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Considerations on Organic Compounds in Solution and Inorganic Ions in Glasses as Fluorescent Standard Reference Materials

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The use of various organic compounds in solution and inorganic ions in glasses has been investigated as possible fluorescence Standard Reference Materials. Emphasis was placed on measuring physical and chemical parameters such as stability, reproducibilities of absorbance and fluorescence measurements, relative quantum efficiencies as a function of excitation wavelength, etc., for quinine derivatives and selected organic compounds. A brief discussion is included on the use of rare earth and non-rare earth inorganic ions in glasses as standards.

Key words: Emission spectra; excitation spectra; fluorescence; fluorescence standards; glass standards; quinine derivatives; rare earths; relative quantum efficiencies; solution standards.

I. Introduction

The rapid growth of publications in fluorimetric research and applications during the last two decades, and the variability of data and results in those publications, underline the need for standardization of fluorescence nomenclature and data presentation. These needs were discussed previously by investigators [1–3][1], who collectively presented a proposal for fluorescence standardization [4]. The main objective of this proposal was to supply a firm basis for reporting fluorimetric results so that comparisons of data among laboratories would be meaningful.

There are basically two methods which can be used for the presentation of data: (a) in absolute radiative or energy units, and (b) by comparing fluorescence data of the compound under study with data obtained for an accepted standard. Very few laboratories are equipped to make absolute radiant energy measurements; however, comparative spectra of standards run under the same experimental conditions on the same instrument can be easily done. This latter method, which effectively corrects for instrumentally dependent perturbations on the true fluorescence spectra is most widely used and applicable provided the users know the limitations, not only of their instrumentation, but also of the standard.

Not included here are fluorimetric measurements obtained on "uncorrected" instruments which are presented without units of intensity or benefit of comparative measurements. Many of these types of published results have led to confusion and are often of no quantitative value.

Fluorescence standards can be used to (a) correct spectra for instrumental parameters such as the wavelength dependence of exciting lamp intensity, monochromator transmission and detector response, (b) determine comparative quantum efficiencies, (c) compare data among laboratories, (d) determine periodically "in house" instrumental stabilities, (e) calibrate fluorescent lifetime determinations, and (f) calibrate polarization value measurements.

The requirements for a "general" fluorescence standard are quite specific. It should:

(a) have broad fluorescence spectra,
(b) be easily purifiable,
(c) be stable in solution or as the solid,
(d) have as little overlap as possible between the excitation (absorbance) and emission spectra,
(e) not be subject to oxygen quenching,
(f) have a constant quantum efficiency as a function of exciting wavelength,
(g) have isotropic emission,
(h) have the same emission spectrum shape independent of exciting wavelength,
(i) be soluble in aqueous and organic solvents, and
(j) absorb and emit in the same general regions as the compound under study.

No single compound exhibits all these desirable characteristics. In fact, the last requirement precludes the use of a single standard, for although absorbance may occur over a wide range (e.g. rhodamine B absorbs from 200 nm to about 560 nm), emission is usually limited to one small wavelength interval (for rhodamine B, 550 nm to 650 nm with maximum at 573 nm).

For this reason, in addition to the fact that fluores-

[1] Figures in brackets indicate the literature references at the end of this paper.

cence spectra are composites of true spectra and instrumental parameters dependent on wavelength, it is necessary to have available a series of fluorescence standards with emission maxima that covers the wavelength range of current interest. With the wide use of lasers and interest in radiative measurements in the near-infrared, this range now extends from 250 nm to 1100 nm.

One of the major objectives of the fluorescence program at the National Bureau of Standards is the selection, production, and certification of a series of fluorescence standards (Standard Reference Materials) which can be used for the standardization and comparison of data and methodologies among laboratories [5, 6]. It is essential that these Standard Reference Materials (SRM's) be certified with absolute values. Data reported here, however, are in relative units and not absolute since instrumentation for absolute measurements is not available at the present time. Absolute measurements will be made and values assigned to the SRM's as soon as instrumentation becomes available.

The work at NBS is following two parallel investigative paths: (a) the use of solutions of organic molecules and metal chelates in various solvents, and (b) in close collaboration with Reisfeld, The Hebrew University, Jerusalem, the use of inorganic ions in solid matrices. Group (b) may be further split into rare earth and nonrare earth inorganic ions. The investigative work on the inorganic ions has been covered extensively earlier in this series of papers by Reisfeld [7]. Discussion here, then, will be restricted primarily to the use of comparative standards in solution and some of the factors which affect quantum yields and spectra.

Since quinine and its derivatives are probably the most widely used comparative standards [13], and since a great deal of controversy exists as to their usefulness as standards, the emphasis of this paper is on the reproducibility, stability, and effects of wavelength excitation and acid concentration on the spectra and quantum efficiencies of the quinines.

I would like to digress briefly and discuss some of the equations and assumptions which have to be made in using comparative techniques and standards. Two types of solutions may be employed: optically dense and optically dilute. With optically dense solutions and front surface excitation and emission, Vavilov's experimental set up with some modifications is generally used [1, 8–12]. This method is not as widely utilized as the optically dilute method since stray light is high, compounds with large emission and excitation overlap give erroneous results, and only compounds with a high molar absorptivity can be used easily [13].

The optically dilute method is based on the Beer-Lambert Law and several assumptions. Combining the definition of quantum efficiency [14], eq (1),

$$Q=\frac{I_F}{I_A} \tag{1}$$

where Q = luminescence quantum efficiency
I_F = rate of fluorescence emission in quanta/s.
I_A = rate of light absorption in quanta/s.

with the Beer-Lambert Law, eq (2),

$$\frac{I_T}{I_0}=e^{-\epsilon cl} \tag{2}$$

where I_0 is the radiative flux of the exciting light in quanta/s,
I_T is the radiative flux of the transmitted light in quanta/s.
ϵ is the molar absorptivity in liter, mole^{-1}, cm^{-1},
c is the concentration in moles/liter,
l is the path length of the cell in cm,

and expanding the exponential term in a power series, the expression for the rate of light emission becomes [3, 13, 15]:

$$I_F=I_0(2.3\epsilon cl)\left[1-\frac{2.3\epsilon cl}{2}+\frac{(2.3\epsilon cl)^2}{6}-\cdots\right]Q. \tag{3}$$

For dilute solutions (i.e. absorbances less than 0.05), eq (3) may be approximated with only slight error by eq (4) [15],

$$I_F=I_0(2.3\epsilon cl)\,Q. \tag{4}$$

In determining comparative quantum efficiencies, a term for the index of refraction of the solvent is added [16–18], resulting in the final form of the applicable equation:

$$Q_u=Q_s\left(\frac{FA_u}{FA_s}\right)\left(\frac{A_s}{A_u}\right)\left(\frac{I_{0s}}{I_{0u}}\right)\left(\frac{\eta_u^2}{\eta_s^2}\right) \tag{5}$$

where Q is the quantum efficiency,
FA is the integrated fluorescence area (Σ quanta/unit bandwidth),
I_0 is the radiative flux of the exciting light in quanta/s,
η is the average refractive index of the solvent over the emission wavelength range, and
u and s refer to the unknown and standard, respectively.

In the studies reported here, comparative quantum efficiencies were calculated using eq (6) [20]:

$$Q_u=Q_s\left(\frac{FA_u}{FA_s}\right)\left(\frac{A_s}{A_u}\right)\left(\frac{\lambda_{ex_s}}{\lambda_{ex_u}}\right)\left(\frac{\eta_u^2}{\eta_s^2}\right) \tag{6}$$

where $\lambda_{ex_s}/\lambda_{ex_u}$ replaces I_0 and converts from an instrumentally produced constant energy to quanta/s.

The assumptions have been made, however, that (a) instrumental geometries are the same for the unknown and standard, (b) emission is isotropic, (c) the exciting light is monochromatic if excitation for both samples is

at different wavelengths, (d) no reabsorption and reemission occur, and (e) the absorption is less than 0.04. (Recent work permits solutions with absorbances of 0.2 to 1.0 to be measured by the sideview technique [21, 22].) In addition, use of dilute solutions minimizes factors (d) and (e), and use of the same instrument and cuvette minimizes factor (a). Anisotropic emissions (polarization effects) may cause large measurement errors which may be corrected [13, 23]; but the easiest way to avoid polarization problems is to use a standard which emits isotropically.

Assuming then that corrected spectra may be obtained [11, 12, 15], the fundamental problem in spectrofluorimetry is the lack of well-characterized standards. Table 1 lists various organic compounds which have been suggested for possible inclusion in a series of

TABLE 1. Compounds proposed as fluorescence standards [1, 3, 8–10]

Compound	Emission maxima (nm)
β-Naphthol	354, 402
2-Aminopyridine	368
Anthracene	383, 404, 428, 454
Pyrene	390
Quinine sulfate	454
Quinidine sulfate	454
3-Aminophthalimide	510[a]
Fluorescein	518
N,*N*-Dimethylamino-*m*-nitrobenzene	542
Rhodamine 6G	557
Aluminum(III)-PBBR-chelate	635
4-Dimethylaminonitrostilbene	742

[a] Value is average.

fluorescence standards [1, 3, 10, 24, 25]. Some may not be suitable as general standards. Fluorescein undergoes degradation in sodium hydroxide, and solutions should be prepared and used within 12 hours. The purification of β-naphthol by recrystallization in air results in some discoloration due probably to oxidation. Aromatics such as anthracene and pyrene have narrow emission and/or excitation spectra (25 nm or less) and are not suitable as standards for fluorimeters which use wide slits since fluorescence spectra, as absorption spectra [26], are bandpass dependent [27, 28]. Other factors, such as acid concentration or excitation wavelength, are reported to affect the fluorescence of quinine and quinidine sulfates [21, 28–37] and 3-aminophthalimide [38]. The presentation and discussion of some of these factors and their effects, follow. The experimental section is presented as addendum A.

The following abbreviations are used in this paper: Q – quinine; QS – quinine sulfate; QDS – quinidine sulfate; 3-APT–3-aminophthalimide; N,N-DMAMNB–*N*,*N*-dimethylamino-*m*-nitrobenzene. Quinine samples are designated A, B, C, etc. and are listed in addendum A.

II. Results and Discussion

A. Quinine Sulfate and Related Compounds

1. Physical and Chemical State

It has been reported that the history of the quinine determines its optical characteristics [24, 35]. A report by Fletcher [28], however, suggests this is not the case, and that the observed differences were probably due to the presence of varying amounts of hydrated water. Although quinine sulfate is obtained as the dihydrate, varying amounts of water are definitely present. Table 2 summarizes the weight losses as determined by thermogravimetric (TGA) measurements for various quinine derivatives before and after vacuum drying. It can be seen that with no

TABLE 2. Water weight losses of various quinine derivatives as a function of pretreatment

Sample[a]		Percent weight loss	Calculated molecular formula
F	No drying	4.24	$QS^{b} \cdot 5.4H_2O$
C	No drying	4.96	$QS \cdot 5.7H_2O$
E	No drying	[c]4.37	$QS \cdot 5.0H_2O$
E	Vacuum dried, in desiccator for 6 months	0.18	$QS \cdot 2.1H_2O$
E	Vacuum dried within 24 hours	.03	$QS \cdot 2.0H_2O$
H	No drying	4.36	$QDS^{d} \cdot 5.4H_2O$
H	Vacuum dried within 24 hours	0.02	$QDS \cdot 2.0H_2O$
J	No drying	.00	$Q^{e} \cdot 2.0H_2O$

[a] See experimental section for designation.
[b] QS = quinine sulfate.
[c] Average of two determinations.
[d] QDS = quinidine sulfate.
[e] Q = quinine.

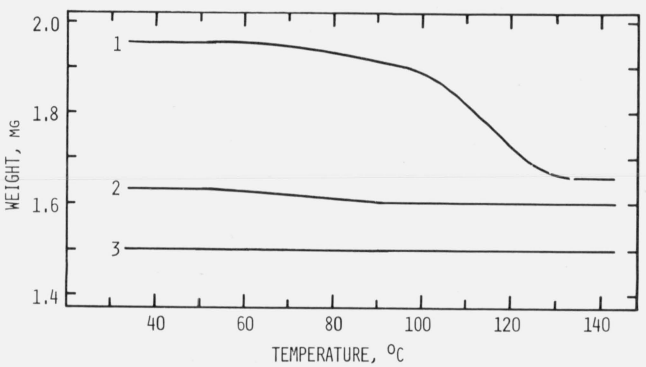

FIGURE 1. Thermogravimetric analyses of quinine sulfate (Sample E). 1. undried after recrystallization; 2. dried in vacuo at 55 °C for 18 hours, placed in desiccator, removed periodically to obtain sample; 3. freshly dried as 2. Heating rate was 5.0 °C/min in dry air. Weight scale offset 10 mg.

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drying, approximately 5 to 6 waters of hydration per molecule rather than 2 are actually present. Figure 1 shows three TGA curves obtained for $QS \cdot 2H_2O$, sample E. There is a slight loss of water for the vacuum dried sample that had been kept in a desiccator for six months and removed periodically to obtain samples which shows the hygroscopic nature of $QS \cdot 2H_2O$. As pointed out by Fletcher [28], insufficient drying can cause variations in absorbance values, leading investigators to propose the inequivalence of quinine derivatives. For this reason, samples used for the following studies were predried as outlined in the experimental section.

2. *Absorbance Measurements*

Average weight absorptivities for various $QS \cdot 2H_2O$ samples from different suppliers with varying recrystallizations were calculated from eq (7), and the results are summarized in table 3.

$$WA = \frac{A}{cl} \tag{7}$$

where WA is the weight absorptivity.
A is the absorbance.
c is the sample concentration in grams solute/gram solvent.
l is the path length of the cell in cm.

Sample F gave consistently high weight absorptivities, but was included in the statistical analyses. The average weight absorptivities at 317.0, 347.5, and 365.0 nm for samples A to F differ from previous data [28] by −1.2 percent, +0.5 percent, and −2.3 percent in that order. Values for the average of all samples, including E, differ from previous data by −1.0 percent, −0.5 percent, and −1.0 percent in that order.

TABLE 3. Determination of weight absorptivities for quinine sulfate with varying recrystallizations in 0.1 N H_2SO_4 from different suppliers

Sample[a,b]	Rx[c]	Weight absorptivities × 10^4			
		365.0 nm	347.5 nm	317.0 nm	250.0 nm
A	0	0.922	1.430	1.148	7.720
B	0	.916	1.418	1.146	7.700
C	0	.918	1.428	1.145	7.710
D	3	.922	1.438	1.140	7.820
F	4	.941	1.454	1.162	7.920
E	6	.925	1.436	1.152	7.820
E'[d]	6	.947	1.445	1.146	7.717
Average[e]		0.924	1.434	1.149	7.782
σ		.009	0.012	0.008	0.086
Percent σ		.97	.84	.66	1.12

[a] All samples vacuum dried at 50–60 °C for 24 hours.
[b] See experimental section for sample designation.
[c] Times recrystallized.
[d] Absorbances measured on Cary 16, others on Cary 14, see addendum A.
[e] For samples A–F only (excluding E').

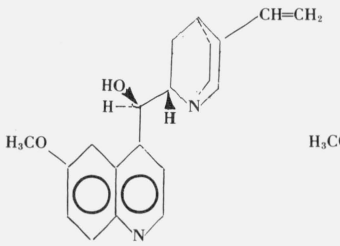
Determination of the weight absorptivities at 347.5 nm for three individual weighings of sample E yielded a value of 1.436 ± 0.005. Weight absorptivities and molar absorptivities are also given for $QDS \cdot 2H_2O$ in

TABLE 4. Molar and weight absorptivities (× 10^4) of quinine and quinidine sulfates in 0.1 N H_2SO_4

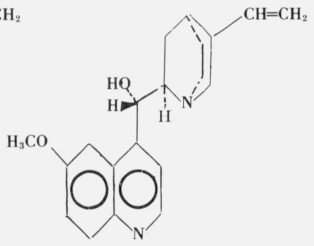
Compound	Concentration $M \times 10^{-5}$	Sample	Wavelength							
			365.0 nm		347.5 nm		317.0 nm		250.0 nm	
			ϵ[c]	WA[d]	ϵ[c]	WA[d]	ϵ[c]	WA[d]	ϵ[c]	WA[d]
Quinine sulfate[a]	2.554	E	0.7235	(0.924)	1.1228	(1.434)	0.8996	(1.149)	6.0930	(7.782)
Quinidine sulfate[b]	3.269	H	.7189	(0.918)	1.1257	(1.438)	.8902	(1.138)	6.0110	(7.676)

[a] Average for 6 determinations, samples A–F, table 2.
[b] Average for two determinations.
[c] l, mol^{-1}, $cm^{-1} \times 10^4$.
[d] $g_{solvents}$, $(g_{solute})^{-1}$, $cm^{-1} \times 10^4$.

table 4. As expected, the molar and weight absorptivities for QS·2H_2O agree with those for QDS·2H_2O since they are stereoisomers (structures for quinine and quinidine are I and II, respectively).



I. Quinine



II. Quinidine

A single determination for the molar or weight absorptivities for quinine dihydrate gave results which, as a function of wavelength, were consistently 3 to 5 percent low as compared to the values for QS and QDS after correction for the difference in molecular weights. Plotting relative absorbances, i.e. A_λ/A_{250nm} versus wavelength, however, resulted in good agreement among the three spectra, figure 2, indicating the probable presence of a systematic error in the determination of the absorptivities for quinine dihydrate. These values are being redetermined.

Values of the ratios for several absorbance peaks of QS in 0.1 *N* sulfuric acid have been calculated from the literature [28–30, 33, 36, 46–48], and these values and those from the present work are reported in table 5. Although systematic errors exist since various spectrophotometers were used, comparison of the average of the literature values and the present work show differences to be +2.77, +0.25 and +0.62 percent. The first value is for the A_{250}/A_{347} ratio, and measurements in the ultraviolet are usually not as accurate or precise as those in the visible. Statistical evaluation (again

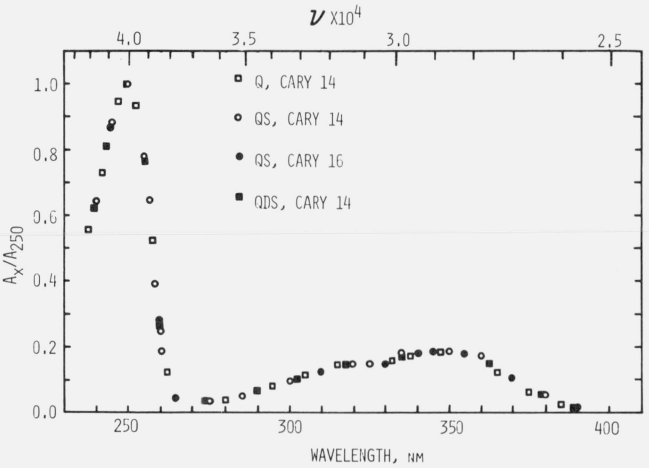


FIGURE 2. Normalized absorbance spectra of quinine (Q), quinine sulphate (QS), and quinidine sulfate (QDS) in 0.1 *N* H_2SO_4 using absorbance at 250.0 nm as 1.0. Temperature, 25.0 °C.

TABLE 5. Absorbance ratios for quinine sulfate dihydrate in 0.1 *N* sulfuric acid

Spectrophotometer	A_{250}/A_{346}	A_{317}/A_{346}	A_{365}/A_{346}	Ref.
Cary 16	5.340	0.793	0.655	this work
Cary 14	5.43	.80	.64	this work
Cary 14	5.38	.78	.65	[33]
Cary 14	5.36	.80	.62	[30]
Beckman D.U		.82	.63	[47]
Beckman D.U	5.43	.80	.59	[29]
Bausch and Lomb	5.23	.80	.63	[30]
Turner 210	5.4	.82	.66	[28]
	5.0	.75		[46]
	5.25	.80	.65	[48]
	5.10	.82	.69	[36]

including systematic errors) of all the ratios shows, however, that any individual ratio may be as much as 4 or 5 percent high or low at only the 68 percent confidence level. It is essential that accurate data be obtained for absorbance measurements, especially since the determinations of relative quantum efficiencies depend on these measurements, eqs (5) and (6).

The instruments used for the absorbance measurements in the present work were checked for accuracy by comparison against optically neutral filters supplied by Mavrodineanu [26]. Absorbance measurements were also made on solutions of QS and 3-APT in 0.1 *N* sulfuric acid at peak maxima using various instruments. The results are summarized in table 6. Good agreement was observed for these solutions. No

TABLE 6. The determination of absorbances of quinine sulfate and 3-aminophthalimide in 0.1 *N* H_2SO_4 using various spectrophotometers

QS A, 250.0 nm	3-APT A, 385.0 nm	Instrument
	0.9164	NBS [26]
0.7506	.9158	Cary 16
.751	.919	Cary 14
.749	.914	Turner 210

apparent fluorescence artifact was observed as shown by comparing absorbances from the first three instruments with those of the last instrument which has a monochromator between the sample and the detector [20, 23].

Differences as large as 8 percent, however, were observed when absorbances were measured on slopes in absorbance spectra. Care must therefore be used when absorbance measurements are made on slopes of peaks since small errors in wavelength (and also large bandwidths if the slope is steep) result in inaccurate absorbance values.

3. *Fluorescence Measurements*

Studies were made to determine fluorescence reproducibility of measurements on quinine sulfate dihydrate (sample E) in 0.1 *N* sulfuric acid. Table 7 summarizes results of repetitive measurements on five different quinine sulfate solutions.

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TABLE 7. Reproducibility of repetitive fluorescence measurements on different solutions of quinine sulfate dihydrate

(Sample E) in 0.1 *N* sulfuric acid[d]

Concentration (ppm)	Average emission peak area	σ	Percent σ
0.5[a]	0.053	0.002	2.83
1.0[a]	.104	.002	1.92
1.0[b]	.104	.001	1.09
2.5[a]	.260	.002	0.85
5.0[a]	.706	.003	.42
5.0[b]	.518	.001	.22
10.0[a]	.915	.007	.78
10.0[b]	.911	.003	.32
5.0[c]	.517	.003	.59

[a] Dilution by volume, four sample weighings.
[b] Dilution by weight, three sample weighings.
[c] Quinidine sulfate dihydrate (Sample H) dilution by weight.
[d] λ_{ex}, 350 nm.

Fairly large standard deviations were obtained with the 0.5 and 1.0 ppm solutions, probably due to instrumental noise resulting from increased instrumental sensitivity. As can be seen, dilution by weight yields results for which standard deviations are approximately two times lower than those for dilution by volume. Similar results were obtained for solutions of quinidine sulfate [38]. All weighings of the vacuum-dried solid samples were made in air. Weighing in a dry nitrogen atmosphere should result in lower percent standard deviations since the dried material is somewhat hygroscopic.

Similar fluorescence measurements made on solutions of quinine sulfate samples from two different sources (samples A, B, D, and F), results summarized in table 8, show that the relative integrated area under the fluorescence peak may be considered to be the same if percent standard deviations of 1 to 3.0 percent are acceptable.

TABLE 8. Measurement of fluorescence peak areas using quinine sulfate dihydrate from two sources[a]

Sample	Emission peak areas: One day average	Emission peak areas: Three day average
A	0.555	0.554
B	.542	.544
D	.552	.556
F	.564	.558
Average	.553	.553
σ	.009	.006
Percent σ	1.64	1.12

[a] λ_{ex}, 350.0 nm; °C = 25.0.

Studies were also initiated to determine the stability of quinine sulfate (sample E) in 0.1 *N* sulfuric acid since little definitive data exist concerning the stability of QS in solution [43, 44, 49]. Table 9 summarizes results for "stabilities" during two "short time" (1 to 10 h) tests, while table 10 and figure 3 summarize results for longer periods of time (25 to 35 days). As might be expected, the results in table 9 show stabilities are better for the 1 hour determinations than for those stretching over a 10 hour period. From table 10, it is observed that glasses give more repeatable values and thus lower percent standard deviations. It is evident from the data in tables 9 and 10 and figure 3 that the results are actually composites of quinine sulfate solution stability and instrumental stability. The latter was maximized by using the overlapping solution method, and the results from this study are graphically illustrated in figure 4.

TABLE 9. Statistical analyses for fluorescence determinations[c] of quinine sulfate dihydrate (Sample E) in 0.1 *N* H_2SO_4 during two "short" time intervals

Concentration (ppm)	1.0[a]	5.0[a]	10.0[a]	1.0[b]	2.0[b]	2.0[b]
H_2SO_4, *N*	0.1	0.1	0.1	0.1	0.1	0.01
$\bar{A}$, peak area	.105	.557	1.116	.411	.816	.848
σ	.004	.003	.005	.001	.002	.001
Percent σ	4.34	.59	.49	.21	.24	.14

[a] Eleven determinations during a 10 hour span (same solution).
[b] Four determinations during a 1 hour span (same solution).
[c] λ_{ex} = 350.0 nm; °C = 25.0.

TABLE 10. Fluorescence peak areas of quinine sulfate and a uranium doped glass as a function of time on corrected and uncorrected instruments

	Instrument A, corrected [20] QS, Sample E[a,b]	Instrument B, uncorrected QS, Sample E[a,c]	Instrument B, uncorrected Uranium glass[c]
Average peak area	0.739	0.398	0.383
σ	.011	.027	.018
Percent σ	1.49	6.89	4.80

[a] 2.0 ppm in 0.1 *N* H_2SO_4.
[b] Fifteen measurements covering 26 days.
[c] Fourteen measurements covering 37 days. The uranium glass was too concentrated for use in the corrected instrument.

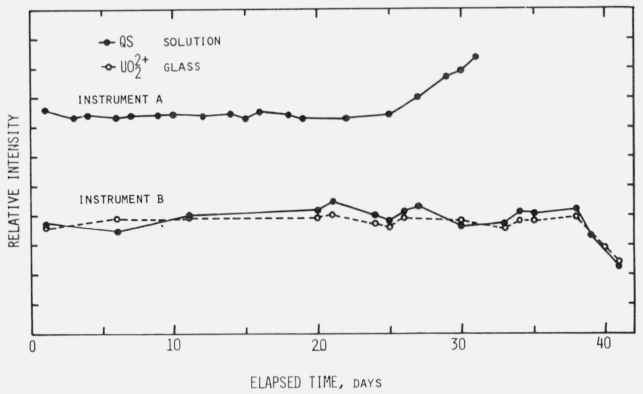

FIGURE 3. Instrumental and compound stability determinations using 2 ppm (μg solute/g solvent, 2.55×10^{-6} *M*) quinine sulfate in 0.1 *N* H_2SO_4 and a uranyl glass on corrected (instrument A) and uncorrected (instrument B) spectrofluorimeters.

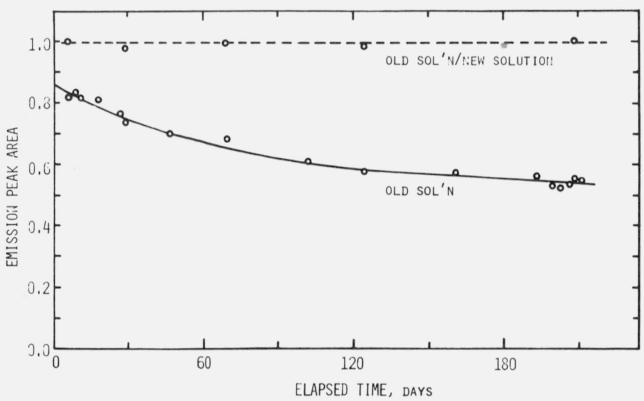

FIGURE 4. 2 ppm (2.55×10^{-6} *M*) quinine sulfate stability test in 0.1 *N* H_2SO_4 using relative emission peak areas as a function of time, designated by solid line and overlapping solution method (peak area original solution/peak area fresh solution), designated by broken line.

A 37 percent decrease in relative peak area as a function of time was observed (solid line) which, on face value, would indicate instability of the quinine sulfate in solution. Preparing new solutions, however, and comparing the peak areas to those of the "aged" solution gave ratios of one (designated by dashed line) proving quinine sulfate stability in solution over this period of time. The assumption was made that dried QS in a desiccator in the dark was stable over the 6-month period of time. Absorbance measurements for the freshly prepared QS solutions agreed within the standard deviations presented in table 3. The observed 37 percent decrease in peak area was due therefore to an instrumental factor and not QS instability.

No long term data as to the stability of quinine sulfate in a 0.1 *N* sulfuric acid solution in light have been established; however, preliminary results indicate that a 2 ppm solution is stable for 2 weeks if irradiated by typical laboratory fluorescent lighting [38]. Excitation in this study was done at 350 nm and emission peak areas were measured in the usual manner. However, Melhuish [50] reports a 25 percent decrease in fluorescence intensity upon irradiation of the QS solution with 313 nm light (and excitation at 313 nm). Excitation at this wavelength also resulted in a deviation of quantum efficiency as a function of excitation wavelength [13, 21]. The problems which arise when this wavelength is used should be investigated.

4. *Corrected Excitation and Emission Spectra*

To insure consistency in relative quantum efficiency measurements with instruments that give corrected spectra, the excitation spectrum should be compared with the absorbance spectrum and the emission spectrum compared with corrected spectra obtained by other investigators.

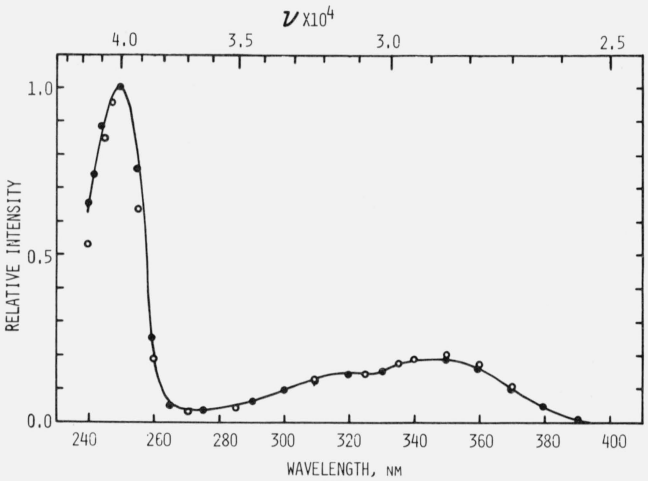

FIGURE 5. Normalized intensities which show equivalency of absorbance spectrum (solid line) and excitation spectrum (circles). Quinine sulfate solution 20 ppm (2.55×10^{-5} *M*) for absorbance spectrum and 2 ppm (2.55×10^{-6} *M*) for excitation spectrum, both in 0.1 *N* H_2SO_4; excitation bandwidth, 25 Å, emission bandwidth, 100 Å; temperature, 25.0 °C.

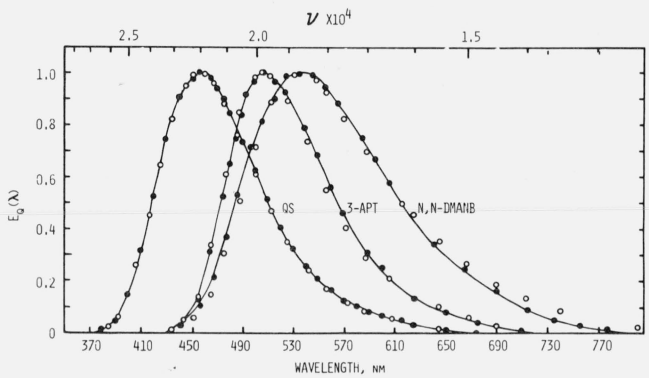

FIGURE 6. Relative $E_q(\lambda)$ versus wavelength for quinine sulfate [2 ppm (2.55×10^{-6} *M*) in 0.1 *N* H_2SO_4], 3-aminophthalimide [5.64 ppm (2.68×10^{-5} *M*) in 0.1 *N* H_2SO_4], and N,N-dimethylamino-*m*-nitrobenzene [2.76 ppm (9.34×10^{-6} *M*) in 70 percent *n*-hexane: 30 percent benzene, V/V] ●= this study, ○=Lippert, et al., [1] corrected from $E_q(\nu)$ to $E_q(\lambda)$ [50].

The equivalency of the excitation spectrum and absorbance spectrum is shown in figure 5, which has, as a direct consequence, the constancy of quantum efficiency as a function of excitation wavelength (discussed later). Some deviation is seen at wavelengths >350 nm as might be expected with the reported shift in emission maximum as a function of excitation wavelength [29, 31]. The emission spectra of QS and 3-APT in 0.1 *N* sulfuric acid and N,N-DMAMNB are given in figure 6 as $E_q(\lambda)$ versus λ in which $E_q(\lambda)_{max}=1$. Data show good agreement with those taken from Lippert, et al. [1] which were corrected to $E_q(\lambda)$ from $E_q(\nu)$ by multiplying $E_q(\nu)$ by λ^{-2} [50].

5. *Relative Quantum Efficiencies*

a. Intercomparative Study.—To verify the validity of the relative quantum efficiency (QE) measurements, an intercomparative study was undertaken using QS, fluorescein, pyrene, rhodamine B, and 3-APT in various solvents as comparison "standards." Table 11 summarizes the results of this study. The values of the quantum efficiencies for quinine or its derivatives in sulfuric acid have been reported to be in the range 0.40 to 0.70 [12, 21, 32, 34, 35, 37, 43–45]. Melhuish [43] and Dawson and Windsor [21] have measured the absolute quantum efficiency for QS in 1.0 *N* sulfuric acid and obtained excellent agreement (0.546 and 0.54 ± 0.02, respectively, $\lambda_{ex}=365$ nm). Measurements by Eastman [35] tend to support these findings. Systematic errors [13] have been suggested to explain low values of 0.46 or less by Rusakowicz and Testa [24] and Drobnik and Yeargers [34]. The quantum efficiencies chosen for this comparative study are the widely used and accepted values of 0.55 and 0.51 in 1.0 and 0.1 *N* sulfuric acid, respectively [43].

It is difficult to reconcile the results of this intercomparative study and many others with those of Scott, et al. [37], who recently reported the quantum efficiency of QS in 0.1 *N* sulfuric acid, based on lifetime measurements, as 0.70. It has been reported that QS has an anomalous lifetime [45, 51], and thus basing quantum efficiencies on this measurement alone is questionable. In addition, Scott, et al., suggest that the discrepancies in quantum efficiencies may be explained if previous investigators had ignored the red "tail" (>550 nm) of the corrected emission spectrum. An examination of the corrected emission spectra, however, figure 6 [1], shows that the spectra extend quite far into the red (>650 nm).

Quantum efficiencies for fluorescein in sodium

TABLE 11. Results of the intercomparative quantum efficiency (QE) study [e]

	Solvent	QE	QE (lit.)
QS[a]	0.1 *N* H_2SO_4	0.51 ± 0.01	0.508[43]
QDS[b]	.1 *N* H_2SO_4	$.52\pm0.01$	
Fluorescein[c]	.1 *N* NaOH	$.87\pm0.03$	[d] .88
Pyrene[b]	Toluene	$.57\pm0.04$	.60[21], 0.32[51]
Rhodamine B[b]	Ethanol	$.72\pm0.02$	.69[3], 0.71[13]
3-Aminophthalimide[b]	0.1 *N* H_2SO_4	$.47\pm0.02$	

[a] Determined by intercomparison study with fluorescein, pyrene and rhodamine B.
[b] Duplicate determinations, duplicate measurements.
[c] Quadruplicate determinations, duplicate measurements.
[d] Average of 16 literature values, see text.
[e] Temperature, 25.0 ± 0.1 °C.

TABLE 12. Dependence of the relative quantum efficiencies of quinine and quinidine sulfates on sulfuric acid concentrations [a]

	Quantum efficiencies				
	$QS\cdot 2H_2O$				$QDS\cdot 2H_2O$
$[H^+]$, *N*	This work	[21]	Percent [c]	Percent [32]	This work
0.01	0.52 ± 0.01		+1.97		
.02	.52		+1.57		
.10	[b] .51	0.50	—		0.52
.50	.54		+5.71		
1.02	$.55\pm0.01$	.54	+7.68	6	.55
2.00	.57		+9.84		
3.56	$.60\pm0.01$	.60	+17.72	13	.59

[a] Temperature, $25.0\pm.1$ °C, $\lambda_{ex}=350.0$ nm.
[b] Reference.
[c] Using 0.51 as 100 percent.

hydroxide have been reported to cover the range 0.76 to 0.96 [3, 9, 21, 23, 52–61]; however, the most widely accepted value is 0.90 plus or minus five percent [13]. The values quoted for pyrene and rhodamine B are less widely accepted: e.g. values of 0.97 and 0.92 have been reported for rhodamine B [23, 57], although it was reported the measurements in these cases were not made with a red-sensitive photomultiplier [13].

b. Acid Concentration.—The variability of quantum efficiency of quinine sulfate as a function of three sulfuric acid concentrations has been reported [21, 32]. These studies were extended to cover eight acid concentrations, as summarized in table 12. The results for the 0.1, 1.0, and 3.5 *N* sulfuric acid concentrations agree well with those reported by Dawson and Windsor [21] and Eisenbrand [32], although the latter reported a constant quantum efficiency for sulfuric acid concentrations from 0.01 to 0.2 *N*. The increases for acid concentrations less than 0.1 *N* are small, but apparently real, since Chen also observed this same phenomenon and suggested that this was due to less quenching by the sulfate ion at low acid concentrations [62]. Although the weight absorptivities of QS and QDS in three different acid concentrations agree within experimental error, table 13, a slight trend towards lower absorbances

TABLE 13. Weight absorptivities [a] of quinine and quinidine sulfates as a function of H_2SO_4 concentration [b]

	WA [a] × 10^4 at 347.5 nm	
$[H_2SO_4]$ *N*	QS	QDS
0.10	1.43	1.44
1.02	1.40	1.41
3.55	1.39	1.40

[a] See equation (7).
[b] Temperature 25.0 °C.

may be noted. Coupled with the increased fluorescence area as $[H^+]$ increases, the observed increase in quantum efficiency may be explained simply by ionization changes; however, discussions by Chen [29, 62] appear to negate this explanation. Further work is being done to resolve these discrepancies.

The increase in quantum yield of QS as a function of acid concentration is paralleled by QDS as might be expected due to their similarities in spectra, figure 2 [30, 47] and molar absorptivities, tables 4 and 13.

c. Excitation Wavelength.—The quantum efficiencies of quinine sulfate, figure 7, rhodamine B, and rhodamine 6G, figure 8 (others discussed later) were found to be independent of excitation wavelength (±5 percent). The largest deviations for QS were found from 260 to 280 nm where the absorbance is at a minimum and larger errors would be expected. Several investigators [21, 29, 36] reported a variation in quantum efficiency of fluorescence intensity as a

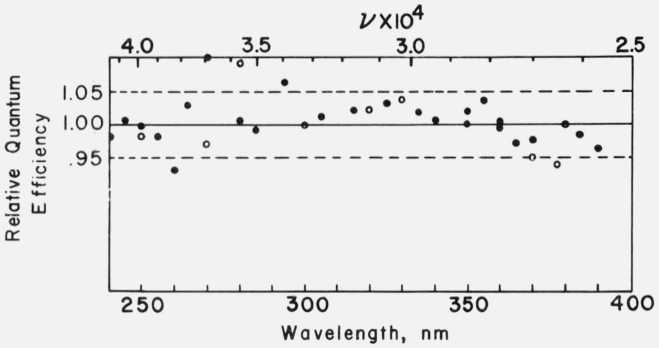

FIGURE 7. Relative quantum efficiencies of quinine sulfate as a function of excitation wavelength (● = this work), quinine sulfate concentrations: λ_{ex} = 240–290 nm, 0.1 ppm (1.28×10^{-7} *M*) and 0.5 ppm in 0.1 *N* H_2SO_4; λ_{ex} = 260–390 nm, 2.0 and 2.5 ppm (6.39×10^{-7} *M*) in 0.1 *N* H_2SO_4; excitation bandwidth, 25 Å; emission bandwidth, 100 Å; temperature, 25.0 °C; ○=Fletcher [28].

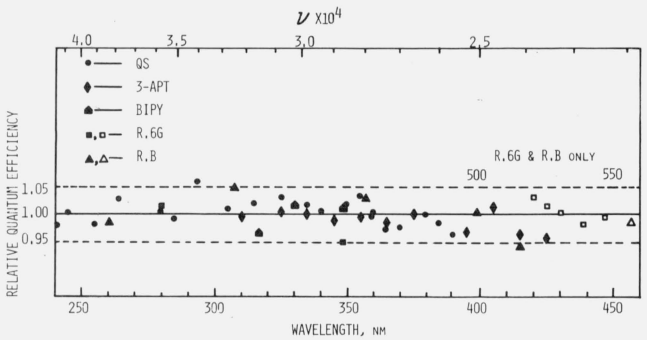

FIGURE 8. Relative quantum efficiencies as a function of excitation wavelength: quinine sulfate—same conditions as figure 7;3-aminophthalimide, 5.64 ppm (2.68×10^{-5} *M*) in 0.1 *N* H_2SO_4; bipyrenyl (BIPY), 0.14 ppm (2.98×10^{-7} *M*) in toluene; rhodamine 6G (R.6G), 0.12 ppm (2.08×10^{-7} *M*) and 0.025 ppm (4.43×10^{-8} *M*) in ethanol; rhodamine B (R.B.), 0.13 ppm (2.15×10^{-7} *M*) in ethanol. Emission bandwidth, 100 Å; excitation bandwidth, 25 Å; temperature, 25.0 °C.

function of excitation wavelength. Fletcher [28], however, found no more than ±5 percent quantum yield deviation for excitation wavelengths from 240 to 400 nm (except for 272 nm region, selected values in fig. 7), and other investigators also report essentially no variation in quantum yield at two excitation wavelengths [33–35].

B. Aromatic Hydrocarbons

Another large group of compounds with relatively high quantum yields are the aromatic hydrocarbons. Series of possible standards may be made by simply adding aromatic rings to the base compound, e.g. benzene and naphthalene fluoresce in the UV, anthracene exhibits blue fluorescence, naphthacene fluoresces in the green, and pentacene in the red. Anthracene has been studied quite extensively in ethanol and benzene, and the quantum efficiencies

reported from these studies vary from 0.25 to 0.33 [3, 18, 21, 23, 43, 57, 63–67].

We have studied pyrene derivatives [42] to determine if these compounds are suitable as fluorescence standards and to determine if substituents on more condensed systems exhibit the same trends in emission wavelengths, intensities and quantum efficiencies as the substituted naphthalenes. Corrected excitation and emission spectra for the pyrene derivatives are presented in figure 9. The 1-chloro-, 1-bromo-, and 1-nitropyrene compounds showed little, if any, fluorescence but would be expected to phosphoresce at low temperatures if the substituent "heavy atom" effect is followed for the substituted pyrenes as in the case of the naphthalenes [68]. The results for the quantum efficiency calculations and data taken from Berlman [51] for the naphthalenes and pyrenes are summarized in table 14. As can be seen, fairly good agreement was obtained with the quantum efficiency values from Berlman for the bipyrenyl. Better agreement for pyrene, however, was obtained with values from Förster and Seidel [69], probably due to the use of the same solvent, toluene, rather than benzene. The value for the quantum efficiency of pyrene by Dawson and Windsor [21] was obtained using ethanol as the solvent. The differences observed here may be due to some of the problems associated in the general use of aromatic hydrocarbons as standards since aromatics (a) usually show oxygen quenching, (b) have fairly large emission-excitation spectra overlap, (c) are very sensitive to extremely small amounts of impurities, (d) have narrow emission bandwidths, necessitating use of narrow slits, and (e) must have large index of refraction corrections made (as in this work) when quantum efficiencies are compared to compounds dissolved in aqueous media.

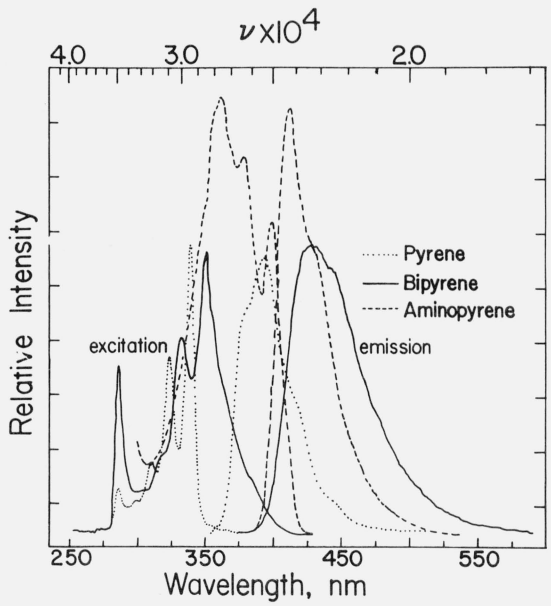

FIGURE 9. Relative intensities (emission spectra: quanta/unit bandwidth) versus wavelength for 0.13 ppm (5.69×10^{-7} *M*) pyrene, 0.47 ppm (1.84×10^{-6} *M*) 1-aminopyrene and 0.14 ppm (2.98×10^{-7} *M*) bipyrenyl in toluene. Excitation bandwidth, 25 Å, emission bandwidth, 100 Å; temperature, 25.0 °C.

Similar trends in quantum efficiencies are noted upon comparing pyrene and naphthalene to bipyrenyl and binaphthalenyl. The dimeric species have quantum efficiencies that are higher than the monomeric species which may be directly attributable to the lifetimes—i.e. 2 to 3 ns for the dimers and 100 to 600 ns for the monomers [51, 70]. The dimeric species have more asymmetry than the monomeric species and thus possess higher transition probabilities.

C. 3-Aminophthalimide

Although the compound 3-APT in 0.1 *N* sulfuric acid has been suggested as a fluorescence standard [1], care should be taken in its use. We have found that the absorption (excitation) spectrum, figure 10, and the quantum efficiency, table 15, are dependent on the acid concentration [38]. The decrease in the weight absorptivities at 385 nm with increasing acid concentration, coincides with the increased absorbance of a peak at 296 nm.

The quantum efficiency of 3-APT in ethanol reported by Alentsov [52] was 0.6. A quantum efficiency of 0.47 in 0.1 *N* sulfuric acid is not unreasonable in view of the difference in solvent. The observed decreases in the quantum efficiencies as a function of acid concentration are consistent with the presence of more than one species, allowing additional radiationless transitions such as energy transfer between the species to occur.

TABLE 14. Quantum efficiencies (QE) of some pyrene derivatives compared to similar naphthalene derivatives

Compound	Solvent	λ_{ex}	$\lambda_{em(max)}$	QE
Pyrene	Toluene	338.5	394	0.48, [a]0.52, [b]0.32, [c]0.53
1-Aminopyrene	Toluene	401	413	.43
1,1′-Bipyrenyl	Toluene	349	428	.78, [b]0.84
Naphthalene[a]	Cyclohexane	276	322	.23
1-Aminonaphthalene [a]	Cyclohexane	319	376	.46
1,1′-Binaphthyl[a]	Cyclohexane	284, 294	361	.77

[a] Ref. [70].
[b] Ref. [51].
[c] Ref. [21].

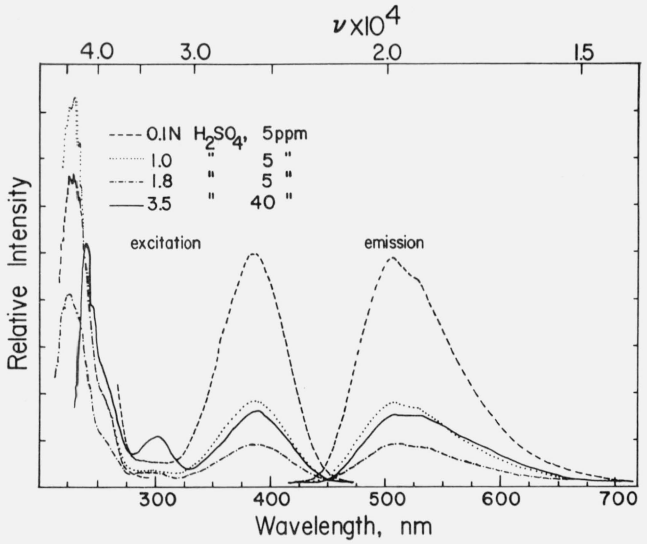

FIGURE 10. Relative intensities (emission spectra: quanta/unit bandwidth) versus wavelength for 5 ppm (2.38×10^{-5} *M*) and 40 ppm (1.90×10^{-4} *M*) 3-aminophthalimide in 0.1 *N*----, 1.0 *N*·····, 1.8*N* -·-·- and 3.5 *N*—H_2SO_4. Excitation bandwidth. 25 Å; emission bandwidth, 100 Å; temperature, 25.0 °C.

TABLE 15. Dependence of absorbance at 385 nm and quantum efficiency for 3-aminophthalimide as a function of sulfuric acid concentration

	WA[a] × 10^4		
$[H_2SO_4]$, *N*	385 nm	296 nm	Quantum efficiencies[b]
0.10	2.36	0.39	0.45 ± 0.01
1.02	1.29	.78	$.31 \pm 0.02$
1.75	0.82	.94	$.23 \pm 0.02$
3.55	.32	1.02	$.15 \pm 0.01$

[a] See equation (7).
[b] λ_{ex}=385 nm; °C = 25.0; reference = QS · $2H_2O$ in 0.1 *N* H_2SO_4.

This work is in progress and will be reported later in more detail. The quantum efficiency for 3-APT in 0.1 *N* sulfuric acid is constant within ±5 percent over the excitation wavelength range of 310 to 425 nm, figure 8.

D. Other Fluorescence Standards

N,*N*-dimethylamino-*m*-nitrobenzene has also been suggested as a standard [1] and, although the quantum efficiency appears to be relatively constant as a function of excitation wavelength [38], it is quite low and not very suitable as a standard. In addition, as mentioned before, a solvent system of 70:30, V:V of hexane:benzene is not desirable since fluorescence analyses in the biochemical areas [71] require use of compounds which are soluble in aqueous media.

Chen [62] has suggested standards which can be used in aqueous solutions and are suitable for biochemical applications. Other compounds which might be added to this list that cover the "red" region, include rhodamine B and rhodamine 6G, both used as quantum counters and both having relatively constant quantum efficiencies over a wide excitation wavelength range (250 to 600 nm), figure 8 [13, 72]. Studies should be made of these compounds in aqueous rather than alcoholic media, although aggregation in water has been reported [73].

E. Inorganic Ions in Glass or Polycrystalline Matrices

The use of inorganic ions in glass matrices as cuvette or disc shaped samples, figure 11, is attractive, especially when one considers ease of handling which may result in decreased methodological errors [74, 75]. The inorganic ions may be split into two classes determined by their absorption and emission spectra.

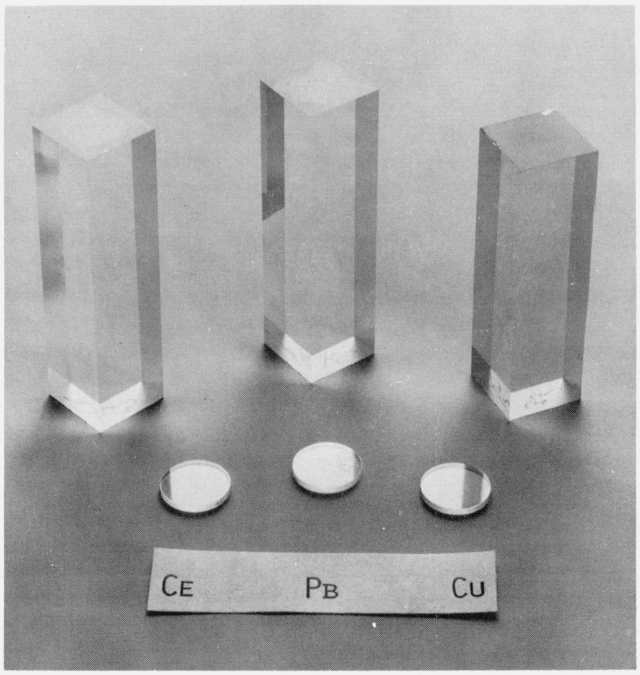

FIGURE 11. Cuvette and disc shaped cerium, lead, and copper doped silicate glasses.

The non-rare earths and cerium(III) have broad spectra with little structure due to interconfigurational electronic transitions, making them prime candidates for fluorescence standards. The rare earths in the +3 oxidation states, on the other hand, have sharp spectra due to intraconfigurational electronic transitions (less than 25 nm bandwidths), and may be used for wavelength calibration, spectral resolution of instruments, and, in special cases, as quantum yield standards.

The non-rare earth inorganic ions interact to a greater extent with the matrix than the rare earth ions. Thus changing the glass matrix from borate to silicate results in fairly large shifts of emission maxima.

Figure 12 shows these shifts for lead-doped phosphate, borate, and silicate glasses.

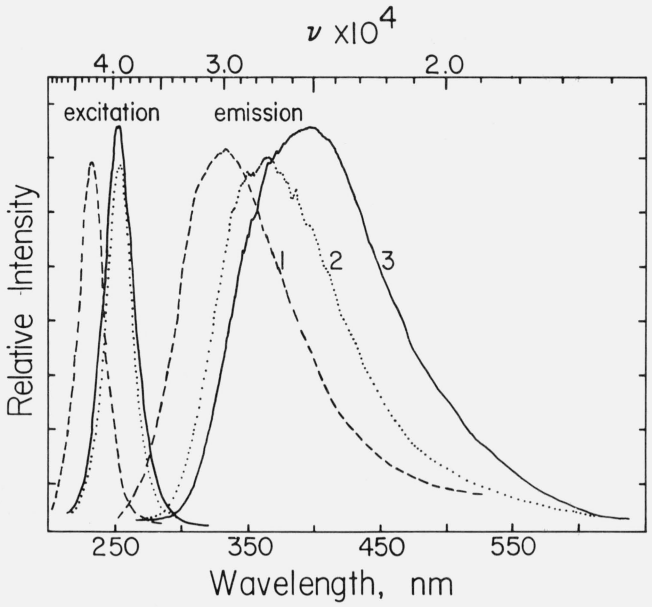

FIGURE 12. Relative intensity (emission spectra: quanta/unit bandwidth) versus wavelength for lead doped phosphate, 1; borate, 2; and silicate, 3; discs. Emission and excitation bandwidths, 100 Å; temperature, 25.0 °C; angle of disc with exciting beam, 15° [74, 75].

Table 16 summarizes ranges of emission maxima for the non-rare earth ions in glass or polycrystalline matrices. For an in-depth discussion of these effects and information concerning the rare earths, see reference [7] and the references listed therein.

TABLE 16. Ranges of emission maxima for cerium and non-rare earth inorganic ions in various solid matrices [a]

Inorganic Ion	Emission Range (nm)
Tl^{+}	290–325
Ce^{3+}	325–400
Pb^{2+}	350–450
UO_2^{2+}	475–550
Cu^{+}	475–550
Mn^{2+}	500–600

[a] For emission spectra, see reference [7].

F. Summary

In summary then, substantiative data are being accumulated as to the applicability of various compounds for use as fluorescence standards. The next major step would appear to be interlaboratory testing and comparison of data leading to the acceptance of either a single or a series of standards which are widely available for general use. Quinine sulfate, due to its desirable fluorescence characteristics, overall applicability, and widespread use and study, should probably be the first material selected for an interlaboratory comparison study.

III. Addendum A. Materials and Instrumentation

In order to adequately describe materials and experimental procedures, it was occasionally necessary to identify commercial products by manufacturer's name or label. In no instances does such identification imply endorsement by the National Bureau of Standards, nor does it imply that the particular product or equipment is necessarily the best available for that purpose.

A. Materials

The following reagents were used in this study:

Perchloric Acid: National Bureau of Standards, purified reagent, double distilled, lot 111103 [39].

Sulfuric Acid: Ultrex Grade, J. T. Baker Chemical Co., Phillipsburg, N.J. Used as obtained.

Sodium Hydroxide: Reagent Grade, J. T. Baker Chemical Co., address above. Used as obtained.

Distilled Water: Distilled water was passed through an ion exchange column (IWT Research Column, Rockford, Illinois), followed by double distillation from a quartz still.

Toluene: Certified, Fisher Chemical Co., Fairlawn, N.J., was shaken with cold concentrated sulfuric acid, water, aqueous 5 percent sodium hydroxide, water, and dried over calcium sulfate in that order. It was then distilled from sodium under nitrogen.

Benzene and *n*-Hexane: Spectrograde, Eastman Chemical Co., Rochester, N.Y. Used as obtained.

Ethanol, 95 and 100 percent: Pharmco Distributing Co., Publicker Industries, Philadelphia, Pa. Used as obtained.

Quinine Sulfate Dihydrate: Sample A—Aldrich Chemical Co., lot 032007; sample B—N. F. Grade, Fisher Chemical Co.; sample C—Pfalz and Bauer, 31–20 College Point Causeway, Flushing, N.Y.; sample D—sample A recrystallized three times from warm water; sample E—sample A recrystallized six times from warm water; sample F—sample B recrystallized four times from warm water.

Quinidine Sulfate Dihydrate: Sample G—Aldrich Chemical Co., lots 032781 and 062607; sample H—sample G recrystallized four times from warm water.

Quinine Dihydrate: Sample J—Aldrich Chemical Co., lot 071491 dissolved in dilute sulfuric acid, precipitated by neutralizing with dilute ammonium hydroxide. Washed with water twice.

All quinine derivatives were dried at 55–60 °C under vacuum for a minimum of 18 h before use [28].

Fluorescein: Aldrich Chemical Co., purified three times by the method of Koch [40].

Rhodamine B and Rhodamine 6G: Samples obtained from R. A. Keller [41]. Rhodamine 6G was purified by repeated recrystallizations, using ethyl acetate:

ethanol followed in one case by elution from a silica column (purification by R. A. Keller).

3-Aminophthalimide: Eastman Organic Chemicals. Recrystallized three times from ethanol, mp-260 °C [1].

N,N-Dimethylamino-*m*-nitrobenzene: Eastman Organic Chemicals. Recrystallized twice from chloroform and once from acetone-benzene [1].

Pyrene, bipyrenyl, 1-aminopyrene, 1-chloropyrene, and 1-nitropyrene prepared as designated [42].

B. Instrumentation

Absorbance spectra were obtained using Cary 14 and Cary 16 spectrophotometers, and a Turner Model 210 "Spectro" in the absorbance mode [20]. The spectrophotometers (except Model 210) were equipped with constant temperature cell blocks. Spectra were recorded or read at 25.0 ± 0.1 °C. Matched quartz cuvettes of 0.1, 1.0, and 5.0 cm were used.

Fluorescence spectra were obtained using a Turner Model 210 "Spectro" which gives corrected excitation and emission spectra [20], and a Farrand spectrofluorimeter which gives uncorrected spectra. Unblackened quartz spectrofluorimeter cells were used. Solvents were always run to determine baselines.

Before quantum efficiencies or band positions were measured, the accuracy of the wavelength scales for the emission and excitation monochromators were checked using a mercury lamp. A polished aluminum block cut at an angle of 45° was placed in the sample compartment. The monochromators were calibrated at 253.6, 435.8, and 546.1 nm. If necessary, the wavelength scales were adjusted so that accuracies of ±0.2 nm over the 253.6 to 546.1 nm range were obtained. A special Schlenk type [76] quartz cell was constructed, figure 13, by H. Deleonibus, Glassblowing Section, 129.06, National Bureau of Standards.

Stopcock and stopper were of polytetrafluoroethylene to avoid contamination. The solution was placed in the cylindrical sidearm and alternately frozen using liquid nitrogen and evacuated and thawed at room temperature at least five times to deoxygenate the solution. A test which repeated this cycle ten times resulted in a solution weight loss of less than 0.1 percent. Fluorescence studies, unless noted, were made on the corrected instrument. Quantum efficiencies were determined in duplicate, except where noted. All fluorescence measurements were made with 25 Å excitation bandwidth, 100 Å emission bandwidth, and at 25.0 ± 0.1 °C, except where noted.

Thermogravimetric measurements were run using dry nitrogen on a Model 950 TGA, E. I. DuPont de Nemours & Co., Wilmington, Delaware, by T. Sterling or S. Wicks, Section 310.04, Analytical Chemistry Division, National Bureau of Standards.

The glasses mentioned were prepared by the Inorganic Glass Section, 313.02, W. Haller, head, National Bureau of Standards. They were cut to size and polished by the Optical Shop, Section 129.05, S. Gerner, National Bureau of Standards.

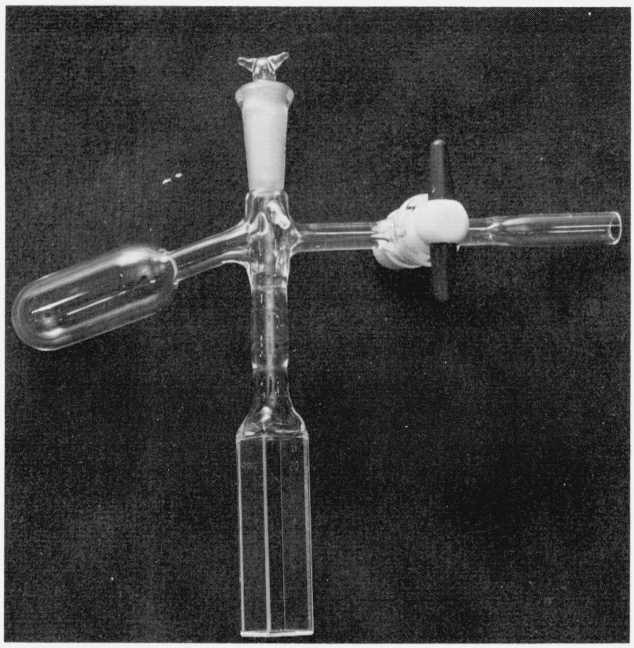

FIGURE 13. Schlenk type [76] spectrofluorimeter cell used with nitrogen manifold for degassing solutions.

The author would like to acknowledge the useful discussions in fluorescence with G. H. Atkinson, NBS, and the encouragement and support of O. Menis and J. I. Shultz, NBS.

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