is needed. Adulteration is detected by measuring the amount of maltotriose present. The characteristics for a food matrix for a sugar SRM were then discussed. The conclusions were that the matrix must be constant in its moisture content, must be free of fatty acid oxidation, must be free from bacteria, must have little or no enzymatic activity, and should be a homogeneous, easily weighed material. A number of food materials were discussed; the dry products were preferred over the non-dry products because hydrolysis of sucrose occurs in materials such as fruit and vegetable purees. Some of the matrices which were discussed were carob powder, pasturized honey, milk chocolate, corn syrup, invert syrup, dried skim milk, and a powdered beverage mix.

Three general industrial groups were cited as having needs for reference materials: the dairy industry, the cereal and grain industry, and the processed fruit and vegetable industry. Based upon the kinds of industries that would use such Standard Reference Materials, the following series were proposed at the end of the workshop: corn syrup, a ready-to-eat cereal made with whey, a milk chocolate preparation made with skim milk, and a fortified skim milk. Of this list the proposed corn syrup SRM was considered by the food industry to have the greatest priority.

4. Workshop on Fat-Soluble Vitamins and Cholesterol

The fat-soluble analytes discussed were vitamin A, beta-carotenes, cholesterol, and tocopherols. Two methods are currently in use for the analysis of vitamin A: an HPLC method and the Carr-Price method (an AOAC method). These often differ by 5 percent or more in their assessment of vitamin A content. The forms of vitamin A which are commonly used for fortifying food materials or as analytical standards are the alcohol, the palmitate ester, or acetate ester. The major problem

with vitamin A samples is that they contain a mixture of the thirteen cis and the thirteen trans isomers. A vitamin A palmitate preparation can consist of anywhere from 5 to 20 percent of the thirteen cis isomer. If the pure thirteen trans vitamin A palmitate is not available, then USP grade vitamin A acetate, which contains essentially no thirteen cis isomer is suitable for use as a reference material. The current method for vitamin A analysis involves a preliminary saponification of the vitamin A followed by ether extraction and chromatographic analysis. The major problems with vitamin A are its relatively easy oxidation and its sensitivity to light. Hence, the ideal storage conditions include low temperature, nitrogen environment, and storage in a low actinic glass in the presence of antioxidants such as tetrabutylhydroquinone or BHA. Two matrices for vitamin A believed to be feasible for certification and useful for nutritional and industrial measurements, are hydrogenated oils or enriched flour, the former being the matrix of choice.

An HPLC method and the AOAC method are being used for the combined analysis of both the alpha- and beta-carotenes. The availability of a primary standard material was questioned. Furthermore, workshop participants pointed out that useful methods for the separation of the alpha- and beta-carotenes were needed, as well as the separation of the carotenes from the xanthenes. The major current problem is the extractions of carotenes from plant material, not their extraction from oil.

For cholesterol, gas chromatography appears to be the best method of analysis. The availability of SRM Cholesterol (SRM 911a) eliminates the need to find a suitable reference material; thus, the discussion focused on the type of matrix which would be most appropriate for a reference material for cholesterol measurement in foods. A hydrogenated vegetable oil or some other stable oil that was not readily susceptible to oxidation was proposed as a matrix. If the NBS GC/IDMS method is used to establish the cholesterol content, then the participants felt there would be no need to free the oil from possible contaminating sterols. Therefore, they proposed that a soybean oil or some other vegetable oil be used as a matrix. Little time was spent on a discussion of the tocopherols, although it was noted that GC and HPLC methods are available for tocopherol analysis and that their levels in the foods usually run about 50 micrograms per 100 milligrams of material.

The question of how to package the fat-soluble material was of considerable interest. The group generally concluded that the gelatin-type capsule used by the USP is the most convenient method of distributing fat-soluble materials because the capsules can be weighed and dissolved relatively easily. However, they do exhibit some degree of permeability to oxygen. This is significant and, therefore, care must be taken to avoid oxidation. Consequently, the capsules have to be stored in some closed system, preferably low actinic glass and in a nitrogen environment. The workshop participants recommend evaluating both the use of gelatin capsules and some sort of glass container noting that the gelatin capsules are much more convenient for the analysts.

The discussion on appropriate matrices led to the posing of four questions. How applicable is the oil matrix to the general needs of the analyst? How measurements of vitamins in an oil matrix relate to the measurements on real samples? How does extraction of oil matrix samples relate to the problems of extraction of real samples? Can a representative food material be selected? With these points in mind, two additional matrices were proposed: dry milk powder and dog food. These two food matrices can be spiked with reasonable confidence in obtaining a homogeneous product and the reference material can contain more than one analyte.

The levels of the various analytes in standards should be equivalent to 30 to 50 percent of the recommended daily allowance. Therefore, the workshop participants were asked to send us their ideas on recommended spiking levels in the report of the workshop leaders. The minimum requirement by the users for accuracy of measurement using a given standard should be ±5 percent at the 95 percent confidence limits. Finally, a relatively high analyte content in the standard was proposed to permit the standard to be used for the assessment of accuracy of analysis by all methods.

National Bureau of Standards Special Publication 635. Proceedings of the Workshop on Reference Materials for Organic Nutrient Measurement held at NBS, Gaithersburg, Maryland, October 23, 1980.

REPORT OF WORKSHOPS

A. Water-Soluble Vitamins

J. R. Kirk and J. Pennington

Materials with Methodologies Acceptable to Workshop Participants

Nutrient	Extraction Method	Analytical Method (Horwitz, Chap. 43)[a]
Vitamin C	Solvent Extraction	Fluorometric with o-phenylenediamine
	Solvent Extraction	Redox titration with 2,4-dichloro-indophenol
	HPLC	Spectrophotometric or refractive index
Thiamin	Ion-Exchange Bio-Rad 70	Fluorometric as thiochrome
Riboflavin	---	Microbiological
		Fluorometric
Niacin	Solvent Extraction	Spectrophotometric cyanogen bromide and barbituric acid derivative
	---	Microbiological

[a]Horwitz, W., Official Methods of Analysis of the Association of Official Analytical Chemists, 13th ed., Association of Official Analytical Chemists, Washington, D. C. (1980).

Priority Rating[b] of Some Water Soluble Vitamins

Vitamin[b]	Method Adequacy	Stability	Public Health Need
Vitamin C	2	3	1
B-1	3	2	2
B-2	3	2	2
Niacin	1	1	3

[b]The numbers indicate the relative position of each vitamin in each category for determination of natural levels as determined by the participants.

Nutrients and Matrices for Proposed SRM's

A. For B-1, B-2, Niacin:

Non-Fat Dry Milk - B-1, B-2, and Niacin
Non-Fat Dry Milk + B-1
Non-Fat Dry Milk + B-2
Non-Fat Dry Milk + Niacin

B. For Vitamin C:

Freeze-Dried Potato Granules
Freeze-Dried Tomato Juice
Dried Drink Mix with Sugar and Vitamin C added

Vitamins with Methodological Problems

For B-6, B-12, Folacin, Pantothenic Acid, and Biotin:

B-6 and Folacin - of public health significance
Biotin - method adequate but of no public health significance
Folacin - methodology problems
B-6 and B-12 - need for reference materials for method development
B-6 - suggest to use bovine liver (SRM) with added B-6
B-12 - of lower priority than B-6

B. Carbohydrate Analysis

W. J. Hurst and R. Schaffer

Materials with Methodologies Acceptable to Workshop Participants

Methods:

Supplement/complement current Association of Official Analytical Chemists (AOAC) methods (Horwitz, Chap. 31, pp. 506-532)[a] by use of:

a) High-Performance Liquid Chromatography
b) Gas Chromatography
c) Enzymes – not AOAC approved but certainly a potentially viable method

The materials should provide analysts with experience in extracting the carbohydrates since there are not any inherent problems in analysis.

Analytes:

Phase I

Common analytes of interest

(1) Sucrose
(2) Glucose
(3) Lactose
(4) Fructose

Phase II

Galactose[b]	-	Dairy industry interest
Maltotriose[b]	-	The primary interest is for identifying corn syrup related products (which contain maltotriose) as adulterants of "pure" products
Maltose	-	

[a] Horwitz, W., Official Methods of Analysis of the Association of Official Analytical Chemists, 13th ed., Association of Official Analytical Chemists, Washington, D. C. (1980).

[b] Need pure reference material for use by NBS.

Matrices for Proposed SRM's

Corn Syrup	- Simple commercially available product, 100 mL units, presenting no major analytical problems - wide number of users - possible suppliers: Staley, CPC - HPLC is readily accepted as method for analysis - certify for glucose, maltose, maltotriose, moisture
Ready-to-eat cereal with skim milk fortification	- 10 g sample size needed for analysis, 250 g units, 100 units/year (est. sales) - low fat matrix - medium extraction difficulty - good number of probable users - Fructose (1.6%), Glucose (1.6%), Sucrose (3.2%), Lactose (?) - possible suppliers: Kellogg, General Mills, General Foods
Milk Chocolate with skim milk	- 10 g sample size, 250 g units, 100 units/year (est. sales) - high fat matrix - medium extraction difficulty - good number of probable users - Sucrose (45%), Lactose (7+%), Moisture (?) - Possible suppliers: Hershey, Nestle, M&M/Mars, Cadbury - chip form

C. Fat-Soluble Vitamins, Cholesterol, and Fat

D. C. Egberg and H. T. Slover

Materials with Methodologies Acceptable to Workshop Participants

The following nutrients are listed in decreasing order of priority and increasing order of analytical difficulty. The currently preferred method of analysis and additional pertinent information are also tabulated.

Nutrient	Method of Analysis	Suggested Source	Key Contact
Cholesterol	GC GC/MS	NBS	H. Slover
Vitamin A Palmitate (all-trans)	HPLC Carr-Price	Hoffmann-LaRoche or USP	J. DeVries (Gen. Mills)
β-Carotene	HPLC AOAC[a]	Eastman Kodak	Stewart (U. of Florida) or S. Reeder (California)
α-Tocopherol	GC	Eastman Kodak	H. Slover
Fat (solid matrix only)	AOAC[b] or best method	Durkee, Anderson, Clayton, or Kraft	H. Lento
Additional Possible Fat Analyses:			L. L. Diosady (U. of Toronto)
Trans-unsaturates	GC or infrared (IR) (in fat)		A. Sheppard
Polyunsaturates	GC or Lipoxidase (in fat)		A. Sheppard

[a] Horwitz, W., Official Methods of Analysis of the Association of Official Analytical Chemists, Chapt. 43, 13th ed., Association of Official Analytical Chemists, Washington, D. C. (1980).

[b] Method is matrix dependent, see footnote a, Chapter 13-18.

Matrices for Proposed SRM's

Two SRM mixtures were proposed for fat, cholesterol, and fat-soluble vitamins: (1) a partially hydrogenated vegetable oil containing cholesterol and the fat-soluble vitamins, and (2) this mixture dispersed on fat-free dry milk powder.

Partially hydrogenated vegetable oil was selected to maximize the stability of the mixture. This matrix is proposed rather than a totally hydrogenated oil because of the high melting point of the latter. Furthermore, a partially hydrogenated oil (same solid-fat index as a common margarine) may serve also as a reference material for trans-saturated fats.

Storage Conditions

The oil (previously degassed to remove O_2) should be put into low-actinic gelatin capsules and stored at -20 °C in a nitrogen atmosphere. An antioxidant (tertiary butyl hydroquinone) should be added to the oil at 200 ppm. The fat dispersed on dry milk powder should also be stored in sealed containers under nitrogen at -20 °C.

Loads of Nutrients in Fat

Nutrient	Hydrogenated Oil, amount 100 gm	Non-Fat Dry Milk, amount 100 gm
Cholesterol	100 mg	25 mg
Vitamin A Palmitate	2500 IU	2500 IU
Tocophenol	15 IU	15 IU
Carotene	2500 IU	2500 IU

Suppliers of Raw Materials and Possible Processors

Non-Fat Dry Milk - Land-O-Lakes, Carnation

To Mix Nutrients - Producers of Dry Infant Formulas - Ross Laboratories, Mead-Johnson

To Fill Capsules - G. D. Searle, Nutrilite Products, Inc.

Summary of Workshop Proposals

S. A. Margolis

The participants in each workshop session established a reference material priority list for the nutrients discussed and the types of matrices to be used. Their recommendations are summarized in the attached reports.

Several general observations were evident upon review of the workshop sessions:

1. With the exception of vitamin B_{12} and folacin, there appears to be minimal need for reference materials of higher purity than USP quality reference materials.

2. There is an immediate need for Standard Reference Materials (SRM's) consisting of food matrices with specific nutrients at certified concentrations.

3. There was some conflict about the nature of the proposed SRM materials. Should they be matrices certified for fortified concentrations of the nutrients or, rather, matrices certified for the natural content of the nutrient? The consensus was to certify for the content of native nutrient, only when the nutrient was present in a free form, e.g., vitamin C. In other cases, the initial effort should be directed toward the preparation of a nutrient fortified food material, e.g., non-fat dry milk.

The levels of the certified nutrients should be between 25 percent and 50 percent of U.S. recommended daily allowance (U.S. RDA). The major reason for not attempting certification of bound nutrients, particularly the B vitamins, is the instability of these nutrients under extraction conditions.

The SRM priority lists proposed by the workshop participants fall into three categories, each with several subcategories. The subcategory assignments do not indicate a preference for order of certification.

1. Standards which are needed and which can be prepared with reasonable hope of success.

   a. Corn syrup certified for glucose, maltose, maltotriose, and moisture: This standard appears to be widely needed; existing isotope dilution mass spectrometry (IDMS) methods can be used for glucose determinations.

   b. Partially hydrogenated vegetable oil certified for cholesterol: Cholesterol can be accurately measured by IDMS methods.

   c. Non-fat dry milk certified for fortified amounts of niacin, thiamin, and riboflavin.

   d. Either non-fat dry milk or a sugar-containing dry drink mix certified for vitamin C.

   e. Finally, of lower priority, a non-fat dry milk certified for galactose and lactose.

2. Standards which are needed but present purity or stability problems.

   Vitamin A palmitate and β-carotene in partially hydrogenated vegetable oil.

3. Other nutrients which present major problems of stability, purity, and/or selection of appropriate vitamers.

   These are listed with their respective problems in the workshop reports.

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This publication is the formal report of the Workshop on Reference Materials for Organic Nutrient Measurement held at the National Bureau of Standards on October 23, 1980. There were seven formal presentations which provided the framework for three workshop sessions. Each workshop session focused on one of three groups of nutrients: (1) cholesterol, fat, and fat-soluble vitamins; (2) water-soluble vitamins; or (3) sugars. Each workshop session reported on the state-of-the-art in measurement techniques, suggested matrices which were appropriate for Standard Reference Materials (SRM's), and indicated areas where there were problems in measurement methodology. These recommendations are included in this publication.

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