Standard Reference Materials:

A Fluorescence Standard Reference Material: Quinine Sulfate Dihydrate

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PREFACE

Standard Reference Materials (SRM's) as defined by the National Bureau of Standards are well-characterized materials, produced in quantity and certified for one or more physical or chemical properties. They are used to assure the accuracy and compatibility of measurements throughout the nation. SRM's are widely used as primary standards in many diverse fields in science, industry, and technology, both within the United States and throughout the world. They are also used extensively in the fields of environmental and clinical analysis. In many applications, traceability of quality control and measurement processes to the national measurement system are carried out through the mechanism and use of SRM's. For many of the nation's scientists and technologists it is therefore of more than passing interest to know the details of the measurements made at NBS in arriving at the certified values of the SRM's produced. An NBS series of papers, of which this publication is a member, called the NBS Special Publication - 260 Series is reserved for this purpose.

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"... The solution of quinine, though it appears to be perfectly transparent and colourless, like water, when viewed by transmitted light, exhibits nevertheless in certain aspects, and under certain incidences of the light, a beautiful celestial blue colour. ..."

Sir George Gabriel Stokes, 1852 [1][1]

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

Abstract

The need, material selection, characterization, certification, and uses of the fluorescence Standard Reference Material, quinine sulfate dihydrate, have been discussed. The emission spectrum for quinine sulfate dihydrate in 0.1 mol/L $HClO_4$ has been measured on the NBS reference spectroradiometer. The spectrum has been reported every 5 nm from 375.0 to 675.0 nm as the relative technical emission spectrum $E^T(\lambda)$ and the relative molecular emission spectra represented by $E(\lambda)$, $E_p(\lambda)$, $E(\tilde{\nu})$, and $E_p(\tilde{\nu})$. The technical emission spectrum has been corrected for the instrumental parameters of the spectral responsitivity of the detection system, photomultiplier tube non-linearity, bandpass, monochromator wavelength error, and further corrections for the sample parameters of solvent refractive index and cell window transmittance were applied to obtain the molecular emission spectra. Estimates of the 95 percent systematic error limits of the emission spectrum values have been made as a function of wavelength. These values are ∿2 percent at the peak maximum (∿455 nm) and ∿5 percent and ∿3 percent at the blue (395 nm) and red (565 nm) one-tenth power points. The experimental replication precision is ∿0.2 percent over the total spectral range. The purity and the stability of the SRM quinine sulfate dihydrate and the effect of solute concentration, solvent acid, acid concentration, excitation wavelength, oxygen quenching, temperature, and polarized exciting radiation on the emission spectrum, and in part, on the absorbance spectrum, photon yield, and fluorescence lifetime also have been discussed.

Key Words: Calibration of spectrofluorimeters; corrected fluorescence spectra; fluorescence; fluorescence lifetimes; fluorescence standards; molecular emission spectrum; photon yields; quinine sulfate dihydrate; spectrofluorimetry; Standard Reference Material; technical emission spectrum; transfer standards.

I. General

The photoluminescence of molecules in solution was noted by Monaides in the early 1500's [2]. In 1852 Stokes correctly described this phenomenon and named it fluorescence [1]. It was not until 1955, however, when Bowman, Caulfield, and Udenfriend published a paper [3] describing a two monochromator, photomultiplier equipped instrument that the fluorescence spectra of small quantities of materials could be measured easily. Since that time, the use of the fluorescence technique in diverse areas such as health, environmental pollution, molecular biology, forensic sciences, species identification and quantitation, etc., has increased significantly [see for example, ref. 4].

Laboratories can report fluorescence spectra that are either uncorrected or corrected for instrument and sample parameters. The uncorrected spectra, although useful for intralaboratory measurements of changes in fluorescence characteristics, should not be used for interlaboratory comparisons since the correction factors for each instrument can be quite different.

Corrected spectra may be determined in two ways. First, in absolute radiometric units, and second, by measuring the spectrum for the compound under investigation relative to that obtained for a standard on the same instrument under similar experimental conditions. Very few laboratories are equipped to make absolute radiant energy measurements; however, comparative spectra obtained from standards run under the same experimental conditions on the same instrument can be done easily. This latter method, which effectively corrects for instrument parameters that affect the fluorescence spectra, is used most widely in practice but is severely limited by the lack of reliable standards. Thus, spectra corrected in this manner by different laboratories cannot be compared with

confidence. This is demonstrated by the large discrepancies in the spectra obtained by different authors for the same substance. For example, the values reported for the corrected emission spectrum of quinine sulfate varied by as much as 50 percent [5-8].

This variability underlined the need for standardization of fluorescence measurements and data presentation [9-11] and in 1963 several investigators collectively published a paper entitled "Proposal for Standardization of Methods of Reporting Fluorescence Emission Spectra" [12]. This proposal established a firm basis for the intercomparisons of data among laboratories, but it did not fulfill the need for fluorescence standards with certified values for the emission and excitation spectra.

The National Institute for General Medical Sciences, National Institutes of Health, recognizing this need and seeing the impact on clinical and biochemical applications of a similar program in spectrophotometry [13], provided partial funding for the development of fluorescence Standard Reference Materials (SRM's) by the Center for Analytical Chemistry of the National Bureau of Standards.

The various types of SRM's available from NBS have been discussed in several publications [14-18]. These SRM's are well characterized, stable materials that are produced in quantity and are available for purchase over a long time period (6-10 years). Their functions are to: (a) help develop reference methods of analyses or tests; (b) calibrate measurement systems; and (c) assure long term adequacy and integrity of a quality control process [14]. SRM's are available for uses ranging from the steel industry to clinical laboratories and include metals as well as organic and inorganic compounds. Meinke [18] discussed the importance of SRM's in Clinical Chemistry, and initiated work at NBS with the objective of providing fluorescence SRM's.

Because of its wide applicability and additional requirements that will be discussed later, the first fluorescence SRM developed is quinine sulfate. This SRM was developed primarily for the calibration of the emission detection systems of luminescence spectrometers. If necessary, the excitation spectrum of quinine sulfate and absolute values of the spectral photon yield could be determined at a later time.

In the development of this SRM, the four categories of need, material selection, material characterization, and emission spectrum certification were considered. The first, need, has already been discussed. The second, SRM selection, depends on the prerequisites and imposed constraints. The third, characterization, compares measured values for photophysical and chemical properties to those of purified materials. Lastly, certification, includes correction of the emission spectrum for instrument and sample parameters so that the corrected emission spectrum is independent of these parameters. Since this study addresses many facets of fluorescence standards, a summary of this work and directions for the use and storage of SRM 936 Quinine Sulfate Dihydrate are presented first in Section II. Additional information on the measurement of the physical and chemical properties and certification of this SRM is included in later sections.

In Section III we present a brief discussion of the suitability of organic and inorganic fluorophors in various matrices as fluorescence standards. Section IV is a glossary of terms and Section V is a general experimental section; however, where relevant and necessary, experimental conditions or data handling techniques are given in each specific section. In Section VI we present results of studies on absorbance and fluorescence parameters of quinine sulfate under various experimental conditions with the objective of further characterizing this material. In this way we hope to provide information on the use of quinine sulfate as a standard and,

more importantly, to make the user aware of the errors associated with making measurements of corrected luminescence spectra. The errors introduced into the emission spectrum by various experimental parameters are discussed in Section VII. In Section VIII we present the values for the normalized, corrected emission spectrum of SRM grade quinine sulfate dihydrate in 0.105 mol/L $HClO_4$ followed by a discussion of the precision and accuracies of these values in Section IX.

II. SRM Use and Summary

A. Use of the SRM Quinine Sulfate Dihydrate

The Certificate issued with SRM 936 is reproduced in Appendix A. To assure the most reliable and accurate results, all glassware used in solution preparation should be "class A" or equivalent. The distilled water used to prepare the 0.105 mol/L $HClO_4$ should have a negligible signal due to fluorescence under the given experimental conditions. The molecular weight of quinine sulfate dihydrate, $(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4 \cdot 2H_2O$, is 782.947[3].

1. Solution Preparation

Prepare a 1.28×10^{-6} mol/L (~1 ppm) solution of quinine sulfate dihydrate ($QS \cdot 2H_2O$) in 0.105 mol/L $HClO_4$ in the following manner:

(a) weigh using an analytical balance 0.100 g $QS \cdot 2H_2O$ and quantitatively transfer it to a 1000 mL volumetric flask;

(b) dilute to the mark with 0.105 mol/L $HClO_4$ giving a solution 1.28×10^{-4} mol/L (100 ppm) in $QS \cdot 2H_2O$;

[3]Atomic weights obtained from Pure and Applied Chem. 47, 75-95 (1976).

(c) Transfer 10 mL of solution (b) to a 1000 mL flask and dilute to the mark using 0.105 mol/L $HClO_4$ to give a final solution that is 1.28×10^{-6} mol/L (1 ppm) solution of $QS \cdot 2H_2O$.

Store solution (b) and (c) in the dark. Solution (c) should be prepared fresh monthly while (b) should be prepared fresh every three months. For critical measurements, all solutions should be prepared fresh from the solid material that has been stored in the dark.

2. Instrumental Conditions

Generally, the instrumental conditions used when recording emission spectra are variable and do affect the spectral distribution of the emitted radiation. Thus, it is recommended to measure the emission spectrum under the following instrumental and experimental conditions:

(a) solution temperature: 25.0 °C;
(b) excitation wavelength λ_1 : 347.5 nm;
(c) bandpass of excitation and emission monochromators : 5.3 nm; and
(d) geometry: 90° viewing, "right angle".

If wide deviations from the above conditions occur, it is recommended that the user refer to the specific section in this report to determine the appropriate corrections to be applied to the emission spectrum.

After obtaining the emission spectrum of 1.28×10^{-6} mol/L $QS \cdot 2H_2O$ in 0.105 mol/L $HClO_4$ under the above conditions, the correction function for your instrument over this wavelength region may be determined by:

$$S = \frac{R_\lambda}{E(\lambda)}$$

where S is the instrumental response,
R_λ is the instrumental reading at λ, and
$E(\lambda)$ are the values from Table 1.

Table 1. The Molecular Emission Spectrum E(λ) of Quinine Sulfate Dihydrate in 0.105 mol/L $HClO_4$.

λ, nm	E(λ)	λ, nm	E(λ)	λ, nm	E(λ)
375.0	0.005	475.0	0.838	575.0	0.076
380.0	.012	480.0	.782	580.0	.065
385.0	.028	485.0	.719	585.0	.057
390.0	.057	490.0	.659	590.0	.050
395.0	.103	495.0	.595	595.0	.043
400.0	.170	500.0	.541	600.0	.037
405.0	.257	505.0	.486	605.0	.032
410.0	.359	510.0	.434	610.0	.028
415.0	.471	515.0	.386	615.0	.024
420.0	.586	520.0	.342	620.0	.021
425.0	.694	525.0	.302	625.0	.018
430.0	.792	530.0	.264	630.0	.016
435.0	.874	535.0	.231	635.0	.014
440.0	.940	540.0	.201	640.0	.011
445.0	.984	545.0	.175	645.0	.010
450.0	.999	550.0	.153	650.0	.009
455.0	.997	555.0	.132	655.0	.008
460.0	.982	560.0	.116	660.0	.007
465.0	.947	565.0	.101	665.0	.006
470.0	.897	570.0	.008	670.0	.005
				675.0	.004

The corrected spectra for other fluorescent materials that emit in the same general spectral region may thus be determined by dividing the R values at particular wavelengths by the instrument response function S determined from the above equation at the corresponding wavelength.

It must be emphasized that this procedure can be used for materials with the same spectral characteristics as quinine sulfate. If the spectral characteristics are quite different, e.g., if the emission peak half-height bandwidth is much smaller (10 nm rather than 90 nm) the monochromator bandpass must be decreased accordingly.

3. Summary

The need, material selection, characterization, and certification of the fluorescence Standard Reference Material Quinine Sulfate Dihydrate are discussed in Sections III-X. The emission spectrum for quinine sulfate dihydrate in 0.1 mol/L $HClO_4$ has been measured on the NBS reference spectroradiometer. The spectrum has been reported every 5 nm from 375.0 to 675.0 nm as the relative technical emission spectrum $E^T(\lambda)$ and the relative molecular emission spectra represented by $E(\lambda)$, $E_p(\lambda)$, $E(\tilde{\nu})$, and $E_p(\tilde{\nu})$. The technical emission spectrum is calculated by correcting the raw emission data for the instrumental parameters of the spectral responsitivity of the detection system, photomultiplier tube non-linearity, bandpass, monochromator wavelength error; further corrections for the sample parameters of solvent refractive index and cell window transmittance are applied to $E^T(\lambda)$ to obtain the molecular emission spectra. Estimates of the 95 percent systematic error limits of the emission spectrum values are made as a function of wavelength. These values are $\sim$2 percent at the peak maximum ($\sim$455 nm) and $\sim$5 percent and $\sim$3 percent at the blue (395 nm) and red (565 nm) one-tenth power points. The experimental replication precision is $\sim$0.2 percent over the total spectral range. The purity and the stability of

the SRM quinine sulfate dihydrate and the effect of solute concentration, solvent acid, acid concentration, excitation wavelength, oxygen quenching, temperature, and polarized exciting radiation on the emission spectrum, and in part, on the absorbance spectrum, photon yield, and fluorescence lifetime also are discussed.

III. Fluorescence Standards

The requirements for an ideal fluorescence standard are that it should: (a) have 'broad' fluorescence spectra; (b) be easily purifiable; (c) be stable in solution or as the solid; (d) have little overlap 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 spectral 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 characteristics. In fact, the last requirement precludes the use of a single standard, since the emission spectra of most fluorophors are limited to relatively small wavelength intervals. For this reason, it is necessary to have available a series of fluorescence standards covering the wavelength range of interest. With the increased use of lasers and interest in radiative measurements in the near-infrared, this range now extends from 250 nm to 1100 nm.

Many types of materials have been proposed or used as fluorescence standards including organic molecules in solvents or plastics, scintillators in radioactive solvents, and inorganic ions in glass and crystal matrices [7,9,11,19-32]. Although we investigated solutions of organic molecules and inorganic ions in glass and crystalline matrices [28,33-36]

as possible candidates for SRMs, only organic molecules in aqueous based solvents were given in-depth consideration because: (a) they can be used in both static and flow systems; (b) a material soluble in an aqueous based solvent was desired; (c) individual calibration is not required (statistical sample testing permissible); and (d) quality control of the material is simplified. Organic molecules in plastic are undesirable since they exhibit large emission anisotropies [37], oxygen diffusion and quenching of fluorescence may be variable, stability of the plastic matrix is questionable, and surface scratching may occur.

Several organic compounds proposed as standards are listed in Table 2.

Table 2. Organic compounds proposed as fluorescence standards.

Compound	Emission Maxima[a], nm
β-naphthol[b]	354, 402
anthracence[c,d]	384, 404, 428, 454
pyrene[c,d]	390
diphenylanthracene[c,d]	408, 427, 458[e], 490[e]
quinine sulfate	455
3-aminophthalimide	506
fluorescein[b]	518
m-dimethylaminonitrobenzene	542
rhodamine 6G	557
rhodamine B	572
aluminum(III)-PBBR chelate[f]	635
methylene blue	∿708
4-dimethylaminonitrostilbene	742

[a] The maxima reported here are approximate.
[b] Unstable in solution.
[c] Narrow emission or excitation spectra.
[d] Oxygen quenched.
[e] Shoulder.
[f] PBBR = pontachrome blue black R.

Considering their characteristics listed in table 1, most of these materials were eliminated as potential SRMs. Of the remaining materials, only quinine sulfate in an aqueous-acidic solvent had been extensively studied and used as a fluorescence standard. Based on the requirements and preliminary investigations [33] quinine sulfate was chosen as the first material to be issued as a fluorescence SRM.

With the recent advent of dye lasers, and research of suitable laser dyes, several additional compounds have been suggested as fluorescence standards [38] including coumarins, oxazines, and two carbocyanines (hexamethylindodi-, and hexamethylindotricarbocyanine). In general, these materials have the best fluorescence characteristics when dissolved in non-aqueous solvents and should certainly be investigated as standards for use in organic solvents.

IV. Glossary

In general, the nomenclature followed in this publication will be a combination of that used by Melhuish [39], Mielenz [40], and Winefordner [41]. Selected terms and definitions used in this text are as follows:

$Y^{P,S}_{\lambda_1,\lambda_2}$ = Compensated fluorescence signal obtained when instrument is operated in the ratio mode (see Section V-B2). The 1st superscript refers to the orientation of the excitation polarizer, (polarizer), the 2nd to the orientation of the emission polarizer (analyzer) where P and S represent geometrical orientations perpendicular and parallel to the grating grooves of the detection monochromator. The letter X denotes the absence of a polarizer. The subscripts λ_1 and λ_2 refer to the excitation and emission wavelengths, respectively. Where convenient, $Y^{P,S}_{\lambda_1,\lambda_2}$ will be abbreviated (Y).

$R^S_{\lambda_2}$ = Emission detector signal obtained for the instrument operated in the detection or "B" mode, usually in conjunction with values obtained by placing the standard lamp in the sample position, S, (see figure 1 in Section V-B2). The superscript and subscript have the same meanings as previously defined.

$M^S_{\lambda_2}$ = Spectral responsivity of the emission detection system. This includes the ellipsoidal mirror EL_2, the analyzer, the detection monochromator, and the detection photomultiplier tube (see figure 1).

$E^T(\lambda)$ = The emission spectrum in radiometric and wavelength units corrected for instrumental parameters.

$E(\lambda)$ = The emission spectrum in radiometric and wavelength units corrected for instrumental and sample parameters.

$E_p(\lambda)$ = The emission spectrum in photon and wavelength units corrected for instrumental and sample parameters.

$E(\tilde{\nu})$ = The emission spectrum in radiometric and wavenumber units corrected for instrumental and sample parameters.

$E_p(\tilde{\nu})$ = The emission spectrum in photon and wavenumber units corrected for instrumental and sample parameters.

V. Experimental Section: Materials, Instrumentation, and Procedures [42]

A. Materials

1. Reagents

The following reagents were used in this study:

Perchloric Acid: National Bureau of Standards, purified reagent, double distilled (non-ebullient distillation process) lot 111103 [43].

Sulfuric Acid: National Bureau of Standards, purified reagent, double distilled (non-ebullient distillation

process) lot 219 [43] and ultrex grade, J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Distilled Water: Three types of distilled water were produced and tested: (a) distilled water was passed through an ion exchange column (IWT Research Column, Rockford, Illinois) followed by double distillation from a quartz still; (b) distilled water was redistilled from a hot potassium permanganate - NaOH mixture; and (c) distilled water was redistilled by the NBS non-ebullient distillation process [44]. The three water samples, upon excitation at 347.5 nm under high sensitivity detection conditions, gave a small peak at ∿395 nm attributable to the Raman spectrum of the solvent [45]. The signal at this wavelength was ∿1 percent of a 1 ppm quinine sulfate signal (y_{max} at $\lambda_{2_{max}}$ = 100). At other wavelengths, however, the signals attributable to the blank were several orders of magnitude less than those of the sample. The water discussed under (a) or (c) above was used to prepare the various acid concentrations needed in these studies.

Dilute Acids: Dilute perchloric acid was prepared by mixing appropriate volumes of concentrated perchloric acid and water. A standard solution of potassium acid phthalate (NBS SRM 84H) was used to determine the concentration of a sodium hydroxide solution (J. T. Baker, Analyzed Reagent, ∿4 g/L). The concentration of the dilute perchloric acid used in the certification process was 0.1049±0.0001 mol/L.

A similar process was used to determine the concentration of the various sulfuric and perchloric acid concentrations used in these studies.

Ethylene Glycol: Fisher Chemical Company, Fairlawn, New Jersey, "Certified Reagent" used as received.

Rhodamine B: Exciton Chemical Company, Inc., Dayton, Ohio. (Laser grade, Rhodamine 610), used as received.

Solvents: All solvents used including chloroform, methanol, acetic acid, n-propanol and formic acid were of reagent grade quality.

2. Quinine Sulfate

(a) General: Samples of quinine or its derivatives were obtained from various suppliers and purified by several recrystallizations from warm water or warm ethanol [33].

(b) SRM Grade: A sample of the bulk, purified, analyzed quinine sulfate obtained from the J. T. Baker Company, Phillipsburg, New Jersey (Appendix B) was compared to purified samples of quinine sulfate by measuring various optical and chemical properties (Section V-A2b). Without further purification, the bulk material was bottled in ∿1.1 g quantities in amber, screw-capped bottles that were numbered consecutively from 1-500. Ten random numbered samples (numbers 27, 96, 122, 177, 230, 284, 311, 370, 421, and 453) were chosen for testing of homogeneity and subsequent certification measurements.

(c) Solution Preparation: Samples of quinine sulfate dihydrate from the ten randomly chosen vials were weighed to ±0.00001 g in glass weighing containers on a calibrated [46a] semi-micro analytical balance. No changes in weight due to water adsorption were noted. The solid samples were rinsed quantitatively with 0.105 mol/L $HClO_4$ into previously weighed 100-mL class "A" volumetric flasks. Solvent was added to the calibrated mark, the flasks were tightly stoppered using polyfluoroethylene stoppers, and the flasks plus solutions were weighed on either the semi-micro balance or on a calibrated top loading analytical balance (to 0.01 g, [46b]) to determine by difference the weight of solvent added and thus, the weight concentration of the quinine sulfate dihydrate. All subsequent dilutions of these quinine-stock solutions were made by weight. Buoyancy corrections were made for the differences in densities of the quinine sulfate

dihydrate, water, and "brass" weights. The stock solutions were $\sim 1 \times 10^{-3}$ mol/L in quinine sulfate dihydrate. A fifty-fold dilution was made to prepare $\sim 2 \times 10^{-5}$ mol/L (20 ppm) quinine sulfate solutions for absorbance measurements. A further 15-fold dilution was made to prepare $\sim 1.3 \times 10^{-6}$ mol/L (1 ppm) quinine sulfate solutions for fluorescence certification measurements.

For certification, one series of ten quinine sulfate solutions was prepared in this manner and the emission spectra were measured during week one. A second series of solutions was prepared during week three by mixing the same ten sample vials, choosing the vials randomly, weighing the sample, diluting, and then, as before, the emission spectra were measured. The emission spectra of the solutions in series one were remeasured during week six.

B. Instrumentation

1. General

Absorbance values were obtained using Cary 14 and 16 spectrophotometers and a NBS high accuracy spectrophotometer [47]. The absorbance scales for the Cary 14 and 16 spectrophotometers were calibrated using NBS SRM 930b, Glass Filters for Spectrophotometry [48]. Comparative and certified fluorescence data were obtained on the NBS Reference Spectroradiometer [49] (hereafter called Reference Spectrofluorimeter) and relative quantum yields were measured on a Turner '210' Corrected Spectrofluorimeter [50]. The wavelength scale of the Turner '210' was calibrated as previously described [33].

The fluorescence lifetimes were measured using a TRW Model 75A Decaytime Fluorimeter System or an Ortec Model 9200 Nanosecond Single-Photon Photometer.

Matched spectrophotometer cells of known pathlength [51] were used for absorbance measurements and a single unblackened quartz spectrofluorimeter cell was used for all fluorescence certification measurements.

All instruments except the Ortec system were equipped with constant temperature cell blocks for sample temperature control to ±0.1 °C. The temperature in the cuvette was measured relative to the bath temperature with a thermocouple type probe (YSI Co., Yellow Springs, Ohio) that was calibrated against a NBS platinum resistance thermometer. All spectra were measured at 25.0±0.1 °C unless otherwise noted.

2. NBS Reference Spectrofluorimeter

Although detailed accounts of the NBS reference spectrofluorimeter [49] and its calibration [52] are being written, a brief description of it will be given here. A scale drawing of the spectrofluorimeter in the right-angle fluorescence mode and an overall photograph of the instrument are presented in figures 1 and 2, respectively.

Excitation radiation is produced by a 450 W xenon source (XL), directed by an ellipsoidal mirror (EL_1) to a 0.3 m Czerny-Turner crossed beam grating monochromator (blaze at 300 nm) equipped with a prism predisperser (PD), stepping motor (SM), and absolute shaft encoder (SE). The slits were 2.0 mm wide (bandpass 5.3 nm) unless noted otherwise. The selected excitation radiation is focussed on the sample (S) by a Cassegrain system (CS, 1:1 magnification, [53]). A small portion of the exciting radiation is reflected by a beam splitter (BS) at a 30° enclosed angle and focussed at the reference detector consisting of a quantum counter (8 g/L rhodamine B in ethylene glycol [54], cell at 15° to the face of the photomultiplier tube) (QC) and an EMI 9659QB extended red photomultiplier tube (RD). A Glan-Thompson polarizer can be placed directly before the sample (S) and a second Glan-Thompson polarizer, the analyzer (P), can be placed between the ellipsoidal mirror (EL_2) and the detection monochromator. Both polarizers were calibrated to an absolute angular orientation of 0.1° with a Rudolph Research Thin Film Ellipsometer Model 43702-200E using a helium-neon laser as a source [55].

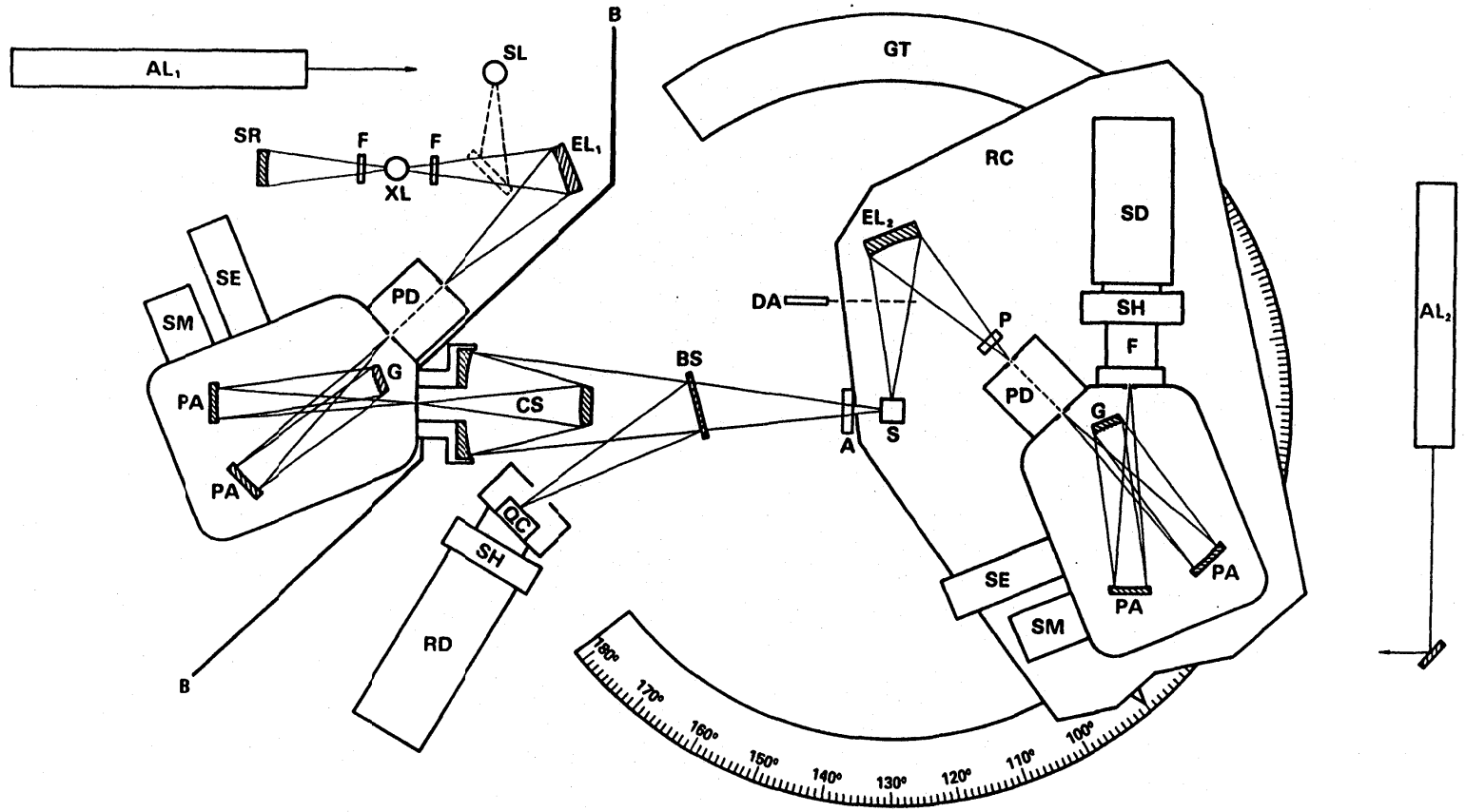


Figure 1. The NBS reference spectroradiometer. Al-alignment laser; B-baffle; BS-beam splitter; CS-cassegrain system; DA-double aperture; EL-ellipsoidal mirror; F-filter; G-grating; GT-goniometer track; P-polarizer; PA-paraboloidal mirrors; PD-predisperser; QC-quantum counter; RD-reference detector; RC-rotary carriage; S-sample; SD-signal detector; SE-shaft encoder; SH-shutter; SL-spectral lamp; SM-stepping motor; SR-spherical mirror; XL-xenon lamp.

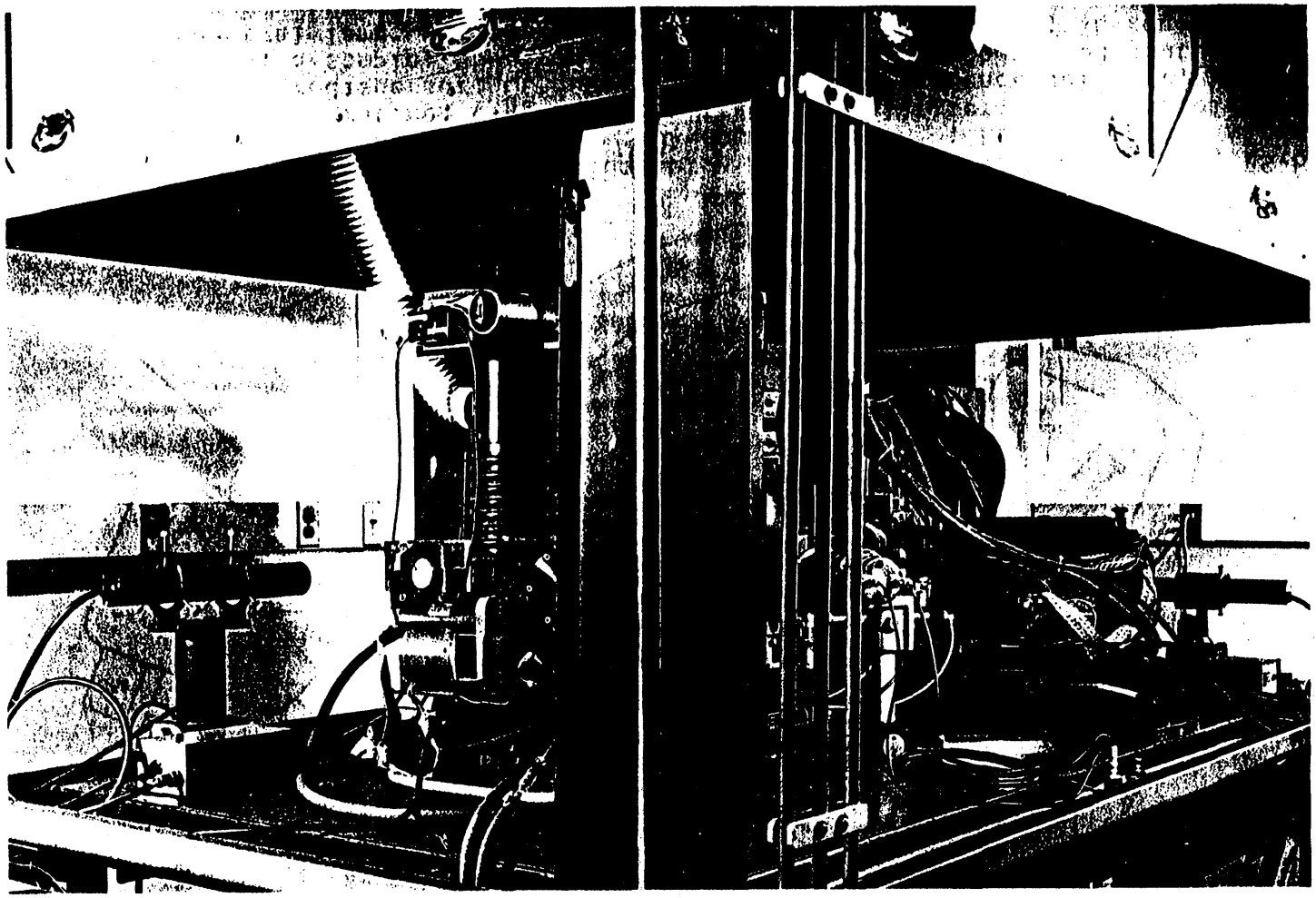


Figure 2. Photograph of the NBS reference spectroradiometer. The excitation side is on the left and emission side is on the right side of the photograph.

The emitted radiation is focussed by the ellipsoidal mirror EL_2 at the slit of the detection monochromator which is identical to the excitation monochromator except that the grating is blazed at 500 nm. The detection photomultiplier is an EMI 9659QB extended red photomultiplier tube (SD), thermoelectrically cooled to -12 °C. The instrument uses analog detection in conjunction with a d.c. amplifier and a digital voltmeter. The instrument is interfaced to a digital computer. Shaft encoders (0.005 nm angular resolution) are used to supply digitized wavelengths of the source monochromator (λ_1) and the detection monochromator (λ_2) to the digital computer. The instrument can be operated in single mode ("B mode" for obtaining emission spectrum, "A mode" for obtaining an excitation spectrum) or ratio mode ("B mode" signal/reference signal). In single mode operation, the signal is sampled one hundred times in 10 s and the values are relayed to the computer where the average and the standard error are calculated. In "R mode" operation (R=B/A) using the reference detector, the emission signal (B) is sampled once, then the reference signal (A) is sampled once, the values are relayed to the computer where the ratio R is calculated and the values B, A and R are stored. This procedure is repeated 50 times in 10 s after which $\bar{B}$, $\bar{A}$, $\bar{R}$, and the standard error in $\bar{R}$ are calculated. These values and additional parameters are transmitted to a teletype or plotter. An example of the teletype output is given in figure 3. The 'X' column represents the operating mode; 'N' represents the number of points (or ratios) measured; 'A'; 'B'; 'R' the computed averages; S.E.(X) the standard error of the value designated in the 'X' column, and WVL(EX) and WVL(EM) the excitation and emission wavelengths. After data are taken at a particular position, the scanning monochromator is changed by a preset wavelength interval (either to longer or shorter wavelengths) and the measurement process repeated until the total spectrum is recorded. The raw data are stored

on magnetic tape and transferred to the NBS Univac 1108 computer where data reduction and analyses are performed.

RUN 9023

X	N	A	B	R	S.E.(X)	WVL(EX)	WVL(EM)
R	49	1.014757	.708699	.698447	.188D-02	440.000	530.000
B	100		.704714		.991D-03	440.000	530.000
A	100	1.013186			.824D-03	440.000	530.000

END OF RUN

Figure 3. Teletype output for the operation of the reference spectroradiometer in the three operating modes: R-ratio, B-emission; A-excitation.

The programs used for data reduction, statistical analysis, curve fitting, and smoothing were written in OMNITAB, a language developed at NBS that is well suited for manipulating columnar data [56].

C. Instrument Calibration

1. Wavelength

Both monochromators were calibrated for wavelength accuracy over the 202.5 to 894.4 nm wavelength range by measuring 37 lines from mercury, zinc, helium, cadmium, neon, cesium, and rubidium low-pressure discharge sources. For calibration of the detection monochromator, the sources were placed at the sample position or attached to the detection monochromator. For calibration of the excitation monochromator, the sources were placed at the xenon lamp position or attached to the excitation monochromator and a mirror was placed in the sample position. The monochromators were stepped in ascending and descending 0.005 nm wavelength intervals over the line position and the wavelength values obtained at the maximum for ascending and descending wavelength intervals were averaged.

The differences between the average measured values and the true values [57] were determined.

2. Detection System

The spectral responsivity, $M^s_{\lambda_2}$, of the emission detection system (ellipsoidal mirror EL_2, analyzer, detection monochromator, and detection photomultiplier) was determined in the following manner (refer to figure 1). The detection photomultiplier was replaced by the alignment laser AL_2 located ∿15 cm from the exit slit of the monochromator. Masks with central holes of ∿5 mm diameter were placed over the paraboloidal mirrors in both monochromators. Both alignment lasers (AL_2 and AL_1) were aligned such that the two beams intersected at 90° at the sample position 30.5 cm (12 inch) above the table.

A NBS calibrated vacuum tungsten ribbon lamp #A7 [58] was placed in the sample position such that the flat portion of the tungsten ribbon was perpendicular to the beam from AL_2 and the alignment notch in the ribbon was on the optic axis, figure 4. The position of the lamp was adjusted until the beam from AL_2 just passed through the notch in the ribbon and contacted the point of an arrow inscribed on the quartz envelope of the lamp. At this point in the alignment procedure, the beam from AL_1 was vertically bisected by the edge of the tungsten ribbon. The masks were removed and the lamp was activated and adjusted to an operating current of ∿8A. A pin diode was placed at the exit slit of the detection monochromator without disturbing AL_2 and the signal maximized using only the horizontal adjustment of EL_2. The pin diode was removed and the lamp current reduced to ∿3A. The beam from AL_2 impinged on the tungsten ribbon as in figure 4. This process was repeated several times with the same results and the lamp was considered to be aligned. The spectra of the lamp at two operating currents were recorded

(see Procedures, Section IV-D) and the spectral responsivity of the detection system was calculated using the calibrated spectral radiance values (see Section VII).

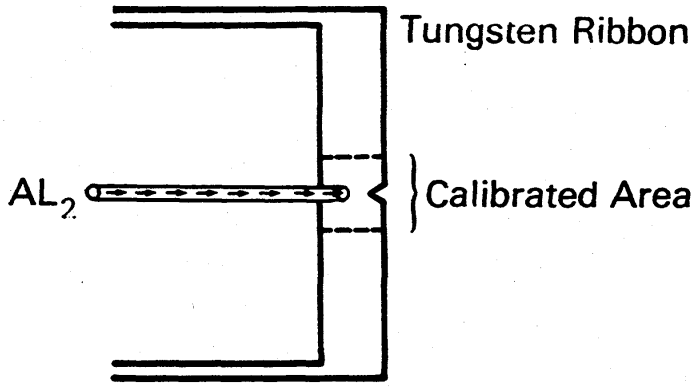


Figure 4. Alignment of the calibrated vacuum tungsten ribbon lamp using the alignment laser AL_2.

3. Photomultiplier Tube Linearity

A tungsten lamp was placed in the sample position S with a double aperture device (DA) [52] located between the sample position and EL_2, figure 1, such that both apertures A and B were included in the 14° angle subtended by EL_2. The photocurrents were measured at varying incident radiant fluxes with aperture A open and aperture B closed, with aperture B open and aperture A closed, and with both apertures open.

D. Measurement Procedures

The measurement procedure used in determining the effect of temperature on the emission spectrum is depicted in figure 5. The temperature of the QS solution was set to T_1, the spectrum recorded in ascending wavelength intervals, the temperature was set to T_2, 30 min were allowed for equilibration, the spectrum recorded in descending wavelength intervals, etc. The spectra were corrected for photomultiplier non-linearity, then averaged as indicated by the

dotted lines, thus eliminating the effects of long term instrumental drift as well as any backlash in the monochromator wavelength drive.

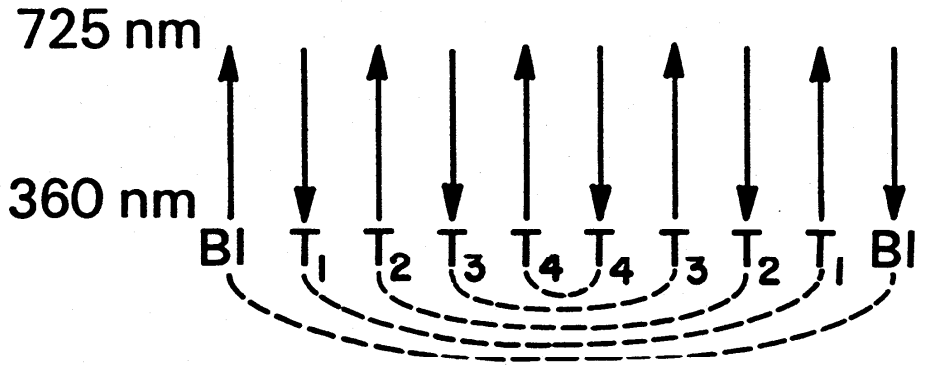


Figure 5. Time symmetrical measurement procedure used for determining effect of temperature on the emission spectrum.

In general, measurements were made with the analyzer (detection polarizer) in the vertical mode (designated S) to avoid "Woods" anomalies from the grating observed with the polarizer in the horizontal mode (designated P) (see Section VI-E).

The measurements on a single series of ten quinine sulfate solutions for the correction and certification of the emission spectrum takes three days as outlined in figure 6. On day 1, the standard lamp was placed in the sample compartment, aligned (Section V-C2), and $R^S_{\lambda_2}$ values were obtained at lamp operating currents of 8.8030±0.0008 and 6.0000±0.0006A from which the spectral responsivities $M^S_{\lambda_2}$ of the detection system were calculated over the 350.0 to 425.0 nm and 400.0 to 900.0 nm ranges, respectively (Section VIII).

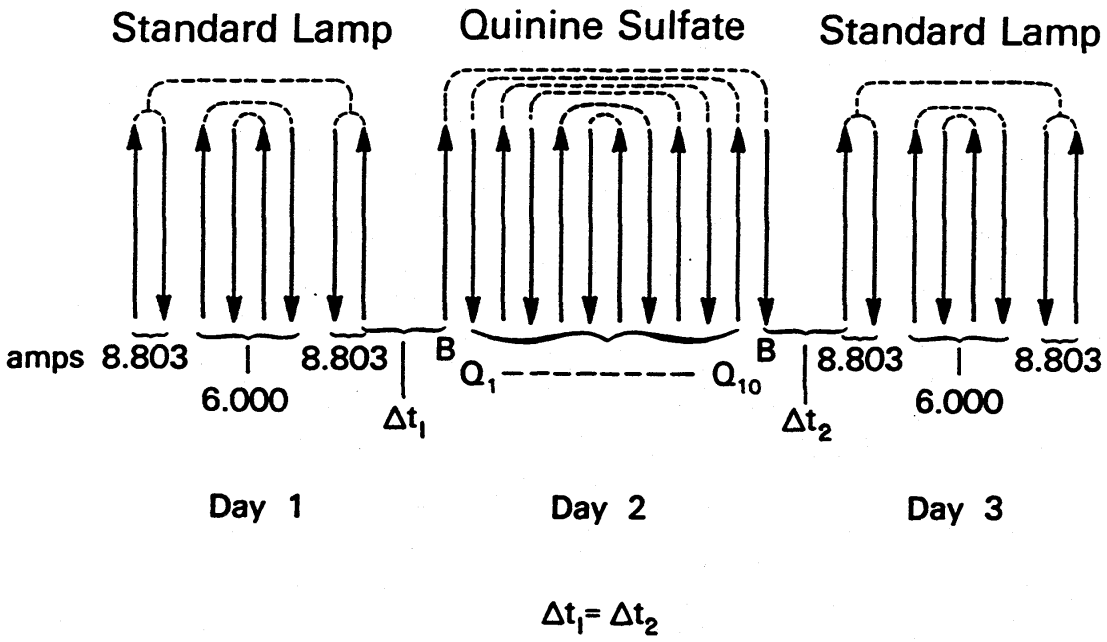


Figure 6. Time symmetrical measurement procedure used for determining the spectral responsivity of the detection system and the uncorrected emission spectrum (Y) for quinine sulfate.

The standard lamp was then replaced by the normal, right-angle (sideview), cuvette sample holder. A clean, quartz, unblackened cuvette was inserted, rinsed a minimum of five times [60] with the blank solution (each rinse solution was removed by suction), and the blank signal $Y^{X,S}_{347.5,\lambda_2}$ was measured every 5 nm over the wavelength region of interest (360-725 nm). The blank solution was removed by suction without cell removal, the cell was rinsed a minimum of five times with solution QS_1, filled with QS_1, and the compensated fluorescence signal $Y^{X,S}_{347.5,\lambda_2}$ was measured every 5 nm from 360 to 725 nm. The cuvette was then rinsed and filled with QS_2 and (Y) for QS_2 was recorded every 5 nm. This procedure was followed until the second blank was measured. Each spectrum took approximately 25 min to record. On day 3 as on day 1, the spectral responsivity $M^S_{\lambda_2}$ of the

detection system was determined to assure that the instrument calibration did not change and to obtain a measure of the stability of the instrument.

A spectrum was recorded in either increasing or decreasing wavelength intervals as designated by the arrow direction in figure 6. The time intervals, Δt_1 and Δt_2 were kept constant so that time symmetry was maintained. The spectra were corrected for photomultiplier non-linearities and then were averaged according to the dashed lines.

VI. Quinine Sulfate

Quinine, an alkaloid extracted from *cinchona* bark, is a nitrogen heterocyclic organic species soluble in acid, alcohol, and ether. It exhibits relatively broad fluorescence and absorbance spectra (figure 7).

Quinine and its derivatives have been the most widely used fluorescence standards but do exhibit some anomalous characteristics. Due to these characteristics, inaccurate values and wrong information on the fluorescence of quinine sulfate can be found in the literature. This, in turn, has cast doubt on using quinine as a fluorescence standard. Therefore, we include here discussions of previous data which suggested problems in the use of quinine sulfate. At the same time, additional results and discussions are included to characterize further the SRM grade quinine sulfate.

A. Memory Effect

It had been stated [6] that the absorbance spectral characteristics depended on whether quinine, quinine bisulfate or quinine sulfate was the original solute used to prepare the solution. Fletcher [25] showed that dissolution of several different samples of quinine and its salts gave equivalent absorbance and excitation spectra [61]. The memory effect

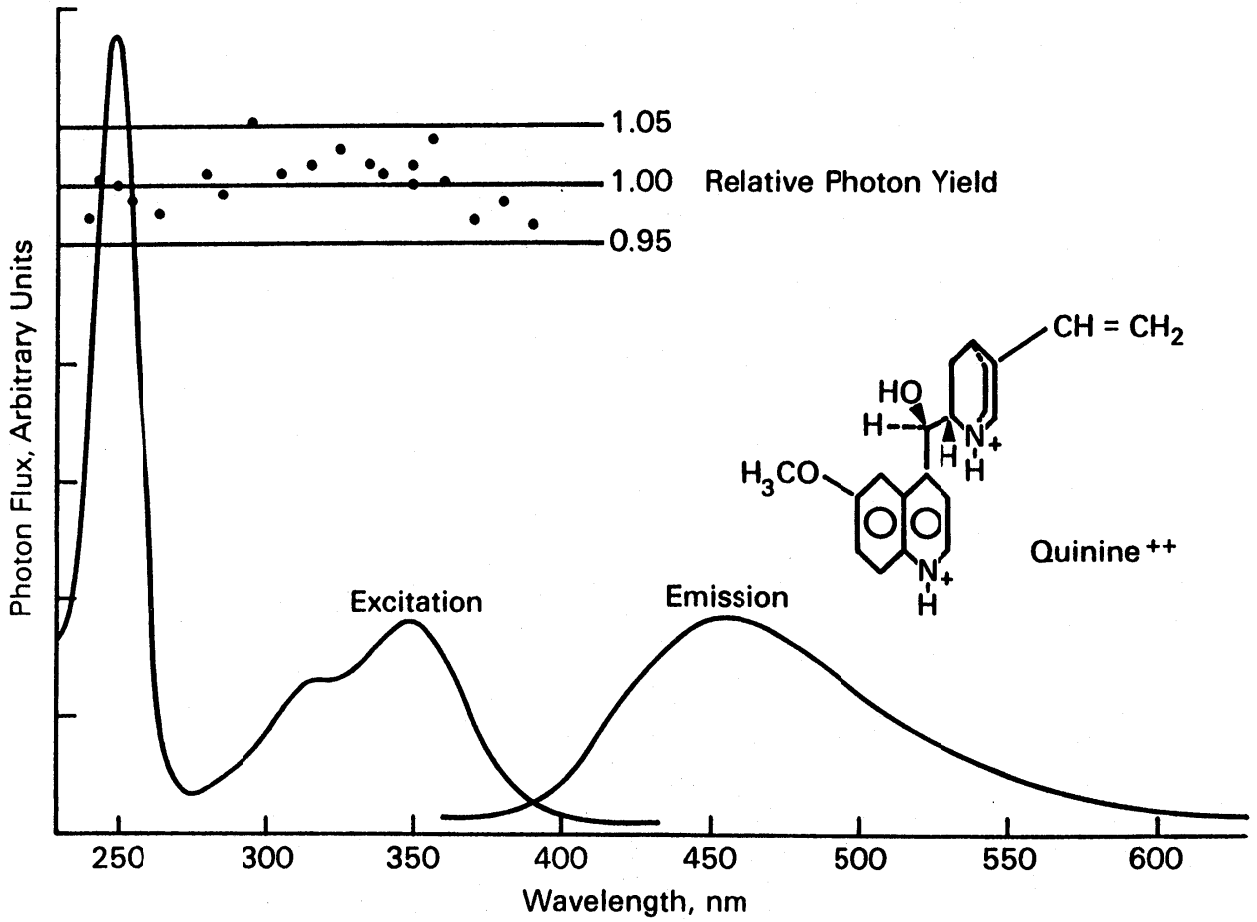


Figure 7. The structure, fluorescence spectra, and relative photon yield as a function of wavelength for quinine in acid media.

observed earlier was attributed to the presence of an impurity, varying waters of hydration, or slit width problems [26,27,33].

B. Purity

1. General

The purity and number of waters of hydration in a material are important factors to consider when measurements of specific molar absorbances and absolute quantum yields are made. However, they are not critical to the determination of a relative spectral photon yield or a corrected spectrum unless the impurity absorbs some of the emitted radiation or itself emits in the same wavelength region as the standard. In both cases, however, the presence of up to 0.1 percent impurity would not significantly affect the measured data if the specific molar absorbance and photon yield of the impurity are similar to those of the standard since the effect is less than the measurement precision and accuracy.

For example, consider an impurity (0.1 percent) that absorbs at 347.5 nm and/or 450 nm with a specific molar absorbance of 10^4 L mol^{-1} cm^{-1} and photon yield of 0.5. The measured fluorescence signal (Y) from the quinine sulfate ($\varepsilon_{347.5} \simeq 10^4$ L $mol^{-1}cm^{-1}$, $Q \simeq 0.6$) in dilute solutions [63] would be decreased by the absorbance of the impurity in solution to give a new measured fluorescence signal (Y)':

$$(Y)' = (Y)\ 10^{-\varepsilon cb} \qquad (4)$$

where

ε = specific molar absorbance ($\sim 10^4$ L mol^{-1} cm^{-1})

b = cell path length (0.5 cm)

c = impurity concentration ($\sim 1.3 \times 10^{-9}$ mol L^{-1} [62])

Upon substitution of these values into equation 4, the fluorescence signal would be decreased by approximately 0.001 percent, a value that is two orders of magnitude smaller

than the observed measurement precision and approximately three orders of magnitude smaller than the stated measurement accuracy (Section IX).

Similarly, for two emitting species, the total fluorescence reading $(Y)_T$ in terms of the signal for the pure compound $(Y)_1$ is:

$$(Y)_T = (Y)_1 (1 + \frac{Q_2 \varepsilon_2 C_2}{Q_1 \varepsilon_1 C_1}), \qquad (5)$$

where Q is the photon yield and the subscripts 1 and 2 refer to the quinine sulfate and impurity, respectively. Substitution of typical values for $Q_{1,2}$, $\varepsilon_{1,2}$ and $C_{1,2}$ show that a negligible error of $\sim$0.1 percent would be incorporated in the emission spectrum.

Under extreme conditions in which the impurity has a maximum ε of $\sim 10^5$ [64], the errors in the absorbance or fluorescence readings increase an order of magnitude to 0.01 and 1 percent, respectively. It is more likely, however, that impurities in quinine sulfate would have specific molar absorbances and photon yields comparable to those of quinine resulting in errors of 0.1 percent or less in absorbance or fluorescence measurements.

2. SRM Grade Quinine Sulfate Dihydrate, $QS \cdot 2H_2O$:

SRM grade $QS \cdot 2H_2O$ was compared to highly purified samples of quinine and quinine sulfate [33]. No impurities that affected the optical characteristics of the material were found in the SRM grade $QS \cdot 2H_2O$ within experimental error as determined by thin layer chromatography (TLC), relative photon yield, fluorescence lifetime, specific weight absorbances, and water content. High performance liquid chromatography (HPLC) did show the presence of a second peak ($\sim$1.7%) that was shown to be dihydroquinine sulfate by high-resolution mass spectrometry; however the optical characteristics of the dihydro species were similar to those of quinine sulfate.

(a) TLC: Fluorescent and non-fluorescent silica gel plates were spotted with eight samples of SRM grade $QS \cdot 2H_2O$ and two samples of purified quinine sulfate. Two different solvent systems (Solvent 1 = $CHCl_3$:MeOH:HOAc-75:20:5; Solvent 2 = i-PrOH:HCOOH-80:20) were used to develop the plates [65]. Both types of plates were irradiated using 265 and 366 nm UV radiation. The fluorescent plates showed a single blue fluorescent spot for each sample (normal green fluorescence of the plate was quenched) and the non-fluorescent plate also showed a single, blue fluorescent spot for each sample. The r_f values for the purified and SRM grade quinine sulfate samples were the same, Table 3. The plates then were developed by spraying with a ten percent H_2SO_4 solution. Again only a single spot was observed for each sample with the same r_f values as those in Table 3.

Table 3. Comparison of SRM Grade Quinine Sulfate with a Purified Quinine Sulfate Preparation Using Thin Layer Chromatography.

	- - - - R_f Values - - - -	
	Solvent 1[c]	Solvent 2[d]
SRM Grade[a]	0.516±0.02	0.625±0.02
Purified[b]	0.515	0.626

[a] Average of eight samples.
[b] Average of two samples.
[c] By volume: 75 $CHCl_3$:20 meOH:5 HOAc.
[d] By volume: 80 i-PrOH:20 HCOOH.

(b) HPLC: The presence of dihydroquinidine in commercially prepared quinidine has been reported [66] and thus suggests the presence of dihydroquinine in commercial quinine. HPLC analysis of SRM grade quinine sulfate showed the presence of a second constituent that was approximately two percent of

the total as determined by UV and fluorescence detection [67]. A microparticulate reversed phase column was used with mobile phases buffered at two pH's. Two of the mobile phases were mixtures of 70 percent aqueous buffer (H_2O:1.36 g/L KH_2PO_4:0.15 mol/L H_2SO_4) and 30 percent acetonitrile at pH's of 4.3 and 2.3, respectively. A third mobile phase was a mixture of 85 percent buffer and 15 percent acetonitrile at a pH of 2.3. Use of all three mobile phases with UV (254 nm) and fluorescence (λ_{ex} = 250, 317, or 350 nm, λ_{em} = 450 nm) showed the presence of a second constituent.

The major peak constituent (labelled A) and the minor peak constituent (labelled B) were individually trapped in a flow cell in an uncorrected spectrofluorimeter. Uncorrected emission and excitation spectra of constituent A and constituent B were identical indicating that constituent B was optically equivalent within experimental error to quinine sulfate. The spectra obtained from the two constituents using the mobile phase buffer at a pH of 4.3 were quite complex. This is not unexpected since 'optical' titration curves, monitored by UV absorbance and fluorescence emission spectra, have mid-points at about pH 4.3 suggesting the presence of complex emitting species [68].

(c) High-resolution Mass Spectrometry: Several samples of SRM grade quinine sulfate were separated by chromatography under preparative HPLC conditions, each time separating the materials responsible for producing peaks A and B. The fractions of 'peak A' were combined and the fractions of 'peak B' were combined. Both fractions were neutralized with sodium hydroxide and the free bases were extracted into methylene chloride. The methylene chloride fractions were separated from the aqueous phases and each fraction was concentrated at least 10-fold by solvent evaporation. Both 'A' and 'B' fractions were analyzed by high resolution,

electron impact mass spectrometry [69]. The mass spectrum of the material in fraction A was identical to the literature spectrum of quinine with molecular ion at m/e 324 [70]. The mass spectrum of the material in fraction 'B' showed a molecular ion at m/e 326. The exact mass measured for constituent B was 326.1990 and the mass required for $C_{20}H_{26}N_2O_2$ is 326.1994; this is consistent with the interpretation that the material separated in peak B is dihydroquinine. It is possible that the peak B constituent could be dihydroquinidine, however, the measured optical rotation (Appendix B) supports the presence of the levorotatory species, dihydroquinine.

(d) Absorbance and Fluorescence Parameters: The optical equivalence of the SRM grade $QS \cdot 2H_2O$ and the purified $QS \cdot 2H_2O$ was demonstrated by determining the specific molar absorbances at 347.5 nm, the relative photon yield Q and the fluorescence lifetimes τ in 0.05 mol/L H_2SO_4. These results are summarized in Table 4 [33].

The normally accepted values for Q are 0.508 and 0.546 in 0.05 and 0.5 mol/L H_2SO_4, respectively [7,74]. The generally accepted value for τ in 0.05 mol/L H_2SO_4 is approximately 19.2 ns [reference 75 and references therein]. As can be seen, excellent agreement within experimental error was obtained for all the parameters measured.

(e) Water Content: As mentioned, waters of hydration, if variable, can be considered an impurity when absolute photon yields or specific molar absorbances are measured. It was reported that quinine sulfate contained variable water content [25,71] but drying at 50-55 °C under vacuum brought the material to the dihydrate, $QS \cdot 2H_2O$ [25]. Thermogravimetric analysis showed that a weight loss of $\sim$4.5 percent occurred upon heating. This was interpreted to mean that the quinine sulfate originally contained 4-5 waters of hydration [33]. However, preliminary mass spectrometry data showed water loss at $\sim$50-80 °C followed by decomposition

Table 4. Equivalence of SRM Grade Quinine Sulfate and a Purified Quinine Sulfate Sample as Shown by Measurements of Specific Weight Absorbances W.A., Relative Photon Yields Q, and Fluorescence Lifetimes τ.

	$(W.A.^a\ g_{sol}(g_{solv})^{-1}cm^{-1})x10^4$	Relative Photon Yields, Q H_2SO_4, mol/L		τ, ns[a]
		0.05	0.5	
Purified $QS\cdot 2H_2O$	1.434±0.012[b,f]	0.508	0.546	19.2±0.1[c,f]
SRM Grade $QS\cdot 2H_2O$	1.432±0.006[f]	0.513±0.005[d,f]	0.544±0.003[e,f]	19.1±0.1[c,f]

[a] Determined in 0.05 mol L^{-1} H_2SO_4.

[b] Reference [33].

[c] Eight determinations.

[d] Three determinations.

[e] Four determinations.

[f] The measures of uncertainty given are the standard deviation of a single measurement.

[72] indicating that the water of hydration was removed at temperatures similar to those suggested for drying the quinine sulfate [25].

The water content of six and four samples of the SRM grade QS were determined by Karl-Fischer (KF) titration and weight loss procedures, respectively. The KF titrations were made using varying amounts of distilled water as the standard and varying amounts of "as is" SRM grade quinine sulfate so that linear regression analyses could be performed on the analytical curves [73]. The weight loss procedure consisted of drying the quinine sulfate at 50-55 °C under vacuum until a constant weight was achieved. The values obtained by these two procedures are summarized in Table 5. The average water content as determined by the KF procedure, 4.74 percent, is a little higher than the 4.57 percent determined by the weight loss procedure. Both values bracket the theoretical value of 4.60 percent. The KF and weight loss values correspond to 2.06 and 1.99 molecules of water per molecule of quinine sulfate.

The dried quinine sulfate samples were found to be hygroscopic when allowed to stand at room temperature in ∿35 percent humidity. After absorbing water and reaching constant weight (∿0.75 h), the weight gained by the four samples corresponded to the weight lost by heating in vacuum to within 0.3 percent by all samples. This indicates that the "before drying stoichiometric, two water molecules per one quinine sulfate molecule ratio" was again reached.

These data show that drying the SRM quinine sulfate at 50-55 °C for 12 hours produces the anhydrous rather than the di-aquo species. Additional evidence for this conclusion is given in the homogeneity and specific weight and molar absorbances sections.

Table 5. Percent Water in SRM Quinine Sulfate·$2H_2O$ as determined by Karl-Fischer Titration and Weight Loss Procedures.

Sample #	mg Taken	Procedure	Percent Water	$\bar{x}$
311	242.7	KF[a]	4.77	
122	153.8	KF	4.78	
284	80.0	KF	4.64	4.74
177	187.6	KF	4.76	
96	303.1	KF	4.73	
453	320.8	KF	4.73	
27	147.56	WL[b,c]	4.52	
311	591.76	WL	4.59	4.57
453	236.59	WL	4.62	
96	273.73	WL	4.55	

[a] The Karl-Fischer titrations were performed by S. Margolis, Organic Analytical Research Division, National Bureau of Standards.

[b] Semi-micro balance calibrated to ±.01 mg using NBS #62730 calibrated weights.

[c] Dried at 55 °C under vacuum for 12 h, put while warm in desiccator over drierite, covered, and weighed when cool.

(f) Homogeneity: The specific weight absorbances WA of twenty quinine sulfate solutions ($\sim 2 \times 10^{-5}$ mol L^{-1}) were measured on an NBS reference spectrophotometer to determine the homogeneity of the SRM. The values obtained are summarized in Table 6.

The average WA values are ~4 percent lower than those reported earlier [33] i.e., 9.24±0.09-, 14.34±0.12-, and 77.82±0.86 x (10^{-5}) $g_{solv}(g_{sol})^{-1}$ cm^{-1}. If it is assumed, however, that the weighed quinine sulfate was the dihydrate, and the dried material in reference 33 was anhydrous, the WA values in Table 6 adjusted to the anhydrous state become

Table 6. Specific Weight Absorbances for Twenty Solutions of Quinine Sulfate Dihydrate in 0.1 mol/L $HClO_4$ at 365.0, 347.5, and 250.0 nm.

	- - - - - (W.A. $g_{solv}(g_{sol})^{-1}$ cm^{-1})x10^{-3} - - - - -					
	λ = 365.0 nm		λ = 347.5 nm		λ = 250.0 nm	
Sample	S-I[a]	S-II	S-I	S-II	S-I	S-II
27	8.8509	8.8854	13.8367	13.8217	72.9934	72.7913
96	8.8504	8.8233	13.8367	13.7926	73.0886	73.3980
122	8.8561	8.8578	13.8536	13.8487	73.0102	72.9393
177	8.8382	8.8543	13.8377	13.7977	73.0297	72.5286
230	8.8426	8.8743	13.8546	13.8656	72.9184	72.9920
284	8.8568	8.8160	13.8547	13.7726	72.9882	72.8797
311	8.8324	8.8426	13.8306	13.7796	73.1418	72.5563
370	8.8698	8.8248	13.8737	13.7797	73.0159	72.9112
421	8.8600	8.8823	13.8798	13.8457	73.2624	72.4397
453	8.8806	8.8762	13.8936	13.8246	72.9898	72.8001
$\bar{x}$[b]	8.854(6.918)[c]		13.834(10.809)[c]		72.934(56.986)[c]	
s[b]	.015		.022		.125	
% s[b]	.169		.159		.171	

[a] Corrected for instrumental bandpass error.

[b] The two values for each sample were averaged and these averages were used to determine $\bar{x}$, s, and % s.

[c] ε (L, mol^{-1}, cm^{-1}) x 10^{-3}.

9.287-, 14.512-, and 76.507 x (10^{-5}) $g_{solv}(g_{sol})^{-1}$ cm^{-1} at 365.0, 347.5 and 250.0 nm, which are in good agreement with those reported earlier. This provides additional evidence that drying quinine sulfate•x hydrate at 50-55 °C under vacuum produces the anhydrous and not the di-aquo species. The relative standard deviations calculated for the data at each of these wavelengths were comparable although they might have been expected to decrease as the WA values increased. This may be offset by the added difficulty in making accurate absorbance measurements in the UV region.

If, in the worst case, the total standard deviation s for the 347.5 nm data is due to sample inhomogeneity (assuming no experimental error), the inhomogeneity of the SRM quinine sulfate can be expressed in terms of a tolerance interval, represented by $\bar{x} \pm Ks$ where $\bar{x}$ is the average value for the specific weight absorbance, s is the standard deviation of the measurements, and K is a factor for two-sided tolerance limits of normal distributions which depends on the number of samples analyzed n, the probability value or confidence level desired γ, and the percent of the 500 samples included in the tolerance interval P. In this case the tolerance interval $\pm$ Ks is designed to cover at least 95 percent of the population with a probability of 95 percent [14]. Thus, for n=10, P=95, and γ=95, the factor K is 3.379 [76], and the inhomogeneity in the samples would be on the order of 0.5 percent since

$$\bar{x} \pm Ks = 13.834 \pm 0.075\ g_{solv}(g_{sol})^{-1} cm^{-1}.$$

However the total percent standard deviation is composed of the contributions due to the inhomogeneity of the SRM and the contributions due to experimental errors:

$$\frac{\sigma^2_{WA}}{WA} = \frac{\sigma^2_I}{I} + \frac{\sigma^2_E}{2E} \qquad (6a)$$

where

$\frac{\sigma_{WA}}{WA}$ = the overall percent standard deviation (0.159 from Table 6);

$\frac{\sigma_I}{I}$ = the percent due to inhomogeneity; and

$\frac{\sigma_E}{E}$ = the percent due to the experimental measurements.

Estimates of the individual components comprising $\frac{\sigma_E}{E}$ are: concentration (several weighings and dilutions) $\sigma_c/C = 0.07$ percent; absorbance - $\sigma_A/A = 0.1$ percent [77]; and cell length $\sigma_b/b = 0.005$ percent. Additional errors in the measurement process are temperature variation $\sigma_T/T = 0.15$ percent and cuvette placement $\sigma_p/p = 0.03$ percent. These values, added in quadrature, give an estimated experimental error for σ_E/E of 0.196 percent. Thus,

$$\frac{\sigma_I}{I} = \sqrt{\frac{\sigma^2_{WA}}{WA} - \frac{\sigma^2_E}{2E}} = 0.078 \qquad (66)$$

and the estimate of SRM inhomogeneity (±Ks) drops to a more realistic 0.26 percent. One major factor in this 'inhomogeneity' could be slightly varying waters of hydration which do affect the measurement of specific weight absorbance values but not the normalized, certified emission spectrum. Thus, although in the worst case, we assign the inhomogeneity of quinine sulfate a tolerance level of 0.5 percent, it is more likely to be on the order of 0.3 percent or less.

The values reported in Table 6 are indicative of vial to vial homogeneity and are not to be used as certified values of the specific weight absorbances. Although preliminary data showed no fluorescence effect when measuring the absorbance of fluorescent solutions on the NBS Reference

Spectrophotometer [33]. Aditional verification would be necessary before certification of absorbance values could be accomplished.

(g) Absorbance Spectrum: It has been reported that the absorbance spectrum of quinine sulfate is independent of the sulfuric acid concentration [75]. This appears to be the case within experimental error when peak ratios are measured over a hydrogen ion concentration range of 0.01 to 1.0 mol/L for H_2SO_4 or $HClO_4$, Table 7. (Note: Ratio values that are within 1 percent of each other are considered equivalent under the measurement conditions if the absorbance values for the individual peaks are approximately equal. Thus, if the values of the ratio of the absorbances at 347.5 nm and 317.5 nm agree to within one percent, the ratios are considered equivalent. On the other hand, one could expect somewhat larger errors for measurements of the absorbances for two disparate peaks such as the 250 nm and 347.5 nm peaks and still consider the ratios to be equivalent.)

Table 7. Ratios of the Absorbances for Several Peaks of Quinine Sulfate in Sulfuric and Perchloric Acids.

	$A_{250}/A_{347.5}$		$A_{347.5}/A_{317}$		$A_{347.5}/A_{365}$
	H_2SO_4	$HClO_4$	H_2SO_4	$HClO_4$	H_2SO_4
(H^+)mol/L					
0.01	5.317	5.400	1.238	1.235	1.561
0.1	5.382	5.382	1.250	1.256	1.648
1.0	5.418	5.404	1.235	1.235	1.570
5.0	5.427	5.626	1.217	1.193	1.510
10.0	5.427	5.558	1.179	1.003	1.414

As can be seen, the range of ratios for a hydrogen ion of 0.01 to 1.0 mol/L for the two acids are 1.2 and 1.6 percent respectively (essentially equivalent) for the $A_{347.5}/A_{317}$ ratios, however, a larger percent is observed for the ratio at these wavelengths over the total hydrogen ion concentration range of 0.01 to 10 mol/L; i.e., 5.7 and 20.2 percent, respectively. A slight increase ($\sim$2 percent) is observed when the $A_{250}/A_{347.5}$ ratios are calculated over the 0.01 - 10.0 mol/L hydrogen ion concentration range.

This trend was also observed in a separate experiment in which the specific weight absorbances were found to be equivalent experimentally in 0.1 and 1.0 mol/L perchloric acid and 0.5 mol/L sulfuric acid, Table 8. However, increasing the $HClO_4$ concentration to 3.5 mol/L resulted in a 4.8 and 5.7 percent increase at 317.5 and 365.0 nm, respectively while the specific molar absorbances at 250.0 and 347.5 nm show only a 0.9 and 0.7 percent decrease, respectively.

Table 8. Specific Weight Absorbances of Quinine Sulfate in Sulfuric and Perchloric Acids.

	- - - (W.A., $g_{solv}(g_{sol})^{-1}cm^{-1})x10^{-3}$ - - -			
(H^+)mol/L	365 nm	347.5 nm	317.5 nm	250 nm
0.1 H_2SO_4	9.25	14.20	11.45	77.85
0.1 $HClO_4$	9.10	14.20	11.35	76.45
1.0 $HClO_4$	9.25	14.20	11.35	76.15
3.5 $HClO_4$	9.70	14.10	11.90	75.55

Thus it does appear that the absorbance spectrum of quinine sulfate is dependent on acid concentration at high $[H^+]$ with the shoulder at 317.5 nm and possibly a peak at ∿365 nm (under the major 347.5 nm absorbance peak) increasing as the H^+ concentration increases. This observation could support the presence of a second emitting level (at 365 nm or higher excitation) that is responsible for the "red edge" effect, (Section VI-F).

C. Stability

It has been reported that quinine sulfate exhibits stability in solution [7,33,71,78]. Several reports, however, have shown that quinine sulfate is unstable in solution upon irradiation [79], but it must be emphasized that in all cases except that reported by Melhuish, the instabilities were observed under extreme conditions of intense radiation, excessive excitation bandpasses (∿30-40 nm), very dilute solutions, and small sample sizes. Melhuish, on the other hand, reported the instability using an excitation wavelength of 313 nm, a location where Dawson and Windsor [80] reported variable quantum yields indicating that excitation at 313 nm may cause problems.

We found [33] that quinine sulfate in 0.05 mol/L H_2SO_4 was stable to ±1 percent when stored in the dark and measured periodically for six months. With similar storage over a six week period, the spectral emission envelope of quinine sulfate in 0.105 mol/L $HClO_4$ did not change by more than ±0.2 percent (equal to the measurement imprecision) at the 67 percent confidence level (see Section VIII on the corrected emission spectrum).

Additionally, studies of 1 ppm quinine sulfate in 0.105 mol/L perchloric acid show stability under normal irradiation (450 W xenon lamp, 5.3 nm bandpass, 347.5 nm excitation) at the ±0.2 percent level for short periods of

time (2 h) and at the ±0.8 percent level over longer time periods (12 h). This latter observation may indicate degradation over long irradiation times.

Nevertheless, to avoid any possible photodegradation, all prepared solutions of quinine sulfate should be stored in the dark and removed only for measurement purposes. Additionally, freshly prepared solutions of quinine sulfate should be used for critical measurements.

D. Oxygen Quenching

Oxygen quenches the fluorescence of many organic aromatic species and may be considered to be an impurity molecule. It is soluble in aqueous solutions to $\sim 1 \times 10^{-4}$ mol/L and aliphatic solvents to $\sim 2 \times 10^{-3}$ mol/L [81]. Every collision of an excited molecule and oxygen leads to quenching by the viscosity dependent equations [82]:

$$^1QS^* + {}^3O_2 \rightarrow {}^1QS + {}^3O_2 \qquad (7a)$$

$$^1QS^* + {}^3O_2 \rightarrow {}^3QS^* + {}^3O_2 \qquad (7b)$$

with the overall effect of reducing the photon yield:

$$Q = \frac{k_{FM}}{k_{FM} + k_{IM} + k_{QM}(O_2)} \qquad (7c)$$

and thus η_2, the spectral photon yield i.e.:

$$Q = \eta_2 d\lambda_2 .$$

No change in the spectral intensity was observed after ten freeze-vacuum-thaw cycles performed in the "Schlenk" adapted [83] spectrofluorimeter cell [33] which agrees with earlier reports [7,26] of non-oxygen quenching of quinine sulfate in 1.0 mol/L H_2SO_4.

E. Polarization

It has been shown that errors in fluorescence measurements can be as large as 80 percent and that errors of 10-20 percent are common under normal operating conditions (90° geometry) if the radiation emitted by a fluorophor is anisotropic [84]. Stokes reported in 1852 [1] that quinine appeared to be an isotropic emitter in sulfuric acid. However, quinine sulfate in glycerol containing sulfuric acid exhibits polarization which is a function of both excitation and emission wavelengths [85].

The dichroic ratio of emission D and the emission anisotropy r, indicative of the polarization of a material, can be determined by measuring the four fluorescence fluxes $Y^{P,P}_{\lambda_1,\lambda_2}$, $Y^{S,S}_{\lambda_1,\lambda_2}$, $Y^{S,P}_{\lambda_1,\lambda_2}$, and $Y^{P,S}_{\lambda_1,\lambda_2}$ and substituting these values in the following equations [84]:

$$D = \frac{\left[Y^{S,S}_{\lambda_1,\lambda_2}\right]\left[Y^{P,P}_{\lambda_1,\lambda_2}\right]}{\left[Y^{S,P}_{\lambda_1,\lambda_2}\right]\left[Y^{P,S}_{\lambda_1,\lambda_2}\right]} \tag{8}$$

and

$$r = \frac{D-1}{D+2} \tag{9}$$

where the superscripts and subscripts have the same meaning as in Section IV.

The ranges of values for D and r are:

$$1/2 \leq D \leq 3$$

$$-1/5 \leq r \leq 2/5$$

and for a material that is not polarized and emits isotropically, D = 1 and r = 0.

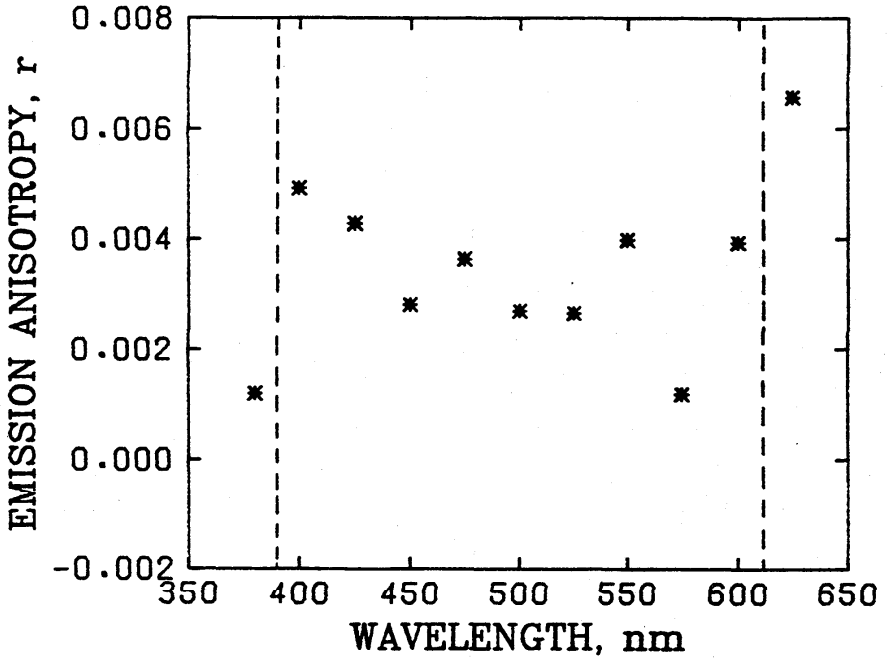
We determined r and D for quinine sulfate in 0.1 mol/L $HClO_4$ at 25 °C using λ_1 = 347.5 nm and a λ_2 range of 360 to 725 nm in 5 nm intervals. The average r and D values obtained by three, time symmetrical determinations are graphed every 25 nm in figure 8a and b. The one percent signal limits are designated by the vertical dashed lines at ∿390 and 615 nm. The averages for all the r and D values obtained between the wavelength range 380 to 630 nm were 0.00354±0.00088 and 1.0107±0.0027, respectively.

Additionally, r and D values were determined as a function of quinine sulfate concentration at λ_1 = 347.5 nm and λ_2 = 460 nm, Table 9.

Table 9. The Dichroic Ratio of Emission D and the Emission Anisotropy r of Quinine Sulfate in 0.1 mol/L $HClO_4$ as a Function of Quinine Sulfate Concentration.

Concentration, ppm	D	r
0.297	1.0167	0.00554
0.513	0.9966	-.00113
1.087	1.0023	.00077
1.121	1.0029	.00087
5.460	1.0023	.00076
10.315	0.9934	-.00221
15.877	1.0026	.00192
	$\bar{D}$ = 1.0024	$\bar{r}$ = .00093

Although care was taken to align the excitation polarizer and analyzer optically so that shifts of excitation and emission radiation were minimized upon rotation of the polarizers, small differences were observed for the average values of D and r in Table 9 and those average values reported over the total emission range for one quinine sulfate concentration (D = 1.0107 and r = 0.00354). These



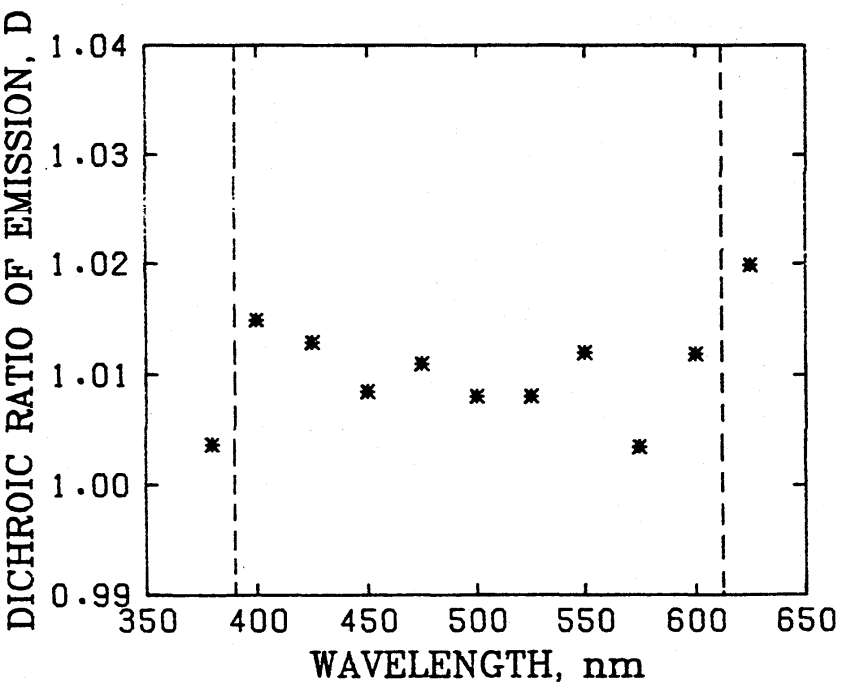


Figure 8. (a,b) Emission anisotropy r (8a) and Dichroic ratio of emission D (8b) as functions of wavelength for quinine sulfate in 0.105 mol/L $HClO_4$.

differences are considered to be due in large part to a small optical misalignment in the excitation polarizer and not isotropic emission. Thus the D and r values determined in these two studies are equivalent and demonstrate the isotropy of the radiation emitted by a solution of quinine sulfate in 0.1 mol/L $HClO_4$.

However, if the values for the emission anisotropy were real and due to a true residual polarization [86], the error introduced in a fluorescence measurement using an instrument with no polarizers, can be calculated by [reference 84, equation 27]:

$$Y^{X,X}_{\lambda_1,\lambda_2}(\alpha) = Y^{S,S}_{\lambda_1,\lambda_2}(\alpha) + Y^{P,S}_{\lambda_1,\lambda_2}(\sigma) + Y^{S,P}_{\lambda_1,\lambda_2}(\alpha) + Y^{P,P}_{\lambda_1,\lambda_2}(\alpha) \quad (10a)$$

$$= \left(Y^{X,U}_{\lambda_1,\lambda_2}\right)_o \left\{1 + r\left[\frac{3\cos^2\alpha - (1 + F + G - 2FG)}{(1 + F)(1 + G)}\right]\right\} \quad (10b)$$

where $\left(Y^{X,X}_{\lambda_1,\lambda_2}\right)$ is the signal recorded without polarizers, $(Y^{X,U}_{\lambda_1,\lambda_2})_o$ would be the signal if the sample emitted isotropically, F is the polarization ratio of the exciting radiation, G is the polarization ratio of the detection system, and α is 90° for right angle viewing.

In the worst case in which measurements are made with no polarizers, F and G $\approx 10^4$, and $\bar{r} \approx 0.002$, then: $(Y) = (Y)_o\ [1 + (.002)(-2)]$ and the error in the measurement is approximately -0.4 percent. Usually, the G and F values are ≈ 0.6 and the error would be on the order of + 0.1 percent which is negligible.

In certification measurements, we wanted to remove the excitation polarizer and allow the analyzer to remain in the 'S' orientation to avoid alignment problems with the excitation polarizer and to avoid the Wood's anomaly caused by the grating in the detection monochromator. The

uncorrected spectra for the standard tungsten lamp (unpolarized) with the analyzer in the S and P positions are given in figure 9. The "P" spectrum shows a "cusp" attributable to the Wood's anomaly of the grating. Since, the anomaly, and thus possible decreased measurement accuracy would be present if the analyzer were absent or in the "P" orientation, it was decided to make all certification measurements with the analyzer in the "S" orientation [52].

In this case [reference 79, equations 25a and b modified as in reference 49], the two signal components are:

$$Y^{P,S}_{\lambda_1,\lambda_2} = 1/2 \left(\frac{1}{1+F}\right) \left[Y^{X,S}_{\lambda_1,\lambda_2}\right]_o (1-r) \qquad (11a)$$

$$Y^{S,S}_{\lambda_1,\lambda_2} = 1/2 \left(\frac{F}{1+F}\right) \left[Y^{X,S}_{\lambda_1,\lambda_1}\right]_o (1+2r) \qquad (11b)$$

where $(Y)_o$ corresponds to the signal unbiased by polarization,

F is the polarization ratio of the exciting radiation, and the remaining symbols are as previously designated.

The total signal observed is:

$$Y^{X,S}_{\lambda_1,\lambda_2} = Y^{P,S}_{\lambda_1,\lambda_2} + Y^{S,S}_{\lambda_1,\lambda_2} \qquad (12a)$$

$$= \left[\left(Y^{X,S}_{\lambda_1,\lambda_2}\right)_o\right](1 + r\,\frac{2F-1}{FH}) \qquad (12b)$$

where $\left(Y^{X,S}_{\lambda_1,\lambda_2}\right)_o = 1/3(F+1)\left[(Y^{S,S}_{\lambda_1,\lambda_2}/F) + 2Y^{P,S}_{\lambda_1,\lambda_2}\right]$. (12c)

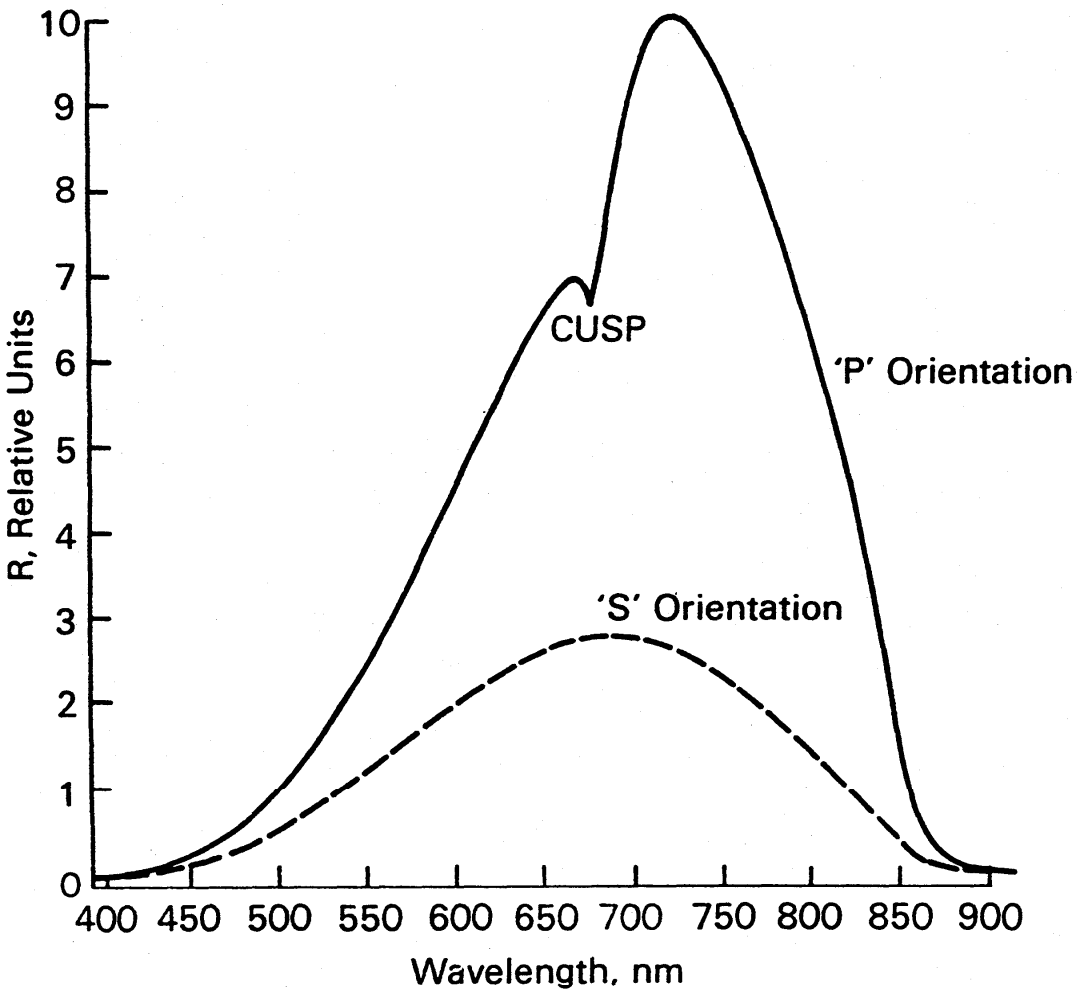


Figure 9. The sensitivity of the detection system to radiation emitted by the calibrated tungsten ribbon lamp that is parallel (P orientation) and perpendicular (S orientation) to the grating grooves in the detection monochromator.

The relative error introduced by removal of the excitation polarizer is [49]:

$$\frac{Y^{X,S}_{\lambda_1,\lambda_2} - (Y^{X,S}_{\lambda_1,\lambda_1})_o}{(Y^{X,S}_{\lambda_1,\lambda_2})_o} = \frac{\Delta Y^{X,S}_{\lambda_1,\lambda_2}}{(Y^{X,S}_{\lambda_1,\lambda_2})_o} = r\left(\frac{2F-1}{F+1}\right). \quad (12d)$$

The average value of $(Y^{X,S}_{347.5,\lambda_2})/Y^{X,S}_{347.5,\lambda_2})_o$, where 380 nm $\leq \lambda_2 \leq$ 615 nm (1% signal limits) is 0.9998±0.006 which would, in the worst case, introduce a negligible error of 0.06 percent due to residual polarization into the certification measurements. Thus the certification data for quinine sulfate dihydrate in 0.105 mol/L $HClO_4$ were obtained without an excitation polarizer and the analyzer in the "S" orientation.

F. Emission Spectrum as a Function of Excitation Wavelength (λ_1)

The luminescence spectrum [87] of quinine sulfate shifts to longer wavelengths if the excitation wavelength is on the "red edge" (greater than 360 nm) of the first absorbance peak [66,85,88-90]. This anomalous effect also has been observed with various fluorescent materials that have similar (quinoline, 6-methoxyquinoline, 6-ethoxyquinoline) and dissimilar (2-aminopurine, luminol) molecular structures compared to quinine sulfate.

The red shift does not appear to involve a change in the emission shape [85,90] nor photon yields (Section I-G), but does give a fairly significant change in the location of the emission peak maximum going from an emission maximum of 453 nm using excitation wavelengths from 230 to 360 nm to an emission maximum of 470 nm using an excitation wavelength of 440 nm [88]. We found that changing the excitation wavelength from 347.5 nm to 366 nm shifts the emission maximum 1.8 nm (451.5 nm to 453.1 nm) and errors of up to 20 percent in fluorescence measurements on the "blue edge" of the

emission peak were observed as shown by the ratio $(Y^{X,S}_{347.5,\lambda_2})/(Y^{X,S}_{366,\lambda_2})$ plotted as a function of λ_2, Figure 10.

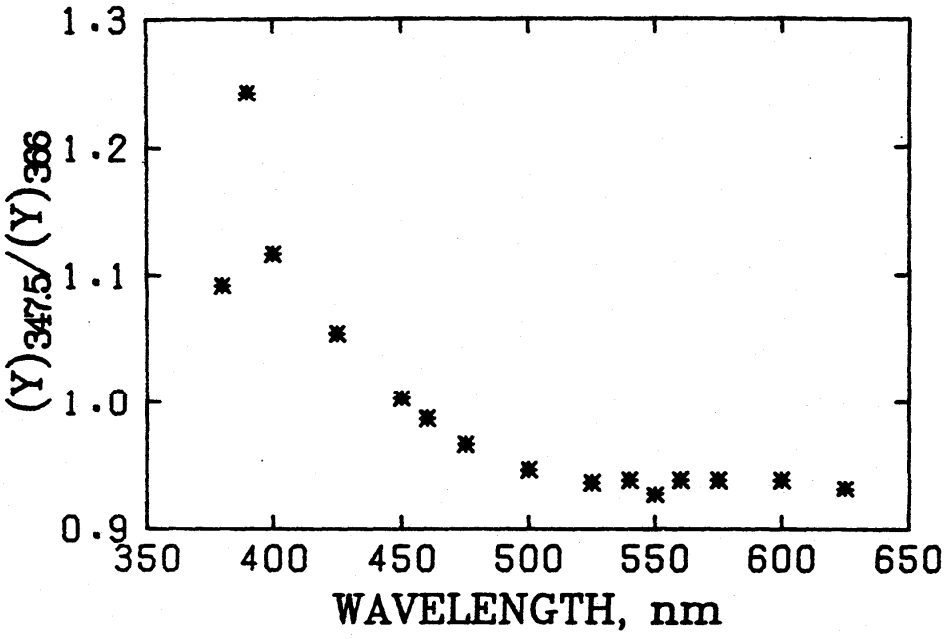


Figure 10. The ratio of the uncorrected emission spectra of quinine sulfate in 0.105 mol/L $HClO_4$ using exciting radiation at 347.5 and 366 nm to demonstrate the 'red edge' shift.

This anomalous effect has been attributed to emissions from two excited singlets [85], a rotatable auxochrome-solvent effect [88], and emission from several molecular configurations differing in solvent orientation [90]. Although the reason for the shift has not been unambiguously established, care must be exercised in using literature values for the emission spectrum since they obviously depend on excitation wavelength.

The certification values of the emission spectrum given in this report (Section VIII) are for 347.5 nm excitation and

the emission spectrum should be determined using this excitation wavelength.

G. Photon Yields

1. Absolute and Relative Values

The photon yield values for quinine sulfate of 0.508 in 0.05 mol/L and 0.546 in 0.5 mol/L H_2SO_4 first measured by Melhuish [7,74] and later by Dawson and Windsor [80] have been challenged [5,6,8,85,91,92]; however, the values still appear to be the best determined photon yields and those most used as relative standard values [25,26,71,75]. An intercomparative study using quinine sulfate as the basic reference standard and fluorescein, pyrene, and rhodamine B as the comparison materials was reported [33] and some of the data are summarized in Table 10. These data show that using the photon yield of 0.508 for quinine in 0.05 mol/L H_2SO_4 results in good agreement between the values measured for fluorescein, pyrene, and rhodamine B and those average values reported in the literature.

Table 10. Comparative Photon Yields Using Quinine Sulfate as the Reference Material.

Compound	Solvent	Q	Q (Lit.)
QS	0.05 mol/L H_2SO_4	---[a]	0.508[7]
Fluorescein	0.1 mol/L NaOH	0.87±0.03	0.88[b] [17]
Rhodamine B	Ethanol	0.72±0.02	0.69[11], 0.71[26]
Pyrene	Toluene	0.57±0.04	0.60[80]

[a] Standard, 0.508 as photon yield.

[b] Average of 16 values (see reference 33).

Other intercomparative studies have been performed using quinine sulfate as the standard with a photon yield of 0.508 with excellent results [75,78b,80], all of which support the values reported by Melhuish [7,74] and Dawson and Windsor [80].

2. Photon Yield as a Function of Excitation Wavelength

It was reported that the photon yield of quinine sulfate in sulfuric acid is dependent on excitation wavelength [80,85,91]; however, careful measurements have shown that the photon yield of quinine sulfate in 0.05 mol/L H_2SO_4 [25,26, 33,71,92] or 0.5 mol/L H_2SO_4 [21] is constant within experimental error (±5%). Our results [33] are summarized in figure 7 and show that the relative photon yield is essentially constant over the 224-390 nm excitation wavelength range which agrees with the summary of the past data [26].

Thus, although the emission spectrum of quinine sulfate shifts to longer wavelengths upon excitation at wavelengths longer than 360 nm, the quantum yield remains constant, an important fact when considering quinine as a standard.

3. Photon Yield as á Function of Acid Concentration

It was reported that the photon yield of quinine sulfate changes with varying sulfuric acid strength [24,80]; an observation that has since been verified [33,75]. Additionally, Chen reported that the fluorescence lifetime was also dependent on the sulfuric acid concentration [75].

In a recent study [93] we found that although the photon yield and fluorescence lifetime were functions of the sulfuric acid concentration attributable to quenching by the sulfate ion, use of an acid with a "non-quenching" anion such as perchloric, phosphoric, or nitric resulted in a photon yield and lifetime that were essentially independent of acid concentration, Table 11. Increasing the $HClO_4$ concentration to 10 mol/L may result in a slight increase in Q (0.59 to 0.62), but over the acid concentrations that are commonly used

(0.1 to 1.0 mol/L), Q is constant and does not exhibit a 10 percent increase as is observed over the same acid concentration range in H_2SO_4.

Table 11. Fluorescence Photon Yields Q and Lifetimes τ of Quinine Sulfate in Various Acids [88].

(H^+)	H_2SO_4		$HClO_4$		HNO_3		H_3PO_4	
mol/L	Q	τ,ns	Q	τ,ns	Q	τ,ns	Q	τ,ns
.01	0.52	20.1	--	--	--	--	--	--
.1	0.51	19.2	0.59	21.6	0.61	22.0	0.59	22.1
1.0	0.55	20.2	0.59	21.2	0.61	22.4	0.58	22.0
3.5	0.57	20.5	0.61	21.7	--	--	--	--
5.0	0.58	20.8	0.61	21.7	--	--	--	--
10.0	0.60	21.9	0.62	21.8	0.60	22.1	0.60	21.8

Thus, perchloric acid was chosen over nitric and phosphoric acids as the solvent for use in certification measurements because: (a) it forms one anion in aqueous solution as opposed to phosphoric acid which forms two; (b) phosphoric acid is somewhat viscous and difficult to work with; (c) the large absorbance tail due to the nitrate ion where quinine absorbs necessitates large corrections in absorbance measurements when relative photon yields are determined; and (d) nitric acid is a fairly strong oxidizing agent.

H. Emission Spectrum as a Function of Acid

In these discussions, it usually is assumed that changing from H_2SO_4 to $HClO_4$ does not affect the envelope of the emission spectrum. To test this, measurements of the emission spectra were made in the usual time symmetrical mode using the same concentration of quinine sulfate ($\sim$1 ppm) in 0.1 mol/L $HClO_4$ and 0.5 mol/L H_2SO_4 under exactly the same instrumental conditions. A small difference in the maxima for both curves

in the uncorrected spectra $Y^{X,S}_{347.5,\lambda_2}$ was observed: 451.5 nm for the 0.1 mol/L $HClO_4$ and 452.7 nm for the 0.5 mol/L H_2SO_4. Additionally, plotting the ratio of the normalized uncorrected spectra, $(Y^{X,S}_{347.5,\lambda_2})$ $HClO_4/(Y^{X,S}_{347.5,\lambda_2})$ H_2SO_4 shows differences from approximately +3 percent towards the blue region to -4 percent towards the red region, figure 11. Thus the emission spectrum of quinine in the two acids is different. This observation is not important when measurements of photon yield are made relative to a standard since the area under the emission curves are used; however, when using the emission spectrum to check instrumental operation, correct instruments, or determine the emission spectrum of other materials relative to the standard, the emission spectrum of the standard is important and care should be exercised in the choice of the acid as solvent and in data comparisons to reported spectra.

I. Emission Spectrum as a Function of QS Concentration

Two phenomena that can affect fluorescence spectra are quenching and inner filter effects. Luminescence quenching is a fundamental effect that diverts the absorbed radiant energy such that the photon yield is less than one. In general, it does not affect the shape of the emission spectrum unless the fluorescence lifetime approaches picoseconds or unless a complex is formed with the quencher that emits over a different wavelength region than the uncomplexed fluorophor.

Inner filter effects, conversely, are instrumental and experimental conditions that do affect the fluorescence spectra or measured flux in three ways: (a) the exciting radiation can be absorbed; (b) the emitted radiation can be absorbed; and (c) the absorbed radiation in (b) can be emitted at different or the same wavelengths.

Assuming there are no impurities at the 0.01 percent level (Section VI-B) and the solvent does not absorb, then the concern is mainly with the effect of increasing solute concentration. In general, absorbance of the excitation

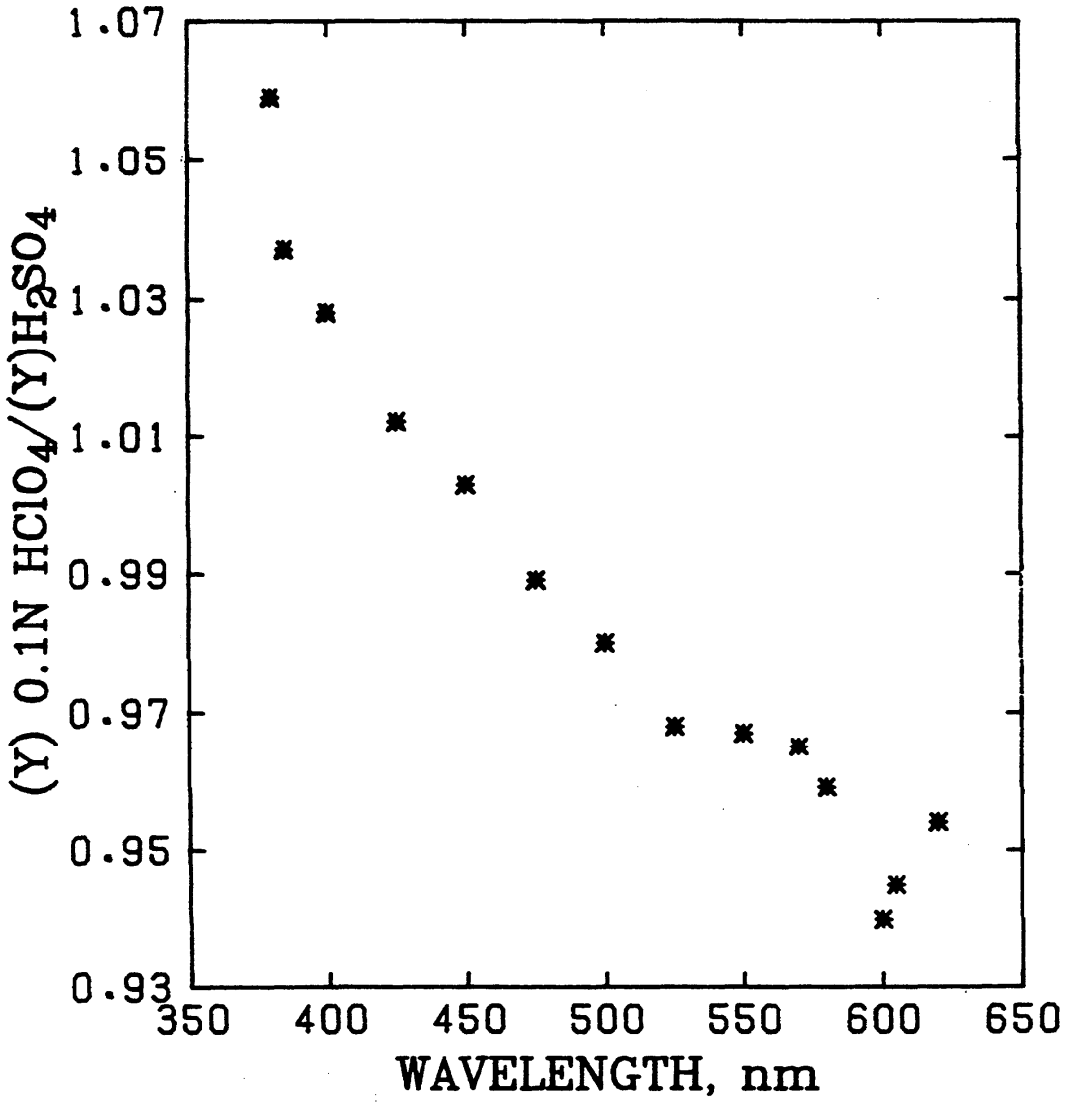


Figure 11. The ratio of the uncorrected emission spectra of quinine sulfate in 0.1 mol/L $HClO_4$ and 0.5 mol/L H_2SO_4 to demonstrate the effect of acid on the emission spectrum.

radiation by the solute (or impurities or solvents) results in errors in the determination of fluorescence flux, excitation spectra, and photon yields, since, as the absorbance of the radiant flux I_o increases, the dilute solution approximation, equation 4, no longer applies. This effect is depicted in figure 12 as a plot of the compensated fluorescence reading ($Y^{X,S}_{347.5,450}$) versus quinine sulfate concentration. Deviations from linearity of greater than 1 percent are not observed until the concentration of quinine sulfate is ∿2-3 ppm. A least squares fit of this data using $(\frac{1}{\sigma})^2$ as weighting factors produced a straight line in which the standard predicted values differed from the experimental values by an average of ∿0.8 percent over the 1 to 0.001 ppm range. The problem of excitation radiation absorption by the solute has been discussed previously and the following equation was proposed to correct this effect [94,95]:

$$F_o/F = \frac{2.303A(d_2-d_1)}{10^{-Ad_1} - 10^{-Ad_2}} \qquad (13)$$

where

F_o = true Y (no inner filter effect),
F = measured Y,
A = absorbance in the solution at λ_1, and
d_1, d_2 = the distances the two sides of the emission monochromator slit images are from the cell face.

The absorbance of a 1 ppm solution of $QS \cdot 2H_2O$ at λ_1 = 347.5 nm is 0.0138 (WA = 13.834×10^{-3} $g_{solv}(g_{sol})^{-1}cm^{-1}$, Table 6). Assuming that the emission monochromator slits are 2 mm and a 1 cm cell is used, $d_1 = 0.4$, and $d_2 = 0.6$. Substitution of these values into equation 13 yields a value for F_o/F of 1.016 which shows that the observed Y should be ∿1.6 percent low as compared to the emitted radiation if no effect were present.

The data for the 5, 10, and 100 ppm solutions were corrected according to equation 13 and these values are plotted

in figure 12 as hourglasses that now fall on the linear portion of the curve, thus demonstrating the usefulness of equation 13.

Re-absorption of the emitted radiation by the solute in optically dense solutions [80,96-99], plane parallel luminophors [100], and by other species [94] has been discussed and correction factors have been derived in some of these papers; however, in the case of solute re-absorption, the emission of secondary fluorescence occurs which also distorts the emission spectrum in the red region as well as in the overlap region. These effects are demonstrated by the uncorrected emission spectra for 1 and 10,000 ppm solutions of quinine sulfate, figure 13. A large effect due mainly to re-absorption with some compensation of the effect by re-emission resulting in decreased 'Y' values is observed in the overlap region (350-425 nm), while a smaller effect due to re-emission resulting in increased 'Y' values is observed at longer wavelengths. These and additional data are presented as the ratio of the spectra for X ppm $QS \cdot 2H_2O$ to that for 1 ppm $QS \cdot 2H_2O$ in figure 14 from which the extent of the effect is readily apparent.

Equation 13 can be used as a first approximation to determine the absorption of the emitted light in the overlap region. The one percent fluorescence signal level (relative to the maximum for $(Y^{X,S}_{347.5,\lambda_2})$ occurs at $\lambda_2 \simeq 390$ nm. The absorbance at this wavelength is $\sim 1/10$ that at 347.5 nm, and considering the crosshatched illuminated volume element in figure 15 as a source and the absorbing depth as $(d_1 + d_2)/2$, then $A \sim 0.00069$. Substitution of these values into equation 13 gives an F_o/F ratio = 1.00098 or an error of approximately 0.1 percent which, at the 1 percent signal level, is considered negligible compared to the imprecision and errors in the overall measurement process.

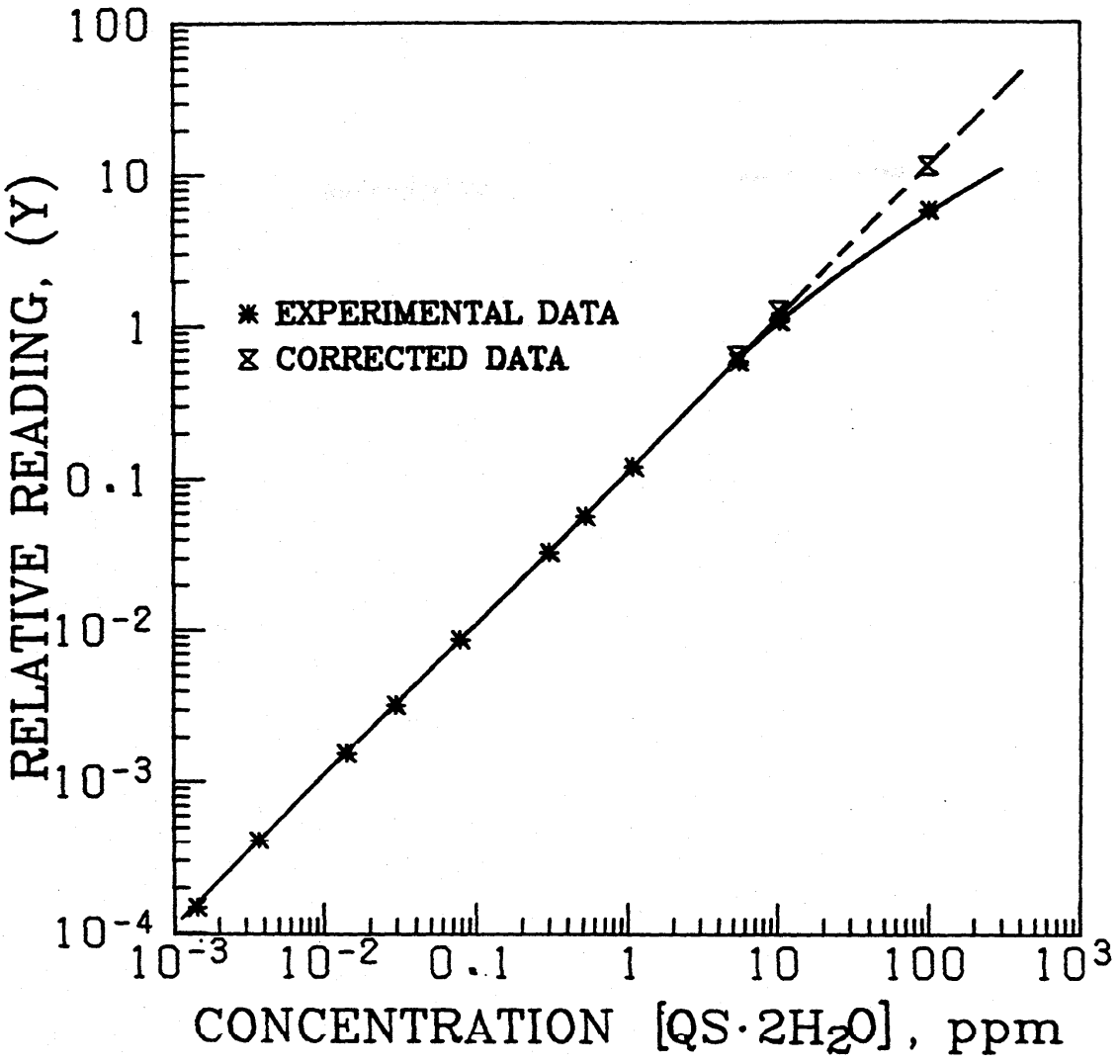


Figure 12. Relative fluorescence flux * and relative fluorescence flux corrected for inner filter effects (absorption of exciting radiation), $\bar{X}$ as a function of quinine concentration.

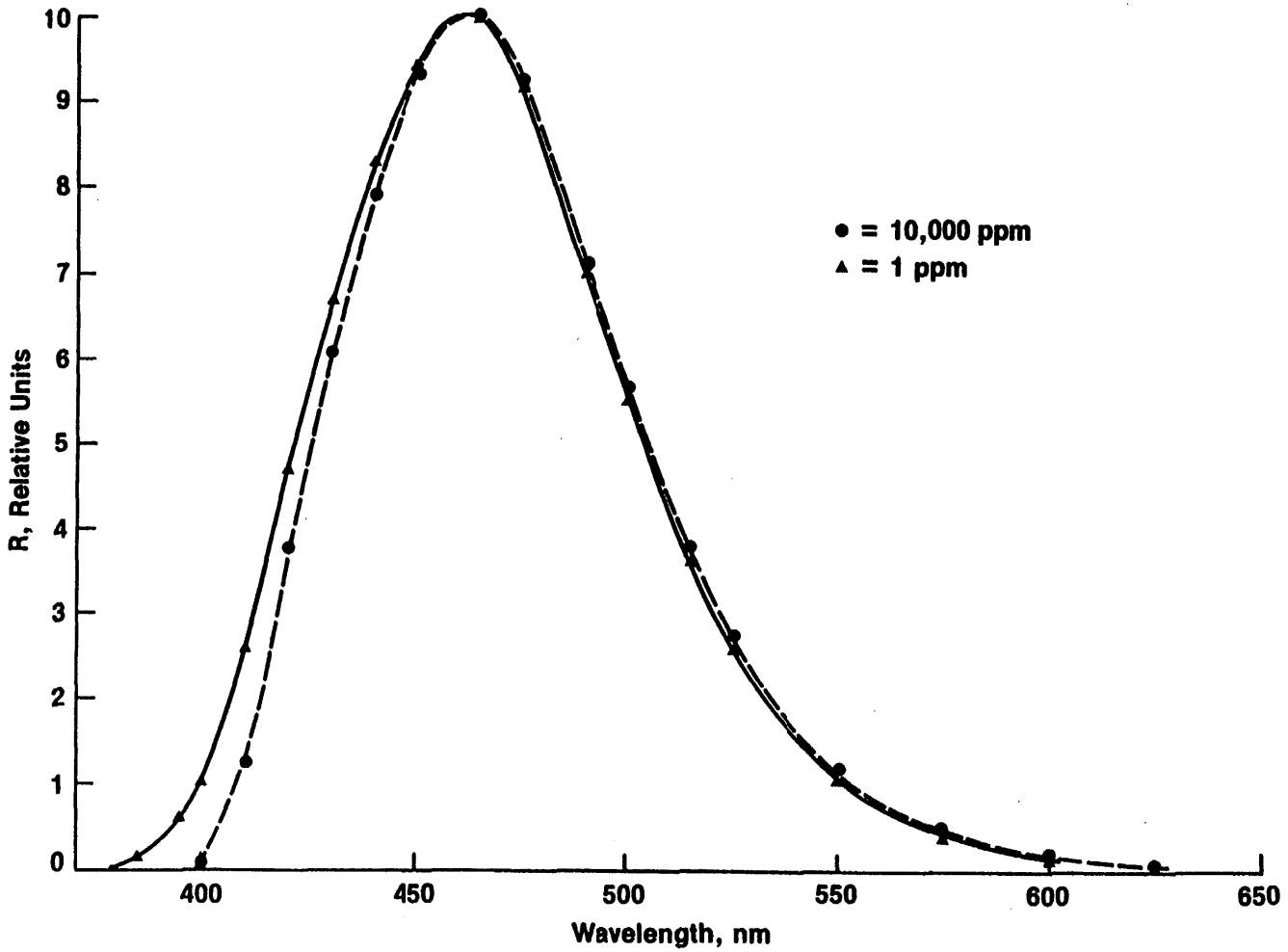


Figure 13. Uncorrected emission spectra for 1.3×10^{-6} mol/L (1 ppm) and 1.3×10^{-2} mol/L (10,000 ppm) quinine sulfate in 0.105 mol/L $HClO_4$.

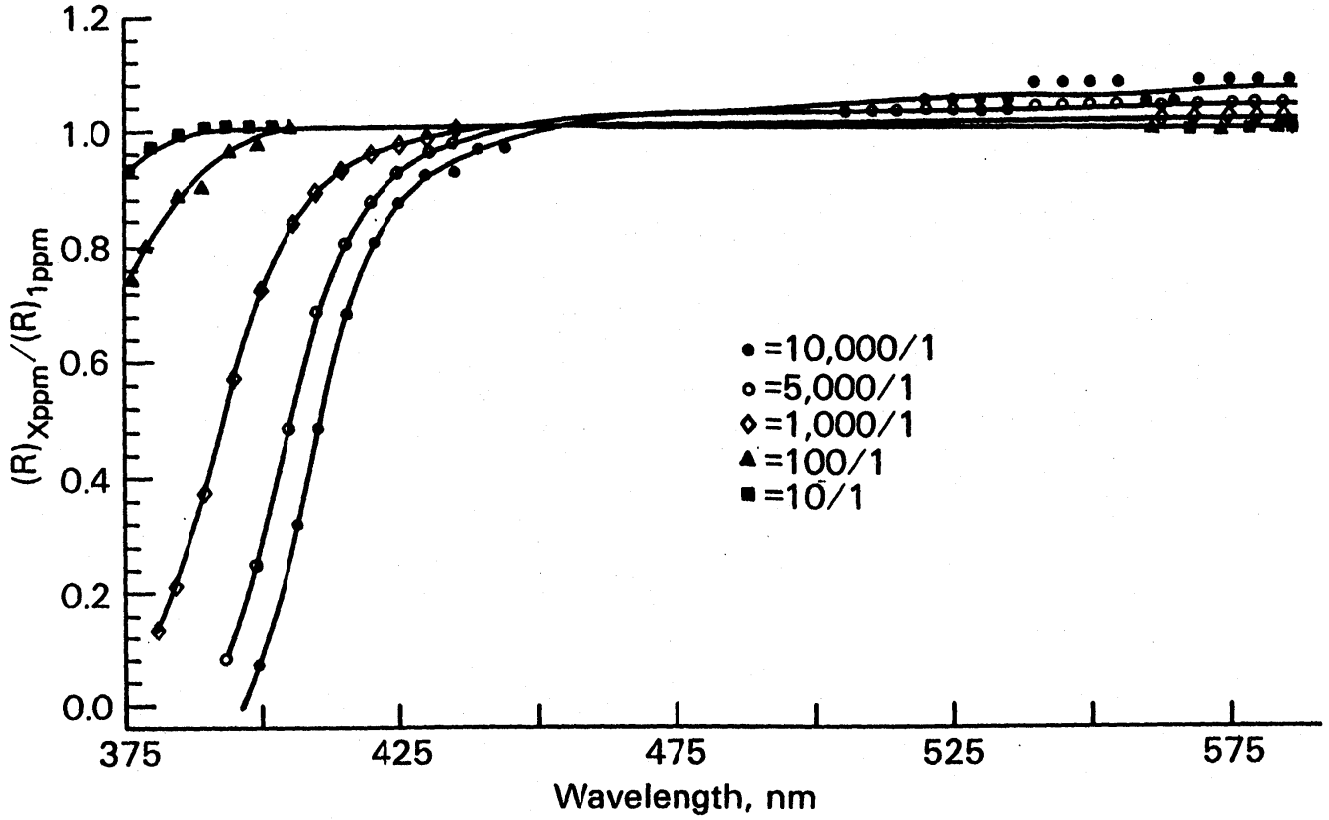


Figure 14. Ratio of the emission spectral data for various concentrations of quinine sulfate (● = 1.3×10^{-2} mol/L; ○ = 6.5×10^{-3} mol/L; ◇ = 1.3×10^{-3} mol/L; ▲ = 1.3×10^{-4} mol/L; ■ = 1.3×10^{-5} mol/L) to the emission data obtained for 1.3×10^{-6} mol/L quinine sulfate as a function of wavelength. Solvent = 0.105 mol/L $HClO_4$.

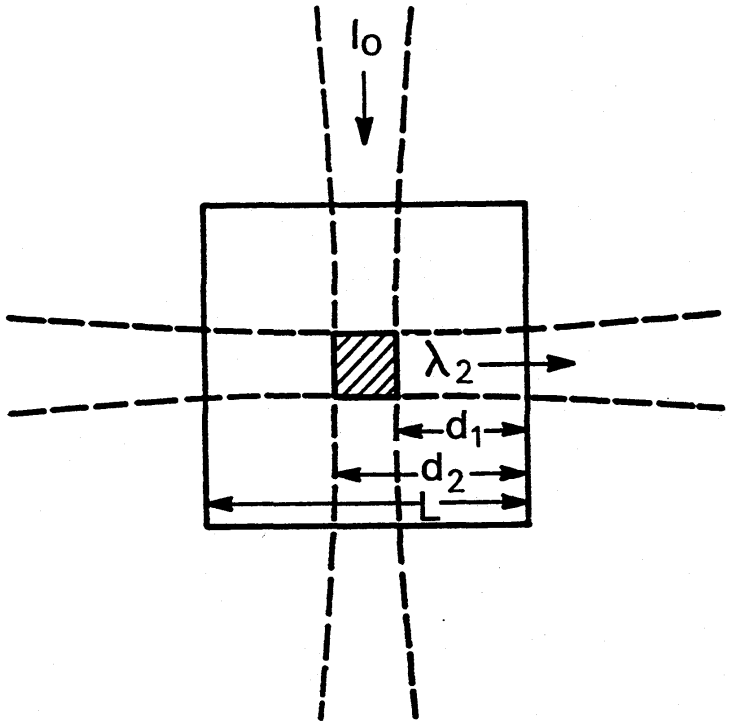


Figure 15. The geometrical representation of the sample cell and illuminated volume element in considering the distortion of the emission spectrum by absorption of the emitted radiation followed by re-emission.

We therefore recommend that emission spectra be obtained for quinine sulfate solutions that have concentrations of 1.28×10^{-6} mol/L (1 ppm) or less.

J. Emission Spectrum as a Function of Temperature

Melhuish reported [7] that a 5×10^{-3} mol/L solution of quinine bisulfate in 0.5 mol/L H_2SO_4 viewed in a front surface mode exhibited a change in "fluorescence intensity" of -0.2 to -0.3 percent per degree in the +10 to +40 °C range. This value represents an average change per degree for the total emission spectrum and not the variation as a function of wavelength since in that case a rhodamine B quantum counter was used to measure the emitted radiation.

The uncorrected emission spectrum of a 1.28×10^{-6} mol/L (~1 ppm) in 0.1 mol/L $HClO_4$ in the side view mode was measured at 16.0, 23.8, 27.8, and 34.6 °C. Plots of the compensated fluorescence signal (Y) at selected wavelengths are given in figure 16 and the $(Y)_{x°C}/(Y)_{23.8\ °C}$ ratios are given in figure 17. The average change, (ΔY) per degree at 25 nm intervals, is summarized in Table 12. The average percent change per degree using the 375-600 nm data from Table 12 (useful wavelength region for rhodamine B - acriflavine quantum counter 220-600 nm) is -0.21 percent/°C, in good agreement with the previously reported value of -0.2 to -0.3 percent [7]. These calculations were made assuming that the change at individual wavelengths is linear as a function of temperature. This assumption generally holds from +20 to +30 °C over the wavelengths of interest, figure 16.

Table 12. Average Percent Change Per Degree Celsius for the Uncorrected Values $(\Delta Y)_{T=23.8\ °C}$ at 25 nm Wavelength Intervals.

λ,nm	(ΔY)/°C
375	+.40
400	-.40
425	-.48
450	-.62
475	-.33
500	-.25
525	-.19
550	-.14
575	-.05
600	-.01
625	-.02

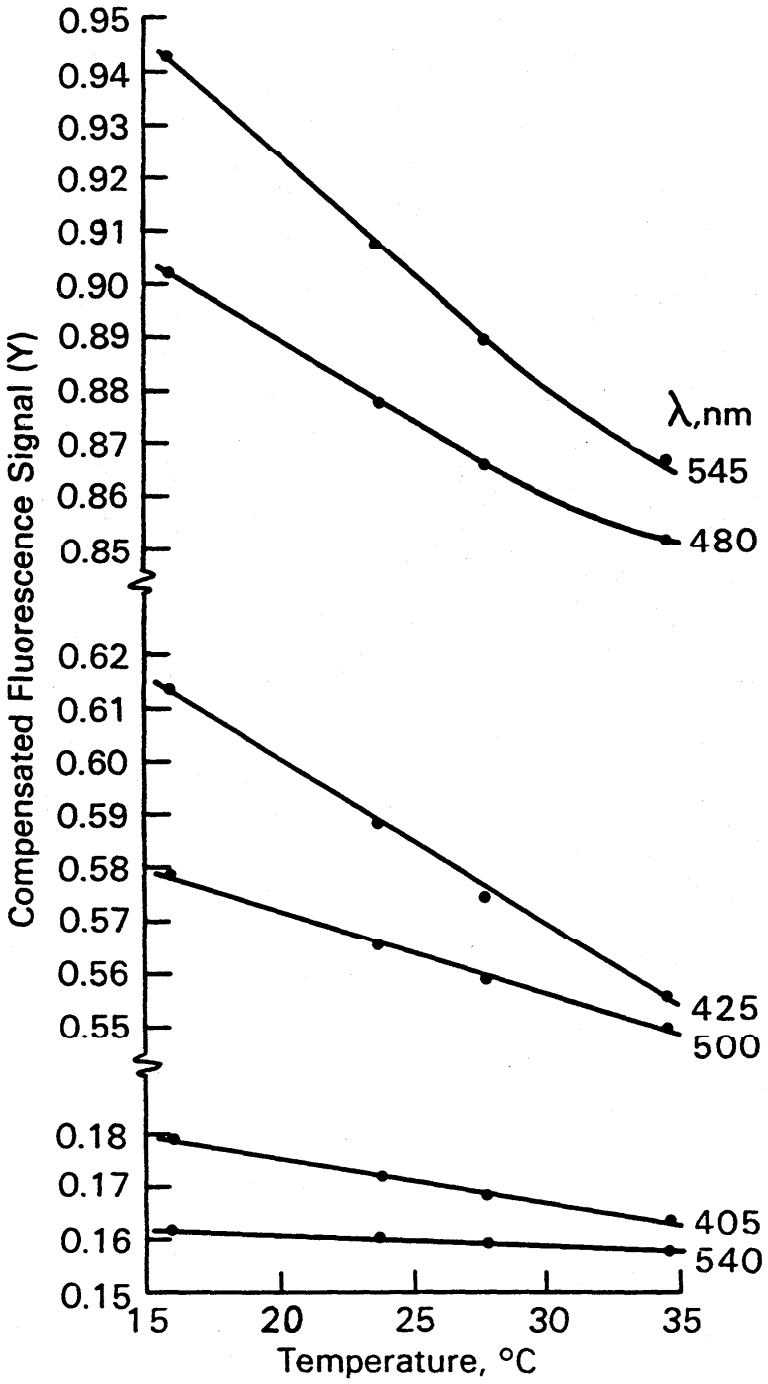


Figure 16. The compensated fluorescence signal (Y) at selected wavelengths as a function of temperature.

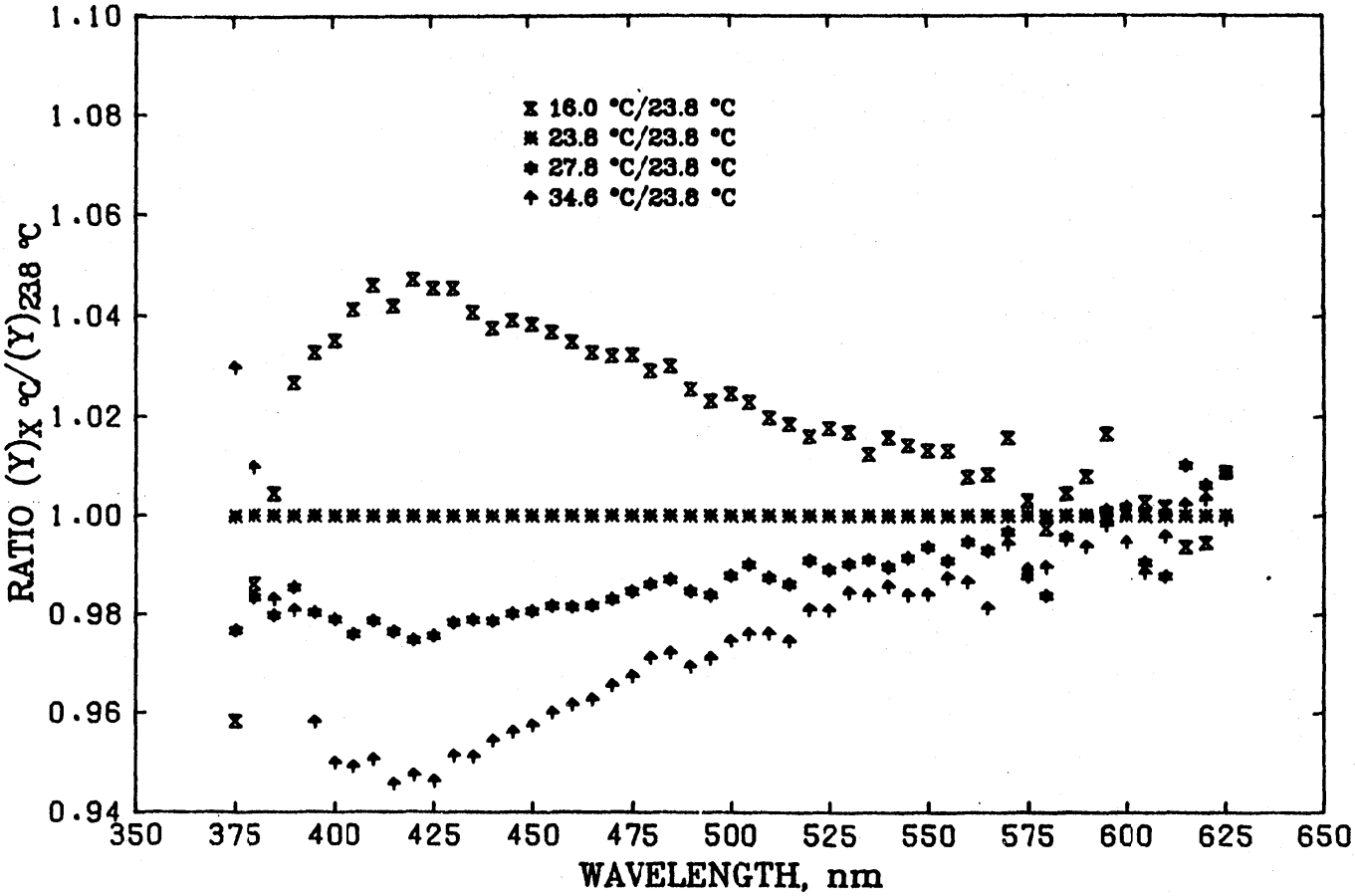


Figure 17. The values of the ratios of the compensated fluorescence signals (Y) at various temperatures to those at 23.8 °C as a function of wavelength. $\overline{X}$ = 16.0 °C; * = 27.8 °C; ↑ = 34.6 °C.

We are concerned, however, with the temperature effect on the normalized spectrum measured at 25 °C and not changes in absolute values. The spectra at the four temperatures were normalized to the (Y) value at their peak maximum, Table 13, and ratios of the normalized readings $(Y)_T/(Y)_{T=23.8\ °C}$ were plotted as a function of wavelength, figure 18. The solid lines represent the best fit to these data using 7th order polynomials [56]. Other fits using lower order polynomials gave plots of the standardized residuals which showed cyclical variations as a function of wavelength, figure 19a, while the 7th order polynomial gave random standardized residuals, figure 19b, indicating a reasonable fit.

Table 13. Emission Peak Maxima and $(Y)_{X\ °C}$ Values for Quinine Sulfate in 0.1 mol/L $HClO_4$ at Various Temperatures.

Temperature °C	Emission Maximum λ, nm	$(Y^{X,S}_{347.5,\lambda_2})$
16.0	461.4	1.0683
23.8	461.8	1.0330
27.8	461.9	1.0140
34.6	462.1	0.9940

It is apparent that the ratios and thus the percent error observed is a function of wavelength as well as temperature. This error is experimentally determined and incorporates various parameters that also change as a function of temperature, i.e., solvent refractive index, absorbance, and solvent expansion with concomitant decrease in solute concentration. With normalization, the last contribution is eliminated.

Although as demonstrated by figure 16 the plots of the changes in the absolute values as a function of wavelength and temperature are essentially linear, normalizations of the emission spectra to peak maxima and 23.8 °C magnify small

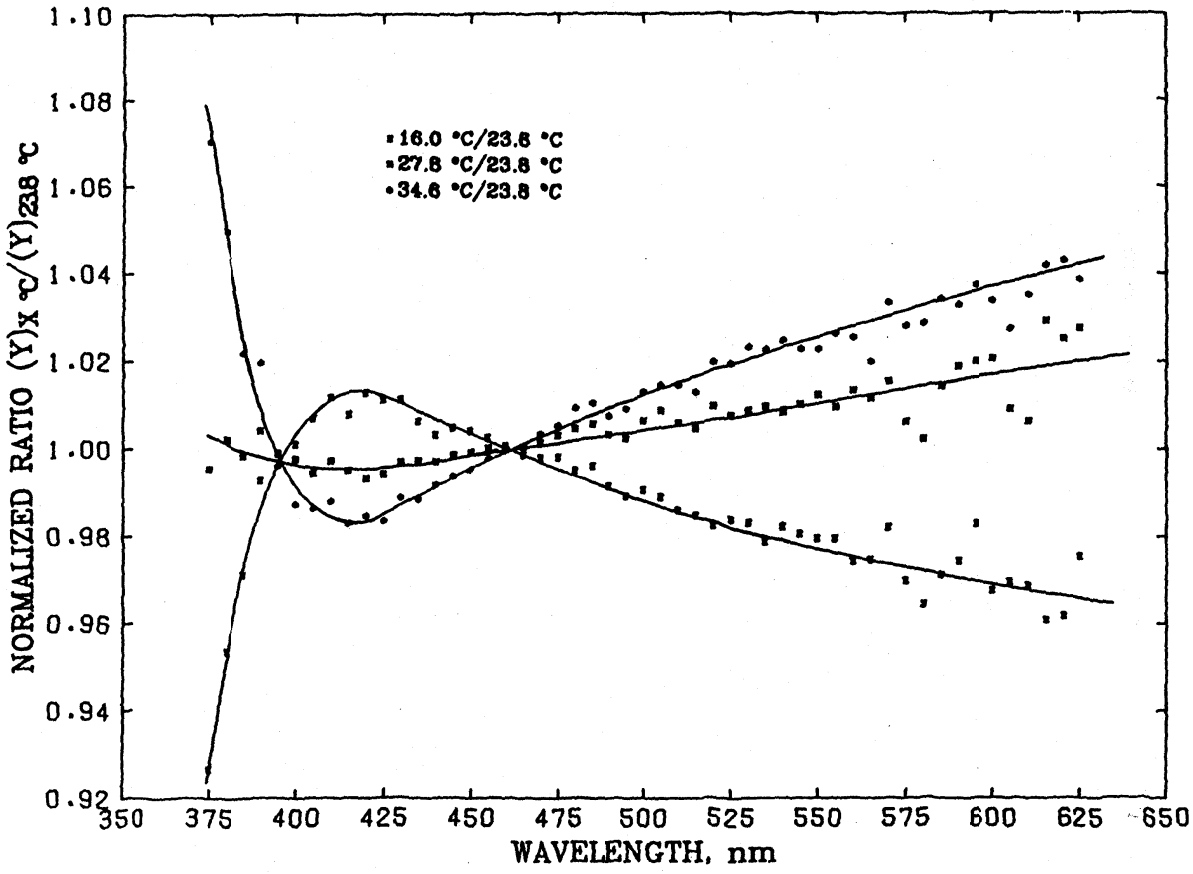


Figure 18. The values of the ratios of the normalized compensated fluorescence signals (Y) at various temperatures to those at 23.8 °C as a function of wavelength. The solid lines are the best fit through the points.

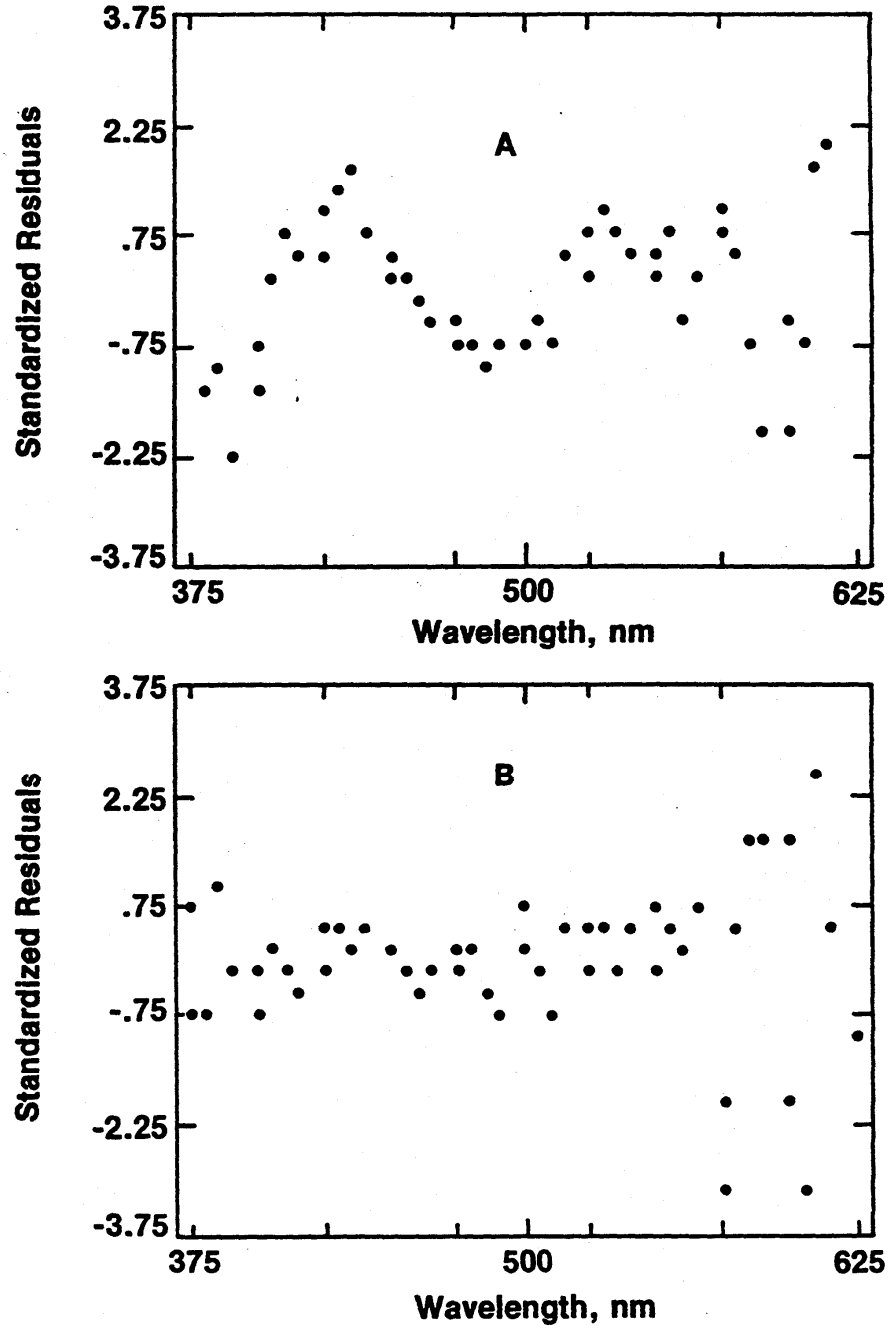


Figure 19. (a,b) Standardized residuals for a fifth order polynomial fit (19a) and a seventh order polynomial fit (19b) for the ratios of the normalized compensated fluorescence signals (Y) at various wavelengths.

deviations from linearity and plots of the normalized ratios $\{[(Y)_T/(Y)_{T=23.8}]-1\}$ versus temperature at selected wavelengths produce figures 20a and b that demonstrate this magnification.

Since a 10-15 percent error in linearity produces a small error in a small correction, linearity can be assumed between 20 and 30 °C and the percent change per degree Celsius $[(1/Y_T)(\Delta Y_T/\Delta T)]_{T=25\ °C}$ can be graphically approximated by obtaining the slope at 25 °C for each wavelength depicted in figures 20a and b in the following way:

$$\text{Let } \left[\frac{Y_T}{Y_{23.8}} - 1\right] = V_T \text{ and graphically determine}$$

$V_{26.2}$ and V_{25} for each wavelength. Thus

$$V_{26.2} = \left[\frac{Y_{26.2}}{Y_{23.8}} - 1\right] = \left[\frac{Y_{26.2} - Y_{23.8}}{Y_{23.8}}\right] = \frac{\Delta Y_T}{Y_{23.8}} \ . \qquad (14a)$$

Adding 1 to V_{25} gives

$$V_{25} + 1 = \left[\frac{Y_{25}}{Y_{23.8}} + 1\right] = \frac{Y_{26.2}}{Y_{23.8}} \ . \qquad (14b)$$

Dividing (14a) by (14b) and dividing by 2.4(ΔT), we find that

$$\left[\left(\frac{1}{Y_T}\right)\left(\frac{\Delta Y_T}{\Delta T}\right)\right]_{T=25.0} = \left[\left(\frac{1}{2.4}\right)\left(\frac{V_{26.2}}{V_{25}+1}\right)\right] \ . \qquad (14c)$$

Values, calculated using eq. 14c at selected wavelengths, are summarized in Table 14 and are presented graphically in figure 21 and represent the percent error per degree Celsius that would be observed for the normalized emission spectrum of QS in 0.1 mol/L $HClO_4$ as a function of wavelength over the temperature range designated by 25±5 °C.

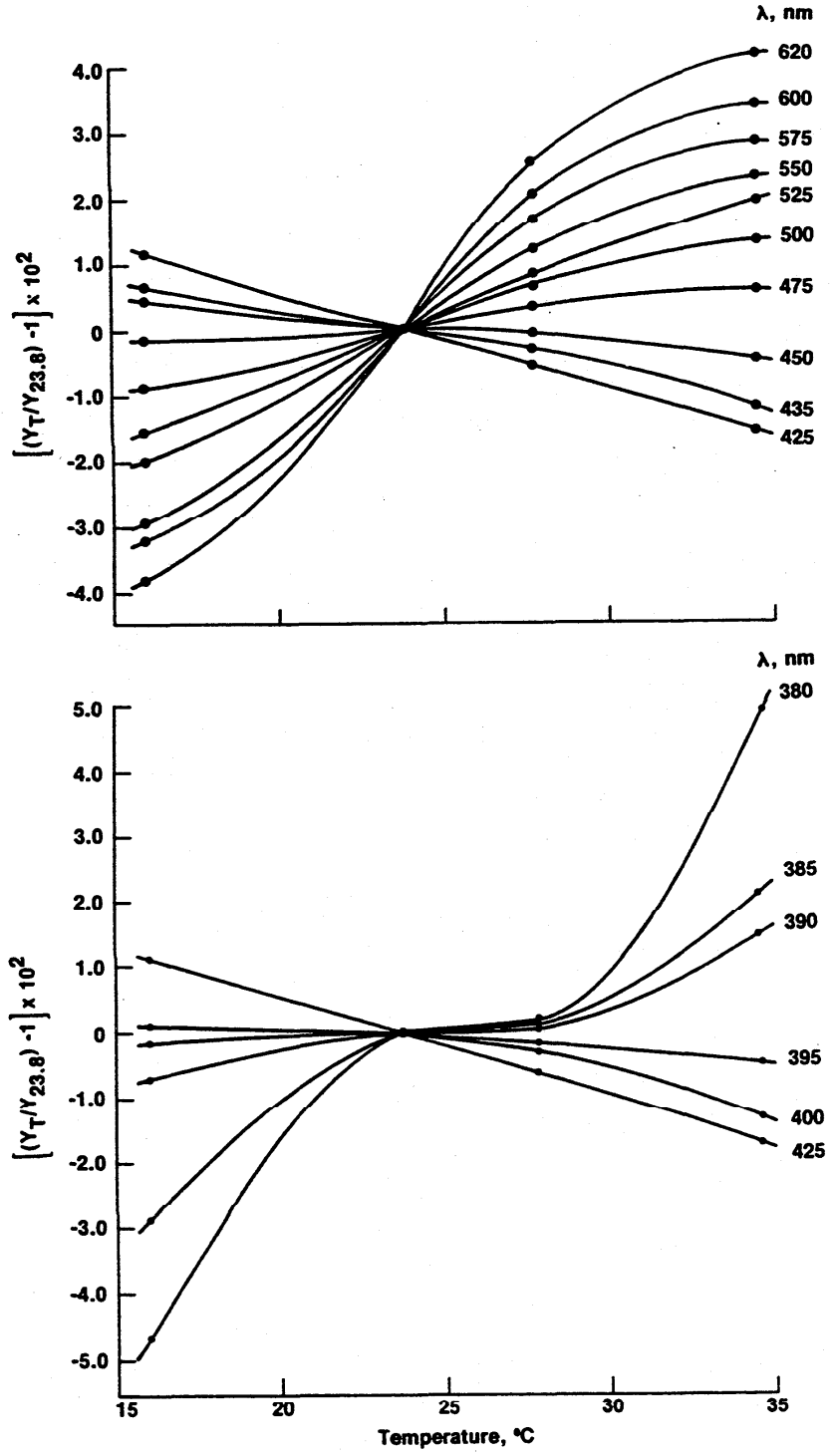


Figure 20. (a,b) The values of $\{[(Y)_T/(Y)_{23.8}]-1\}10^2$ at selected wavelengths as a function of temperature.

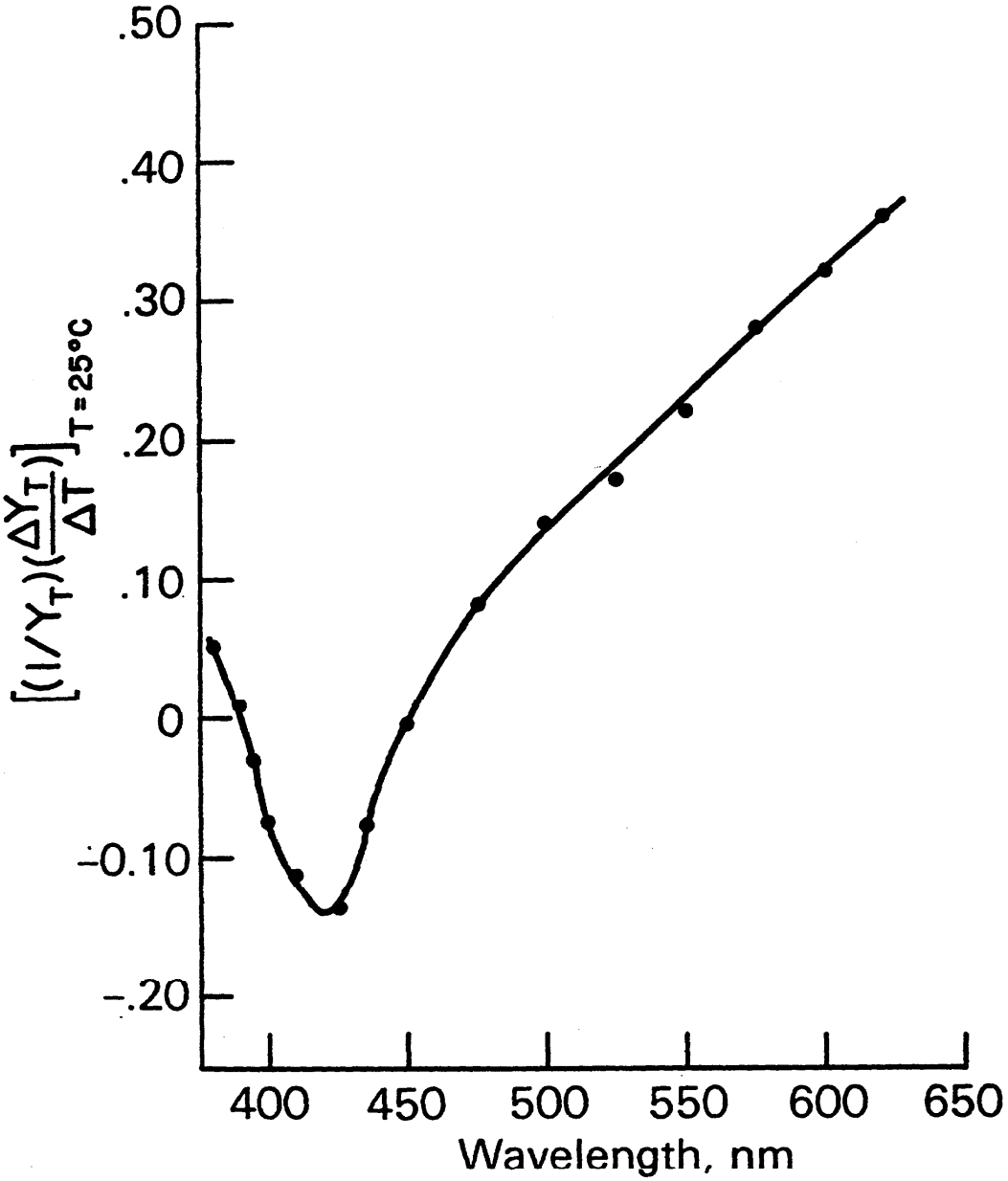


Figure 21. The average percent error per °C observed in the emission spectrum of quinine sulfate over the temperature range 25±5 °C.

Table 14. The Percent Change per Degree Celsius for the Emission Spectrum of Quinine Sulfate in 0.1 mol/L $HClO_4$ at 25 °C at Selected Wavelengths.

λ	$\left[\left(\frac{1}{Y_T}\right)\left(\frac{\Delta Y_T}{\Delta T}\right)\right]_{T=25\ °C}$	λ	$\left[\left(\frac{1}{Y_T}\right)\left(\frac{\Delta Y_T}{\Delta T}\right)\right]_{T=25\ °C}$
380	+.05	450	-.01
385	+.03	475	+.08
390	+.01	500	+.14
395	-.03	525	+.17
400	-.07	550	+.22
415	-.11	575	+.28
425	-.14	600	+.32
435	-.08	620	+.36

VII. Corrections for Instrumental and Experimental Parameters

A. Photomultiplier Tube Linearity

The measurement of the non-linearity of the photomultiplier tube response caused by a dependence of the photomultiplier gain on the incident radiative flux has been reviewed [47,101,102]. The double aperture device and "cascade" measuring procedure was used to determine the non-linearity of the total detection system of the reference spectroradiometer. The photocurrent measured with "A" open plus the photocurrent measured with "B" open should equal the photocurrent measured with "A and B" open (see Section V-C3).

$$\left[(Y^S_{\lambda_1})_A\right] + \left[(Y^S_{\lambda_1})_B\right] = \left[(Y^S_{\lambda_1})_A + (Y^S_{\lambda_1})_B\right] . \qquad (15)$$

The differences obtained between these measurements defines the non-linearity of the detection system, and for amplifier gains of 10^6 and 10^7 V/A, the following equations represent the relative correction factors applied to $(Y)_m$, respectively:

$$(Y)_{corr} = (Y)_m \left\{1+\left[1-(Y)_m/(Y)_{max}\right].0028\ (Y)_{max}\right\}, \quad (16a)$$

$$(Y)_{corr} = (Y)_m \left\{1+\left[1-(Y)_m/(Y)_{max}\right].00028\ (Y)_{max}\right\}. \quad (16b)$$

The correction factors were as large as 0.3 and 0.03 percent for the photomultiplier tube used at amplifier gains of 10^6 and 10^7, respectively and are given in figures 22a and b.

The non-linearity of the photomultiplier tube was determined at 450.0 and 695.0 nm and results verified that the correction is wavelength independent for this type of photomultiplier tube at the 1 part in 10^4 measurement level [49,102]. These corrections were applied to all measurements made on the the NBS Reference Spectroradiometer.

B. Emission Spectrum as a Function of Bandwidth

A rule of thumb in spectrophotometry that is also applied in spectrofluorimetry is that if the bandpass of a monochromator is 1/10 to 1/20 that of the measured peak half-height bandwidth (HHBW), the measured values are accurate to ∿0.1 percent at the peak maximum but only to 5 percent at the wings.

A procedure to correct spectra for bandpass error was developed [49] using numerical differentiation of the emission spectrum based on an earlier method of Hardy and Young [103]. The derived equation is:

$$Y(\lambda) = Y_m(\lambda) - 1/12(\Delta\lambda)^2 Y_m''(\lambda) + \frac{1}{240}(\Delta\lambda)^4 Y_m''''(\lambda) - \ldots. \quad (17)$$

where: $Y(\lambda)$ = true readings,

$Y_m(\lambda)$ = measured readings.

$\Delta\lambda$ = bandpass of the monochromator, and

$Y_m''(\lambda)$ and $Y_m''''(\lambda)$ are the second and fourth derivatives of the emission spectrum calculated by the method of Rutledge [104].

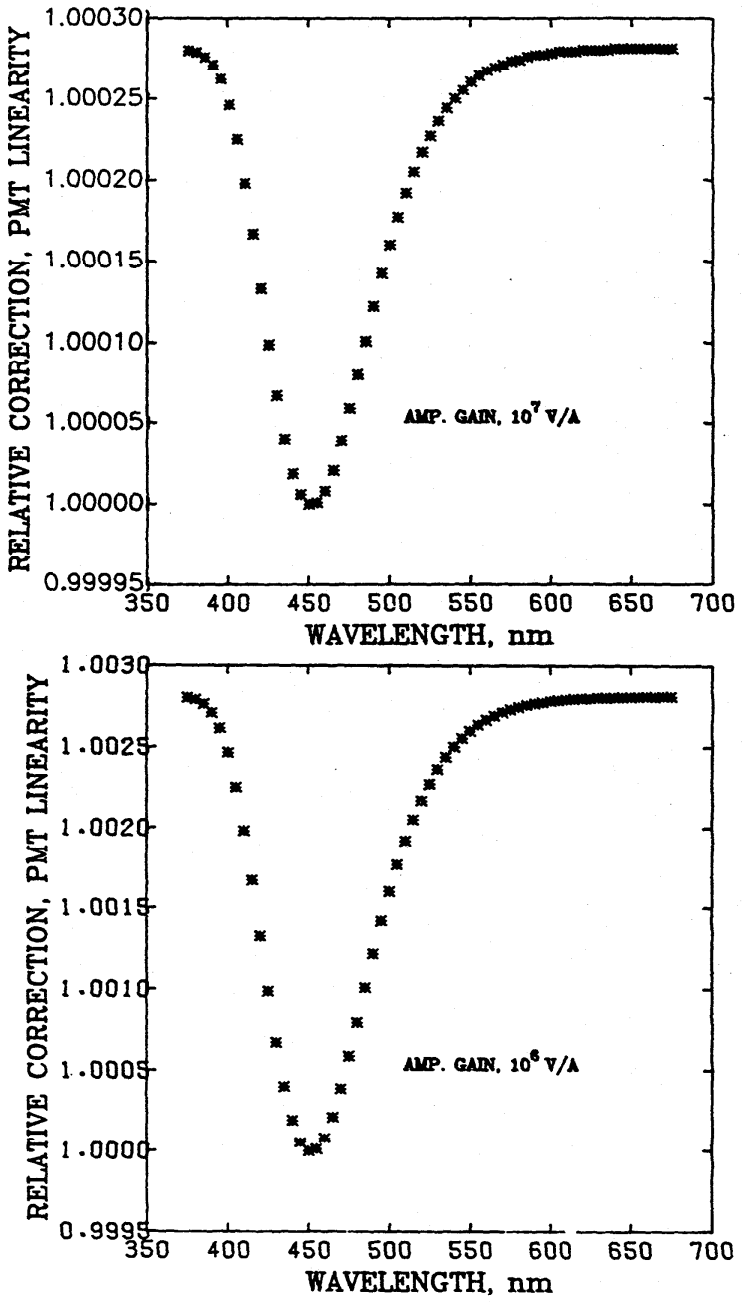


Figure 22. (a,b) Relative photomultiplier tube linearity correction factors for amplifier gains of 10^7 (22a) and 10^6 (22b) volts/amp applied to the compensated fluorescence signal (Y).

The odd derivatives, $Y'_m(\lambda), Y'''_m(\lambda)...$, cancel out due to the triangular bandpass function produced if the width of the two monochromator slits are equal. The bandpass used in the measurement of the tungsten lamp spectral radiant flux was ∿1/450 of the HHBW for the "measured peak" and no correction was necessary. However, the bandpass used in the measurement of the emitted flux from the quinine sulfate in solution was ∿1/18 of the HHBW for the emission peak and the correction factor by the numerical integration procedure was calculated and applied. Equation (17) was truncated after the second order term since the fourth derivative term contributed less than 0.1 percent to the overall correction.

A typical correction derived from the second derivative function of the emission spectrum is plotted in absolute (Y) values versus wavelength in figure 23a and the relative correction applied to the spectrum is illustrated in figure 23b.

C. Wavelength Correction

The detection monochromator wavelength errors (differences between the measured wavelengths and the true wavelengths for the line sources, Section IV-C) and the best polynomial fit for these errors are graphed in figure 24. Selected (Y) data at small wavelength increases (∿0.03 nm) are summarized in Table 15 for the "blue edge" of the uncorrected emission spectrum of quinine sulfate. The difference in (Y), Δ(Y), for a total wavelength change of 0.295 nm is 0.005614 units. From this, the error in (Y) was calculated to be 4.6 percent per nanometer in the wavelength reading. Considering the wavelength region of interest for the quinine sulfate emission spectrum, the largest errors in the wavelength calibration occur at 375 nm (-0.04 nm) and 715 nm (+0.077 nm). Errors in $(Y)_m$ would, therefore, range from -0.18 to +0.36 percent. We are, however, interested in the error produced in the final reported value of E(λ) and not the error produced in $(Y)_m$ since the wavelength error associated with the measured

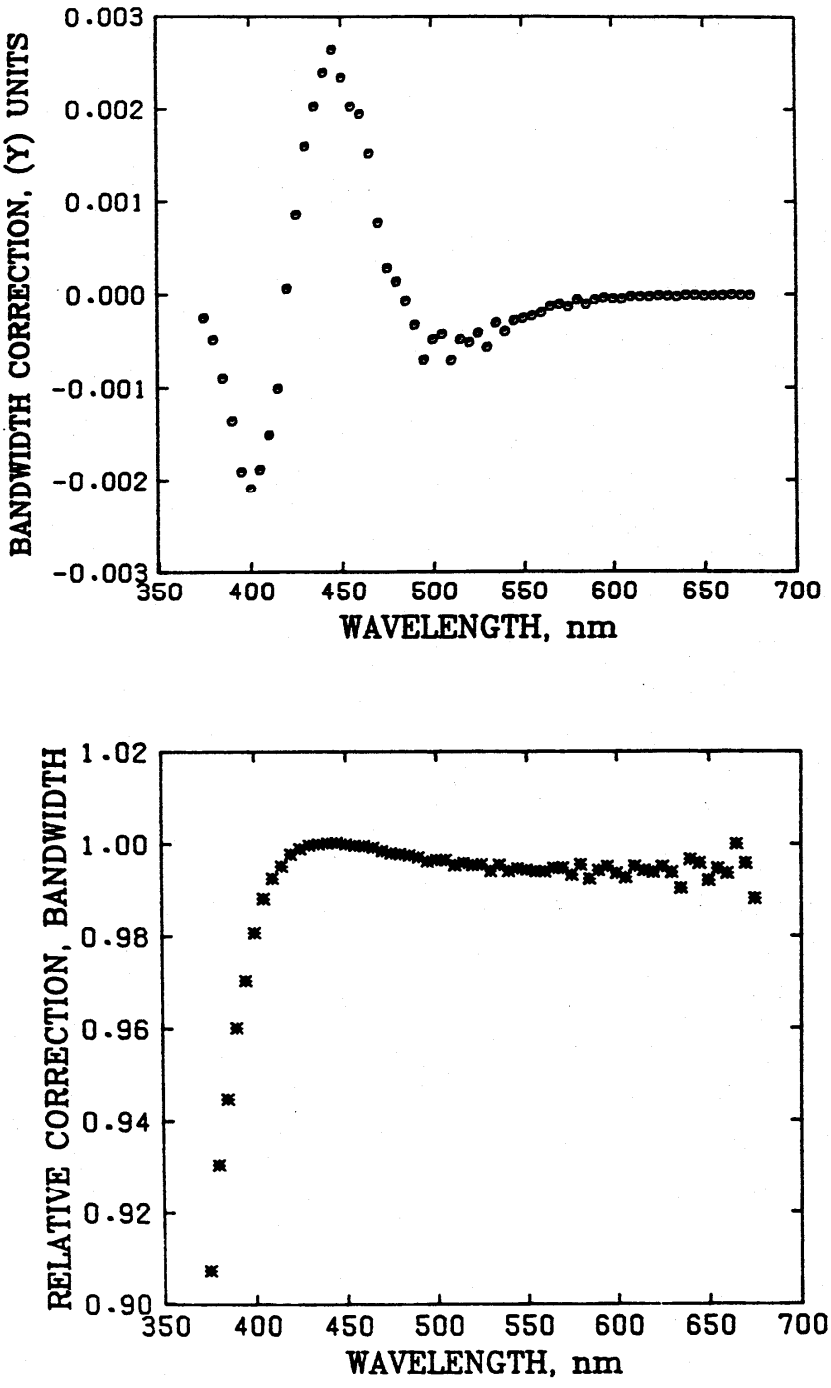


Figure 23. (a,b) Bandwidth correction in compensated fluorescence units (Y) (23a) and the relative correction factor applied to the quinine sulfate emission (23b) as a function of wavelength.

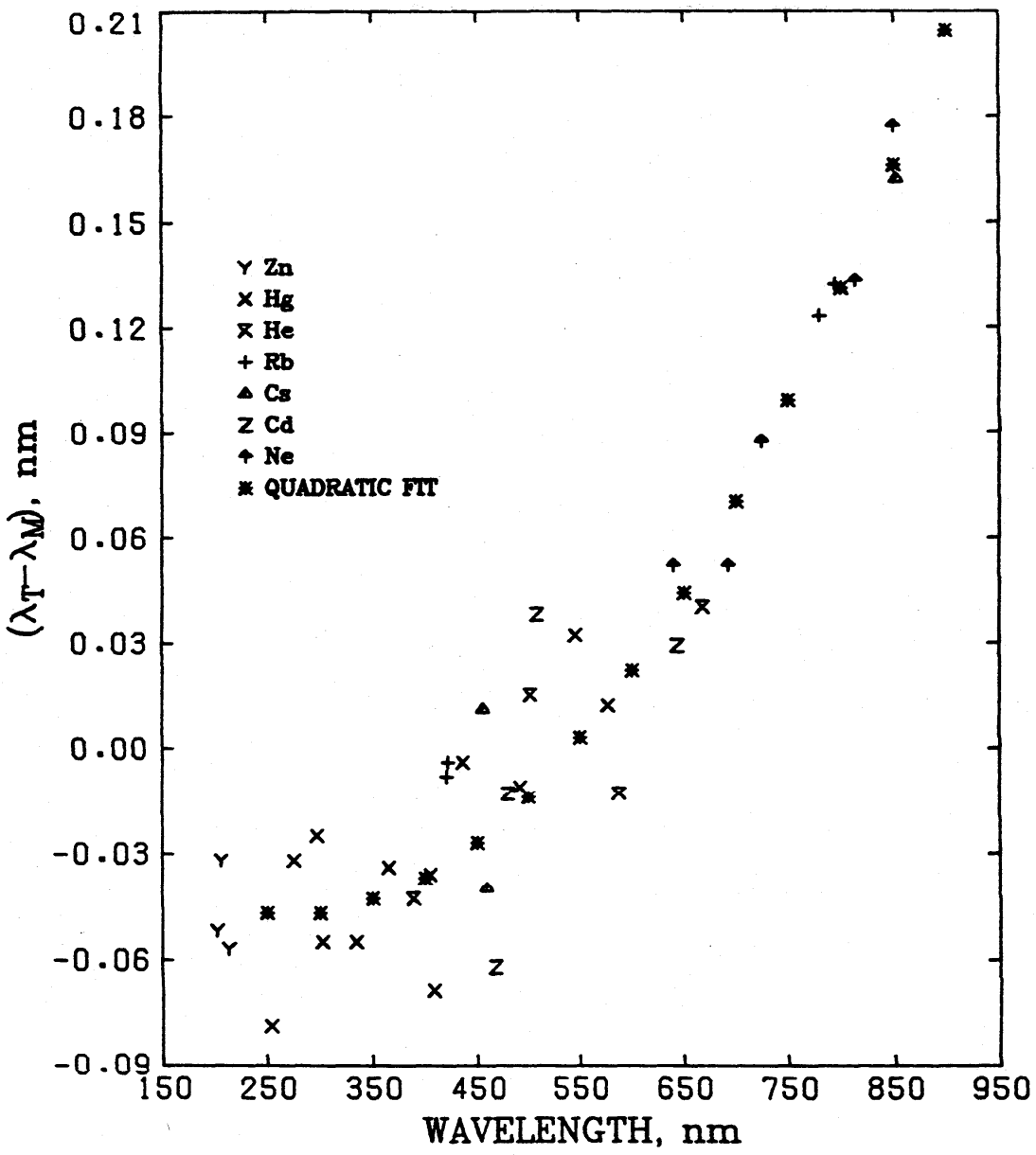


Figure 24. Wavelength deviations of the emission monochromator at 34 wavelengths using line sources. The stars represent the quadratic fit and these values were used to correct the quinine sulfate emission spectrum.

error in the emission detector signal (R), used in calculating $M^{S}_{\lambda_2}$ may be either compensative or additive.

Table 15. Quinine Sulfate Emission Values (Y) as a Function of Wavelength.

λ, nm	(Y)
415.055	.411944
415.115	.412675
415.145	.412784
415.205	.415470
415.235	.415675
415.260	.415808
415.290	.416142
415.370	.417509
415.350	.417558

For measurements of (Y) and (M) we find that:

$$(Y)_T = (Y)_m + (\lambda_E)\left(\frac{d(Y)_m}{d\lambda_2}\right) \qquad (18a)$$

and

$$(M)_T = (M)_m + (\lambda_E)\left(\frac{d(M)_m}{d\lambda_2}\right) \qquad (18b)$$

where $(Y)_T$ and $(M)_T$ are true values,

$(Y)_m$ is the measured compensated fluorescence signal,

$(M)_m$ is the value calculated from the measured detector signal $(R)_m$,

λ_E is the wavelength error, and

$\frac{d(Y)_m}{d\lambda_2}$ and $\frac{d(M)_m}{d\lambda_2}$ are the changes in the $(Y)_m$ and $(M)_m$ values as a function of λ_2 at the wavelength measurement.

The corrected emission spectrum in radiometric units $E^T(\lambda)$ is:

$$E^T(\lambda) = \frac{(Y)_T}{(M)_T} = \left[\frac{(Y)_m + (\lambda_E)\frac{d(Y)_m}{d\lambda_2}}{(M)_m + (\lambda_E)\frac{d(M)_m}{d\lambda_2}}\right]$$

$$= \frac{(Y)_m}{(M)_m}\left[\frac{1 + (\lambda_E)\left(\frac{1}{(y)_m}\right)\frac{d(y)_m}{d\lambda_2}}{1 + (\lambda_E)\left(\frac{1}{(m)_m}\right)\frac{d(M)_m}{d\lambda_2}}\right] \qquad (18c)$$

By Taylor expansion and truncation, using only terms of first order in λ_E (λ_E^2 is $\sim$30 times smaller than the λ_E terms), the relative error in $E^T(\lambda)$ is:

$$\frac{\Delta E^T(\lambda)}{R^T} = \left[\frac{\frac{(Y)_T}{(M)_T} - \frac{(Y)_m}{(M)_m}}{\frac{(Y)_m}{(M)_m}}\right] = (\lambda_E)\left[\left(\frac{1}{(Y)_m}\right)\left(\frac{d(Y)_m}{d\lambda_2}\right) - \left(\frac{1}{(M)_m}\right)\left(\frac{d(M)_m}{d\lambda_2}\right)\right]. \qquad (18d)$$

The errors in $E^T(\lambda)$ were calculated using equation (18d) and were subtracted from 1.0 to give the relative correction values, figure 25, that were applied to the $E^T(\lambda)$ values. The magnitude of the corrections range from $\sim$0.5 to $\sim$0.2 percent with many being close to zero.

The source monochromator was calibrated in a similar manner and found to be in error by +0.07 nm at 347.5 nm which was taken into account when setting λ_1 on the source monochromator.

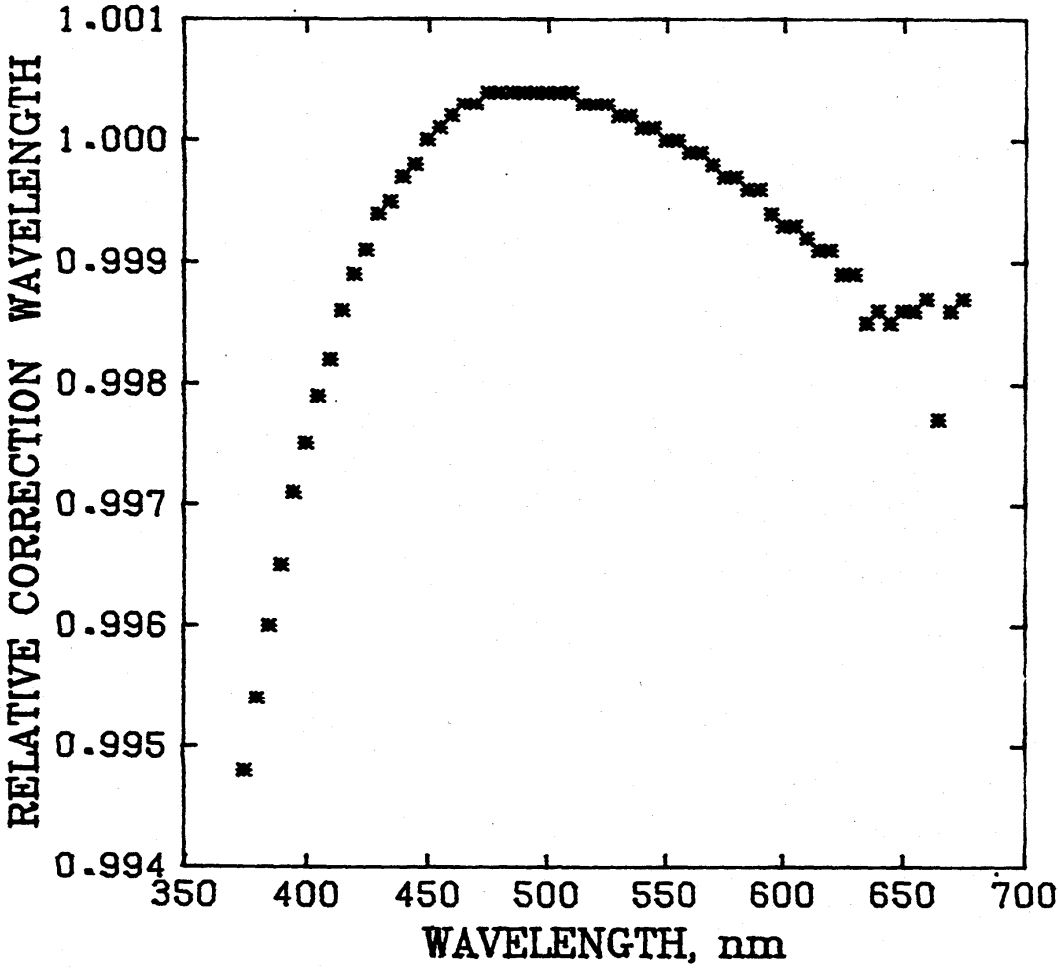


Figure 25. Relative correction factor applied to the compensated fluorescence signal (Y) to correct for the wavelength error.

D. Dispersion Correction

The grating dispersion of a monochromator is wavelength dependent and normally a correction [49] is applied to spectral data since the bandpass, as defined by the grating dispersion and slit width, is a function of wavelength. In this case, however, the correction must be made to both ($Y^{X,S}_{347.5,\lambda_2}$) and ($R^{S}_{\lambda_2}$), and since $E^{T}(\lambda)$ is:

$$E^T(\lambda) = \frac{(Y^{X,S}_{347.5,\lambda_2})}{M^S_{\lambda_2}} \qquad (19)$$

$$\text{where} \quad M^S_{\lambda_2} = \frac{R^S_{\lambda_2}}{L/Lo} \quad ,$$

the correction cancels out.

E. Solvent Refractive Index

Although the radiation is emitted isotropically within the solution and within the cone of radiation subtended by the collecting mirror EL_2, figure 1, the fraction emerging into the surrounding air depends on the square of the solvent refractive index, n^2 [105]. In relative quantum efficiency measurements, the solvent refractive index used is generally the value at the emission maximum [26]; however, the refractive index is a function of wavelength [106,107] and the emission spectrum should be corrected by multiplying the compensated fluorescence signal (Y) by the value of n^2 at λ_2. The difference between the refractive indices of water and 0.105 mol/L $HClO_4$ is $\sim$0.1 percent [106]; thus the index of refraction values for water [49] were used to correct the emission spectrum since the total index of refraction correction is $\sim$2 percent over the emission spectrum of quinine sulfate and the error introduced by this approximation is small (0.002 percent or less).

The values of n^2 for water at different λ_2's were calculated from equation 20 [49]:

$$n^2 = 1.7604457 + 4.03368 \times 10^{-3}\lambda - 1.54182 \times 10^{-2}\lambda^2 + \frac{6.44277 \times 10^{-3}}{\lambda^2 - 1.49119 \times 10^{-2}} \qquad (20)$$

where λ is in nm.

The relative values for the refractive index corrections are presented graphically in figure 26.

F. Cuvette Window Transmittance

In a manner similar to the refractive index correction, a correction was also made for the variation of the transmittance of the cuvette window as a function of wavelength. The equation derived for this correction at a given emission wavelength, λ_2, is [49]:

$$\tau'_{\lambda_2} = \frac{4nn'}{[n^2 + (n')^2] + n'(1 + n^2)} \qquad (21)$$

where τ' = the transmissivity of the quartz window at λ_2,
n = the refractive index of quartz at λ_2, and
n' = the refractive index of water at λ_2.

The refractive index of quartz as a function of wavelength was calculated using the equation given by Malitson [108] and τ'_{λ_2} was calculated by substituting the appropriate n' and n values at given λ_2's. The correction is on the order of 0.2 percent and the relative cell transmittance correction is plotted versus λ_2 in figure 27.

G. Composite Correction

The composite errors as a function of wavelength for the preceding instrumental and experimental parameters are given in figure 28a. To show the magnitude of the corrections applied to either (Y) or E(λ) in section VII, the composite corrections (excluding the correction for spectral responsivity) as a function of wavelength are given in figure 28b.

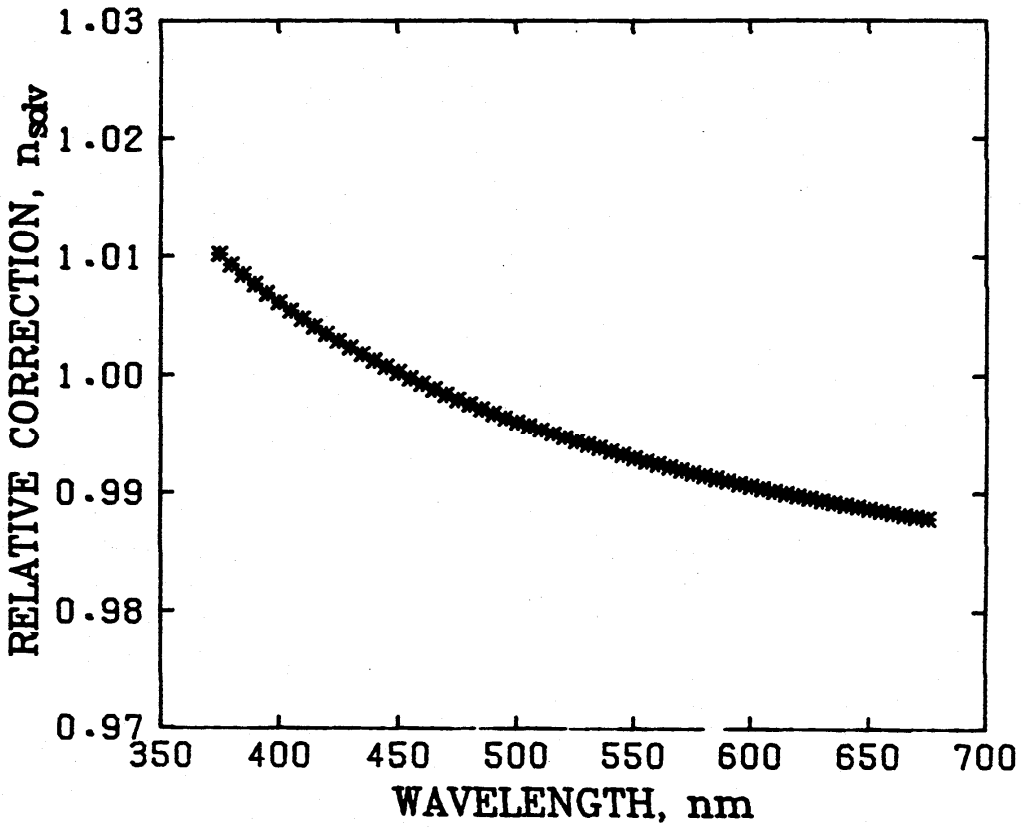


Figure 26. Relative correction factor applied to the emission spectrum to compensate for errors introduced by the refractive index of the solvent.

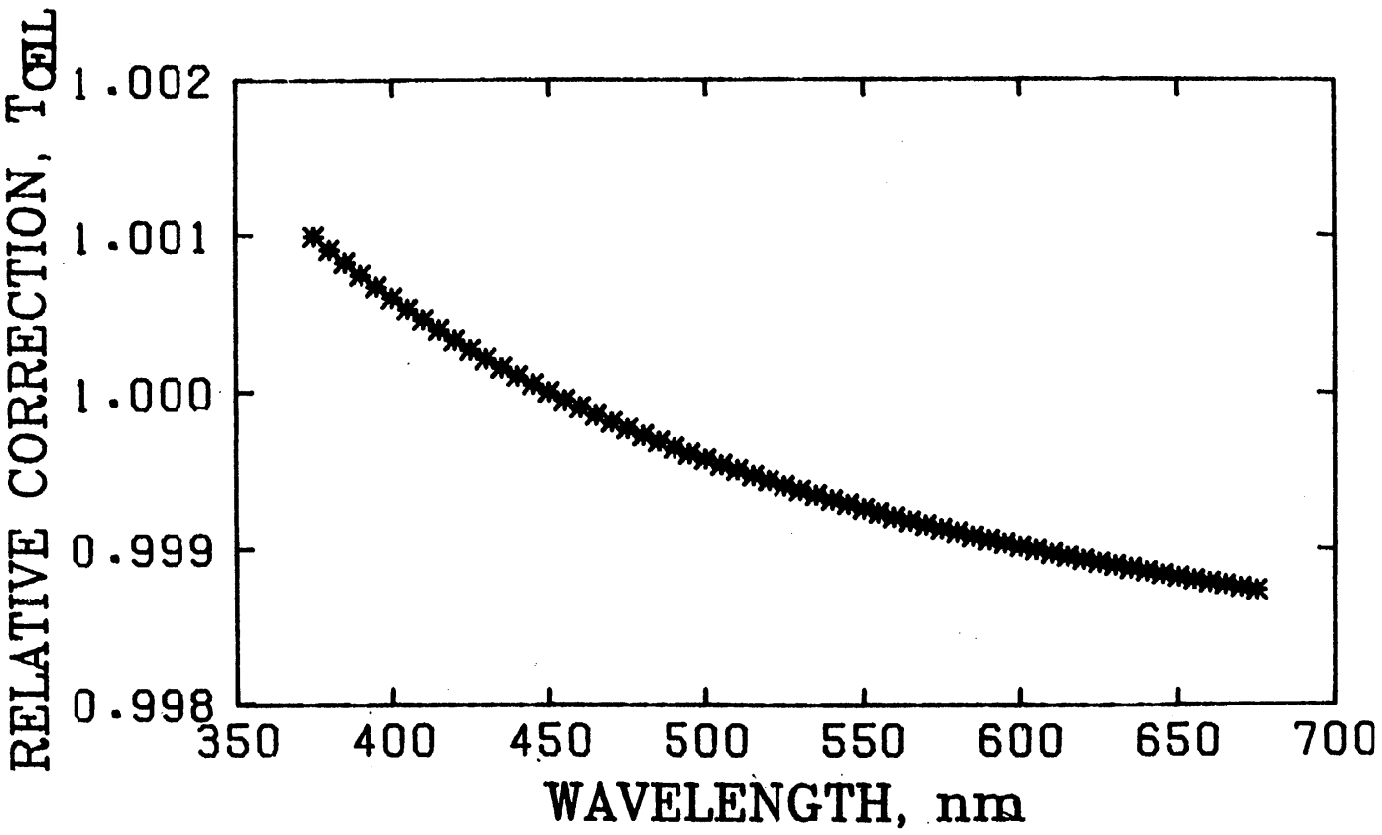


Figure 27. Relative correction factor applied to the emission spectrum to compensate for the variation of cell transmittance as a function of wavelength.

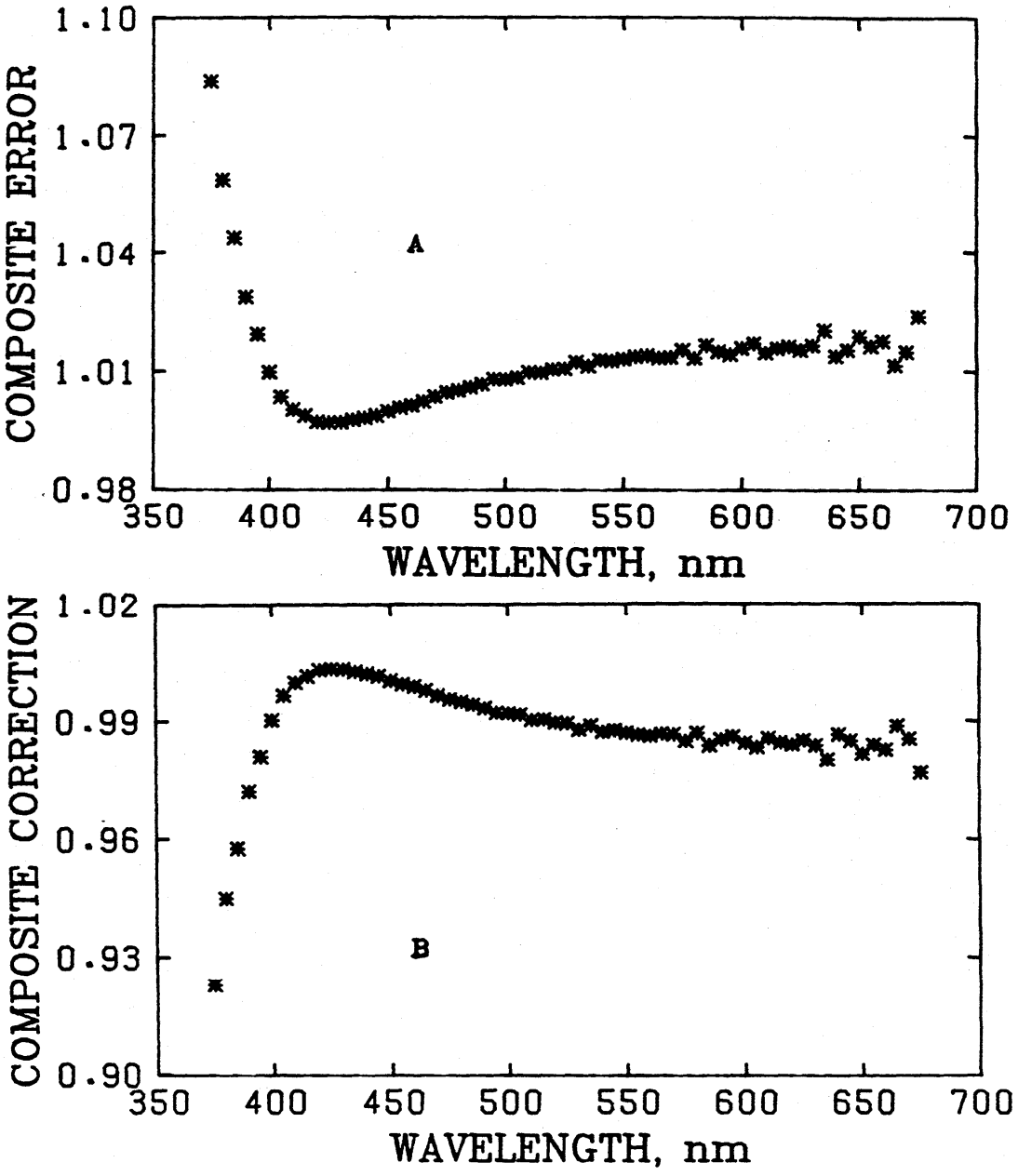


Figure 28. (a,b) Composite errors that occur in emission spectra measurements (28a) and the composite correction factors applied to the emission spectrum of quinine sulfate to correct for various instrumental and sample parameters (28b).

VIII. The Spectral Responsivity $M^S_{\lambda_2}$ of the Detection System, the Technical Emission Spectrum $E^T(\lambda)$, and the Molecular Spectrum $E(\lambda)$ of Quinine Sulfate in 0.105 mol/L $HClO_4$

The determination of the spectral responsivity $M^S_{\lambda_2}$, the technical emission spectrum $E^T(\lambda)$ (the emission spectrum corrected for instrument parameters), and the molecular emission spectrum $E(\lambda)$ (the emission spectrum corrected for instrument and sample parameters) could be discussed in two separate sections since $M^S_{\lambda_2}$ is a correction factor for the response of the detection system and $E^T(\lambda)$ and $E(\lambda)$ are emission spectra for the quinine sulfate. However, we have chosen to combine them because their determinations are closely related with respect to measuring procedures and data reduction.

In general, data reduction was performed with the objective of improving measurement precision by eliminating instrumental drift or by assuring that a maximum measured value of (R) or (Y), used as a normalization factor, was not in error so that the normalized data would not be biased by one inaccurate data point. Additionally, the accuracy of the data was increased by applying theoretically sound corrections to the measured data for instrumental and sample related errors. Attaining increased precision and accuracy are interdependent and it was not possible to average or smooth all data first and then apply all corrections. For example, since the correction for photomultiplier non-linearity must be made on the measured values of (Y) and not on the normalized (Y) readings, the correction was made before time symmetrical averaging, which was, in turn, performed before data normalization. Thus, the following sections will be somewhat chronological in order. Also, discussions will be included where relevant.

A. Measurement and Averaging of Data

Values of $R^{S}_{\lambda_2}$ for the standard lamp at the two operating currents (8.8030 and 6.0000 amps) and $Y^{X,S}_{\lambda_1,\lambda_2}$ for the three series of quinine sulfate solutions were obtained as Runs I, II, and III according to the time symmetrical measurement sequence illustrated in figure 7. The data were corrected for photomultiplier non-linearity and then were averaged as indicated in figure 7.

B. Data Smoothing

To avoid errors in the (Y) or (R) value chosen for normalization, attempts were made to "fit" the emission envelope data by polynomial equations (up to 12th order, reference 56), a modified spline technique [109], or an exponential-polynomial combination [110] to obtain a predicted (Y) or (R) value at the peak maximum, designated λ_n. These procedures were unsuitable since the imprecisions introduced by the fitting techniques were larger than the intra- and inter-run imprecisions.

However, several data points bracketing λ_n [111] could be fitted using a 3rd order polynomial [112] yielding a calculated $(Y)_n$ value that was accurate to better than 0.1 percent. The predicted values of $(Y)_n$ or $(R)_n$ at λ_n for the spectra from Runs I, II, and III then were used to obtain initial spectral normalizations. The normalized spectra were averaged and for Runs I, II, and III the following data were available:

1. the averaged, normalized data $(R^{S}_{\lambda_2})_n$ for the two standard lamp currents before measuring the ten quinine spectra;
2. the averaged, normalized, compensated values $(Y^{X,S}_{\lambda_1,\lambda_2})_n$ for the ten quinine sulfate spectra; and
3. the averaged, normalized data $(R^{S}_{\lambda_2})_n$ for the two standard lamp currents after measuring the ten quinine spectra.

C. Spectral Responsivity $M^S_{\lambda_2}$

The spectral responsivity of the detection system was determined by:

$$M^S_{\lambda_2} = \frac{(R^S_{\lambda_2})_n}{L/L_n} \tag{22}$$

where: L is the spectral radiance of the calibrated tungsten lamp [58] in $W(mm)^{-2}(sr)^{-1}(nm)^{-1}$, and
L_n is the value of the spectral radiance at λ_n.

The values of the spectral responsivities obtained before, $(M^S_{\lambda_2})_B$, and after, $(M^S_{\lambda_2})_A$, measuring the quinine spectra were compared as an indication of instrument stability during a given run. The ratios $(M^S_{\lambda_2})_A/(M^S_{\lambda_2})_B$ for Run III are summarized at selected wavelengths in Table 16 and show agreement that is on the order of the precision for a single data point during a particular run [113]. The ratios at 400 nm (6.0000 A data) and 350 nm (8.8030 A data) show a little more variation, however, this is due to the low signal values at these wavelengths (1% or less of the maximum signal).

In order to obtain a single $M^S_{\lambda_2}$, the two sets of data (350 to 425 nm and 400 to 900 nm) were spliced by using the average ratio of the normalized values in the 400 to 425 nm overlap region and spectral fitting procedures to produce a calculated $M^S_{\lambda_2}$ for the wavelength interval 350 to 900 nm for each run. The $M^S_{\lambda_2}$ values for Runs I, II, and III then were averaged and evaluated statistically to give an average spectral responsivity for the detection system ($M^S_{\lambda_2}$) and the relative standard errors (RSE) in these values. The results are summarized in Table 17 and depicted graphically in figures 29 and 30.

Table 16. Typical Intrarun Instrument Stabilities as Determined by $(M^S_{\lambda_2})$ Values for the Standard Lamp at the Two Operating Currents.

	- - - $[(M^S_{\lambda_2})_A]/[(M^S_{\lambda_2})_B]$ - - -	
λ, nm	6.0000 amp	8.8030 amp
350	--	0.9968
370	--	1.0002
375	--	.9985
400	.9964	1.0003
410	.9983	1.0006
420	.9982	1.0000
450	1.0025	--
475	1.0002	--
500	1.0003	--
525	1.0013	--
550	.9997	--
575	.9995	--
600	.9998	--
625	.9990	--

The shoulder observed at ∿375 nm in figure 30 is not an artifact of the splicing procedure since it is observable in the 8.803A data. The RSE values average ∿0.17 percent over the total wavelength region measured (350-900 nm) and are less than 0.1 percent over most of the wavelength region of interest (375 to 675 nm) which demonstrates excellent system stability.

Table 17. Measured and Calculated Data: The Spectral Responsivity of the Detection System $M^S_{\lambda_2}$ and the Relative Standard Error (RSE) in $M^S_{\lambda_2}$; the Compensated Fluorescence Signal (Y) and the RSE in (Y); the Technical Emission Spectrum $E^T(\lambda)$ and the RSE and Percent Systematic Error Limits (PSEL) in $E^T(\lambda)$; the Corrected Molecular Emission Spectrum $E(\lambda)$ and the RSE and PSEL in $E(\lambda)$.

λ, nm	$M^S_{\lambda_2}$	RSE($M^S_{\lambda_2}$)	(Y)	RSE(Y)	$E^T(\lambda)$	RSE[$E^T(\lambda)$]	PSEL[$E^T(\lambda)$]	$E(\lambda)$	RSE[$E(\lambda)$]	PSEL[$E(\lambda)$]
375.0	0.6663	0.0031	0.003	0.017	0.005	0.019	8.40	0.005	0.019	8.73
380.0	.6841	.0028	.007	.008	.012	.006	7.54	.012	.006	7.83
385.0	.7182	.0035	.017	.003	.028	.003	6.72	.028	.003	7.08
390.0	.7670	.0035	.036	.002	.057	.003	6.20	.057	.003	6.44
395.0	.8364	.0018	.070	.002	.103	.002	5.66	.103	.002	5.88
400.0	.9043	.0010	.123	.001	.169	.002	5.17	.170	.002	5.37
405.0	.9696	.0011	.198	.002	.256	.003	4.73	.257	.003	4.91
410.0	1.0309	.0014	.294	.002	.357	.003	4.32	.359	.003	4.49
415.0	1.0872	.0016	.405	.002	.469	.003	3.95	.471	.003	4.09
420.0	1.1371	.0017	.526	.002	.583	.003	3.60	.586	.003	3.72
425.0	1.1796	.0016	.647	.001	.692	.003	3.29	.694	.003	3.38
430.0	1.2134	.0015	.759	.001	.790	.002	3.01	.792	.002	3.08
435.0	1.2372	.0012	.855	.001	.872	.002	2.77	.874	.002	2.82
440.0	1.2499	.0009	.929	.001	.938	.001	2.58	.940	.001	2.62
445.0	1.2563	.0012	.978	.001	.983	.001	2.43	.984	.001	2.44
450.0	1.2624	.0011	.999	.001	.998	.001	2.33	.999	.001	2.34
455.0	1.2581	.0005	.995	.000	.998	.001	2.32	.997	.001	2.33
460.0	1.2440	.0006	.969	.001	.983	.001	2.35	.982	.001	2.37
465.0	1.2265	.0007	.923	.001	.949	.001	2.40	.947	.001	2.44
470.0	1.2077	.0008	.861	.001	.898	.001	2.47	.897	.001	2.52

continued

Table 17 continued.

λ, nm	$M^S_{\lambda_2}$	RSE($M^S_{\lambda_2}$)	(Y)	RSE(Y)	$E^T(\lambda)$	RSE[$E^T(\lambda)$]	PSEL[$E^T(\lambda)$]	E(λ)	RSE[E(λ)]	PSEL[E(λ)]
475.0	1.1848	0.0007	0.791	0.001	0.840	0.002	2.55	0.838	0.002	2.61
480.0	1.1518	.0008	.717	.002	.784	.002	2.58	.782	.002	2.66
485.0	1.1186	.0006	.641	.001	.721	.002	2.60	.719	.002	2.69
490.0	1.0812	.0003	.567	.002	.659	.002	2.61	.659	.002	2.72
495.0	1.0424	.0002	.496	.002	.598	.003	2.61	.595	.003	2.73
500.0	.9987	.0006	.432	.002	.543	.002	2.61	.541	.002	2.74
505.0	.9578	.0003	.372	.001	.488	.001	2.62	.486	.001	2.77
510.0	.9169	.0002	.318	.002	.436	.003	2.64	.434	.003	2.79
515.0	.8751	.0005	.270	.002	.388	.003	2.65	.386	.003	2.82
520.0	.8331	.0007	.228	.002	.344	.002	2.66	.342	.002	2.84
525.0	.7940	.0008	.192	.001	.304	.001	2.67	.302	.001	2.86
530.0	.7562	.0008	.160	.003	.266	.003	2.68	.264	.003	2.87
535.0	.7202	.0006	.133	.002	.233	.003	2.68	.231	.003	2.89
540.0	.6855	.0007	.111	.002	.203	.002	2.68	.201	.002	2.90
545.0	.6517	.0005	.092	.002	.177	.002	2.68	.175	.002	2.91
550.0	.6185	.0009	.076	.002	.154	.001	2.68	.153	.001	2.91
555.0	.5875	.0004	.062	.001	.133	.001	2.67	.132	.001	2.91
560.0	.5571	.0006	.052	.001	.117	.001	2.66	.116	.001	2.91
565.0	.5271	.0006	.043	.001	.102	.002	2.65	.101	.002	2.91
570.0	.5005	.0006	.035	.002	.089	.002	2.64	.088	.002	2.91

continued

Table 17 continued.

λ, nm	$M^S_{\lambda_2}$	RSE($M^S_{\lambda_2}$)	(Y)	RSE(Y)	$E^T(\lambda)$	RSE[$E^T(\lambda)$]	PSEL[$E^T(\lambda)$]	E(λ)	RSE[E(λ)]	PSEL[E(λ)]
575.0	0.4746	0.0008	0.029	0.002	0.077	0.003	2.64	0.076	0.003	2.92
580.0	.4491	.0006	.024	.002	.067	.003	2.64	.065	.003	2.93
585.0	.4259	.0007	.020	.001	.058	.001	2.65	.057	.001	2.94
590.0	.4032	.0007	.016	.003	.050	.003	2.65	.050	.003	2.95
595.0	.3831	.0008	.013	.003	.044	.004	2.66	.043	.004	2.96
600.0	.3636	.0007	.011	.005	.038	.006	2.66	.037	.006	2.97
605.0	.3445	.0007	.009	.002	.032	.002	2.65	.032	.002	2.97
610.0	.3272	.0008	.007	.005	.028	.006	2.65	.028	.006	2.97
615.0	.3105	.0006	.006	.003	.025	.003	2.64	.024	.003	2.98
620.0	.2945	.0006	.005	.009	.021	.011	2.64	.021	.011	2.98
625.0	.2789	.0007	.004	.003	.019	.003	2.63	.018	.003	2.98
630.0	.2644	.0006	.003	.013	.016	.016	2.63	.016	.015	2.98
635.0	.2502	.0006	.003	.012	.014	.014	2.63	.014	.014	2.98
640.0	.2371	.0007	.002	.031	.012	.037	2.63	.011	.037	2.99
645.0	.2245	.0005	.002	.016	.010	.015	2.64	.010	.015	3.01
650.0	.2122	.0005	.002	.026	.009	.027	2.66	.009	.027	3.03
655.0	.2005	.0005	.001	.035	.008	.035	2.69	.008	.035	3.07
660.0	.1891	.0005	.001	.067	.007	.073	2.72	.007	.073	3.11
665.0	.1782	.0006	.001	.046	.006	.046	2.77	.006	.046	3.16
670.0	.1677	.0004	.001	.045	.005	.053	2.83	.005	.053	3.22
675.0	.1573	.0005	.001	.060	.004	.065	2.89	.004	.065	3.29

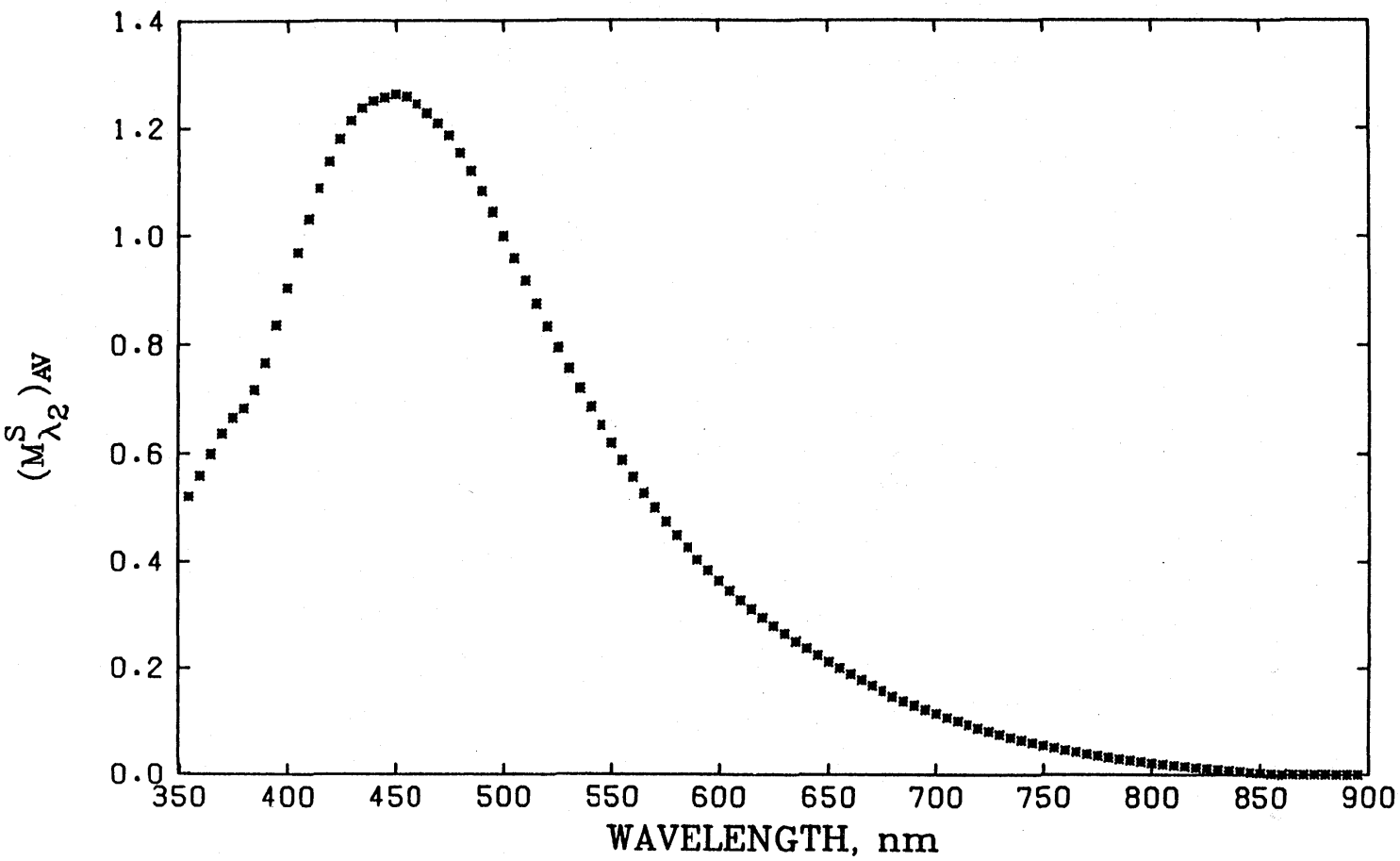


Figure 29. The spectral responsivity $(M^S_{\lambda_2})_{AV}$ for the detection system of the NBS reference spectroradiometer.

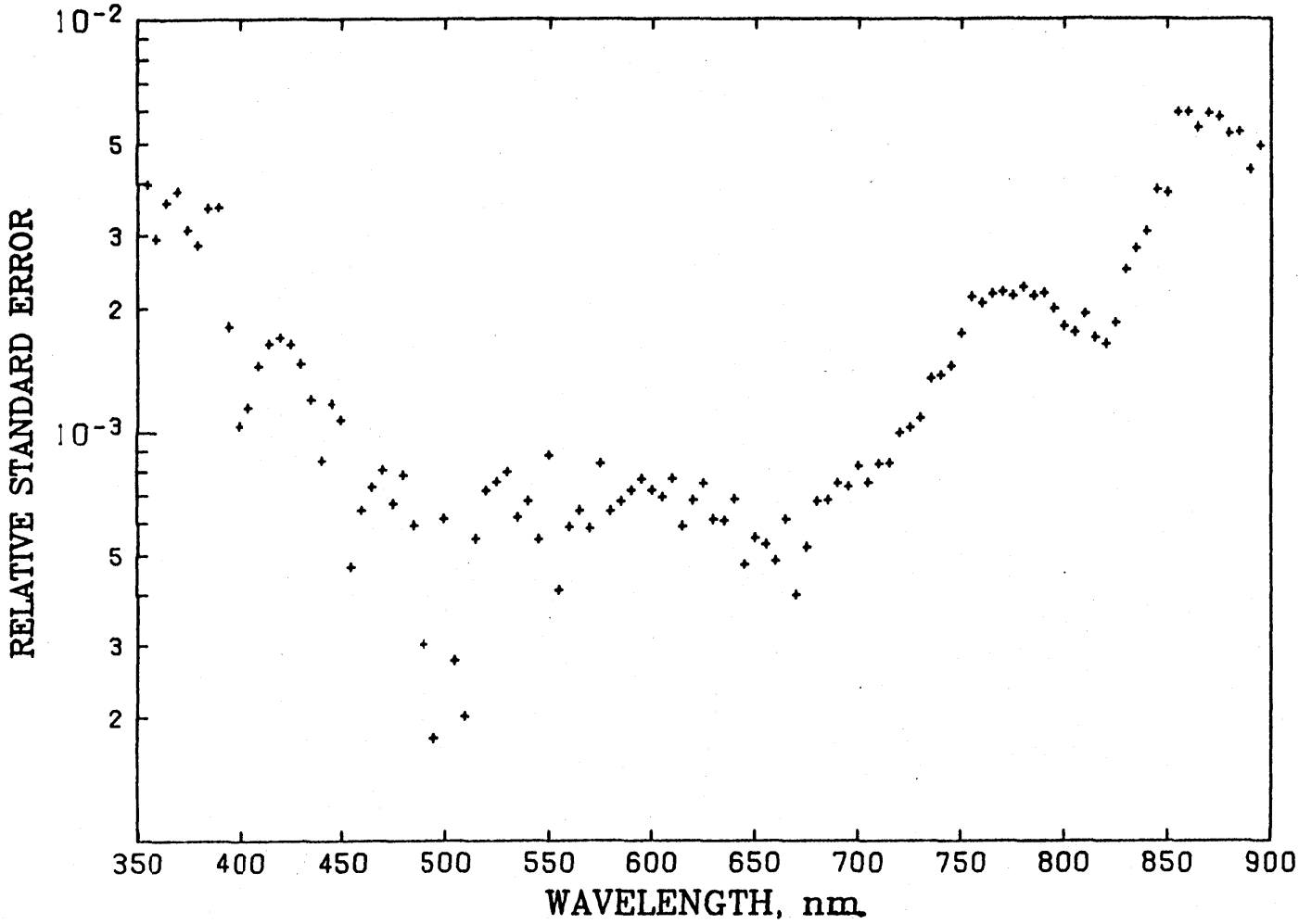


Figure 30. The relative standard error in the values for spectral responsivity of the detection system.

D. Compensated Emission Spectrum ($Y^{X,S}_{347.5,\lambda_2}$)

The compensated, normalized, uncorrected emission spectra for Runs I, II, and III (i.e., $(Y)_I$, $(Y)_{II}$, and $(Y)_{III}$) were averaged and the RSE's in these values were calculated and are summarized in Table 17. As can be seen, the RSE in (Y) varied from 0 percent at the peak maximum values to as high as 6 percent at one of the wings. In general, however, the RSE was less than 0.2 percent over the major portion of the uncorrected emission spectrum.

E. Technical Emission Spectrum $E^T(\lambda)$ of Quinine Sulfate

The normalized (Y) values for Runs I, II, and III were corrected for bandpass errors (Section VII-B) and monochromator wavelength errors (Section VIII-C) and the technical emission spectrum $E^T(\lambda)_x$ for each run was calculated by:

$$E^T(\lambda)_x = \frac{(Y)_x}{(M^S_{\lambda_2})_x} \qquad (23)$$

where the subscript x represents the values from Runs I, II, or III and the other symbols have the same representation as stated previously.

The values for $E^T(\lambda)_I$, $E^T(\lambda)_{II}$, and $E^T(\lambda)_{III}$ were averaged to give $E^T(\lambda)$. The $E^T(\lambda)$ values, the RSE $E^T(\lambda)$, and the percent systematic error limits PSEL $E^T(\lambda)$ (Section IX-A-4) are summarized in Table 17.

F. Molecular Emission Spectrum $E(\lambda)$

The $E^T(\lambda)$ values for each run were then corrected for the sample parameter effects of solvent refractive index (Section VII-E) and cell window transmittance (Section VII-F). The resulting values were averaged and the $E(\lambda)$ values, the RSE in the $E(\lambda)$ values, and the PSEL $E(\lambda)$ values (Section IX-A5) are given in Table 17.

In general, the $E^T(\lambda)$ values differ from the $E(\lambda)$ values by no more than 10 percent with most of the values differing by much less than that.

The values of $E_p(\lambda)$, $E(\tilde{\nu})$, and $E_p(\tilde{\nu})$ were calculated according to [39,114]:

$$\lambda^3 E(\lambda) = \lambda^2 E_p(\lambda) = \lambda E(\tilde{\nu}) = E_p(\tilde{\nu}). \qquad (24)$$

The normalized, calculated values for $E^T(\lambda)$, $E(\lambda)$, $E_p(\lambda)$, $E(\tilde{\nu})$, and $E_p(\tilde{\nu})$ are summarized in Table 18. The values of (Y) and $E(\lambda)$ are plotted versus wavelength in figure 31 so that the extent of the corrections are evident. The relative standard errors, a measure of imprecision in the $E(\lambda)$ values are plotted in figure 32 and vary from ∿1 percent at the spectrum wings to less than 0.1 percent at the center of the spectrum with most of the values being less than ∿0.2 percent. The signal level expressed as the percent of the maximum signal and the RSE for $E(\lambda)$ values are summarized in Table 19. The observed RSE's are of the same order of magnitude as the RSE's determined by the usual propagation of error treatment used throughout the data handling and smoothing.

The emission spectra of quinine sulfate in $E(\lambda)$ and $E_p(\tilde{\nu})$ units as functions of wavelength are also presented in figure 33 to demonstrate the differences in spectral distribution depending on the reporting units [39,78b]. Interpolated $E_p(\tilde{\nu})$ values at even wavenumber intervals are given in Table 20.

Table 18. The Technical and Molecular Emission Spectra of Quinine Sulfate in the Various Radiometric and Photon Units of $E^T(\lambda)$, $E(\lambda)$, $E_p(\lambda)$, $E(\tilde{\nu})$, and $E_p(\tilde{\nu})$.

λ, nm	$E^T(\lambda)$[a]	$E(\lambda)$[a]	$E_p(\lambda)$[a]	$\tilde{\nu}$, $cm^{-1}(10^{-4})$	$E(\tilde{\nu})$[a]	$E_p(\tilde{\nu})$[a]
375.0	0.005	0.005	0.004	2.667	0.003	0.003
380.0	.012	.012	.010	2.632	.009	.007
385.0	.028	.028	.024	2.597	.020	.017
390.0	.057	.057	.049	2.564	.042	.036
395.0	.103	.103	.090	2.532	.078	.067
400.0	.169	.170	.150	2.500	.131	.114
405.0	.256	.257	.229	2.469	.203	.178
410.0	.357	.359	.324	2.439	.290	.259
415.0	.469	.471	.430	2.410	.390	.352
420.0	.583	.586	.542	2.381	.497	.453
425.0	.692	.694	.650	2.353	.603	.557
430.0	.790	.792	.750	2.326	.705	.658
435.0	.872	.874	.837	2.299	.795	.752
440.0	.938	.940	.911	2.273	.875	.836
445.0	.983	.984	.965	2.247	.937	.906
450.0	.998	.999	.990	2.222	.972	.951
455.0	.998	.997	.999	2.198	.993	.982
460.0	.983	.982	.995	2.174	.999	.999
465.0	.949	.947	.970	2.151	.985	.995
470.0	.898	.897	.929	2.128	.953	.973
475.0	.840	.838	.877	2.105	.910	.939
480.0	.784	.782	.826	2.083	.866	.903
485.0	.719	.719	.768	2.062	.813	.857
490.0	.659	.657	.709	2.041	.759	.808
495.0	.598	.595	.649	2.020	.702	.755
500.0	.543	.541	.596	2.000	.651	.707
505.0	.488	.486	.540	1.980	.596	.653
510.0	.436	.434	.487	1.961	.542	.601
515.0	.388	.386	.438	1.942	.492	.551
520.0	.344	.342	.392	1.923	.445	.503

[a]Peak maxima: $E^T(\lambda)$ = 452.4 nm; $E(\lambda)$ = 452.2 nm; $E_p(\lambda)$ = 456.0 nm; $E(\tilde{\nu})$ = 2.178 $cm^{-1}(x10^{-4})$; $E_p(\tilde{\nu})$ = 2.165 $cm^{-1}(x10^{-4})$.

continued

Table 18 continued.

λ, nm	$E^T(\lambda)$	$E(\lambda)$	$E_p(\lambda)$	$\tilde{\nu}$, $cm^{-1}(10^{-4})$	$E(\tilde{\nu})$	$E_p(\tilde{\nu})$
525.0	0.304	0.302	0.349	1.905	0.400	0.456
530.0	.266	.264	.308	1.887	.356	.410
535.0	.233	.231	.272	1.869	.318	.370
540.0	.203	.201	.239	1.852	.282	.331
545.0	.177	.175	.211	1.835	.251	.297
550.0	.154	.153	.185	1.818	.223	.266
555.0	.133	.132	.162	1.802	.196	.236
560.0	.117	.116	.143	1.786	.174	.212
565.0	.102	.101	.126	1.770	.155	.191
570.0	.089	.088	.110	1.754	.137	.170
575.0	.077	.076	.096	1.739	.121	.151
580.0	.067	.065	.085	1.724	.107	.135
585.0	.058	.057	.074	1.709	.094	.119
590.0	.050	.050	.065	1.695	.084	.107
595.0	.044	.043	.056	1.681	.073	.095
600.0	.038	.037	.049	1.667	.064	.084
605.0	.032	.032	.043	1.653	.057	.074
610.0	.028	.028	.038	1.639	.050	.066
615.0	.025	.024	.033	1.626	.044	.059
620.0	.021	.021	.029	1.613	.039	.052
625.0	.019	.018	.025	1.600	.035	.047
630.0	.016	.016	.022	1.587	.029	.041
635.0	.014	.014	.019	1.575	.027	.037
640.0	.012	.011	.016	1.563	.023	.031
645.0	.010	.010	.015	1.550	.021	.029
650.0	.009	.009	.013	1.538	.018	.025
655.0	.008	.008	.011	1.527	.016	.022
660.0	.007	.007	.010	1.515	.014	.020
665.0	.006	.006	.009	1.504	.013	.018
670.0	.005	.005	.007	1.493	.011	.015
675.0	.004	.004	.007	1.481	.010	.014

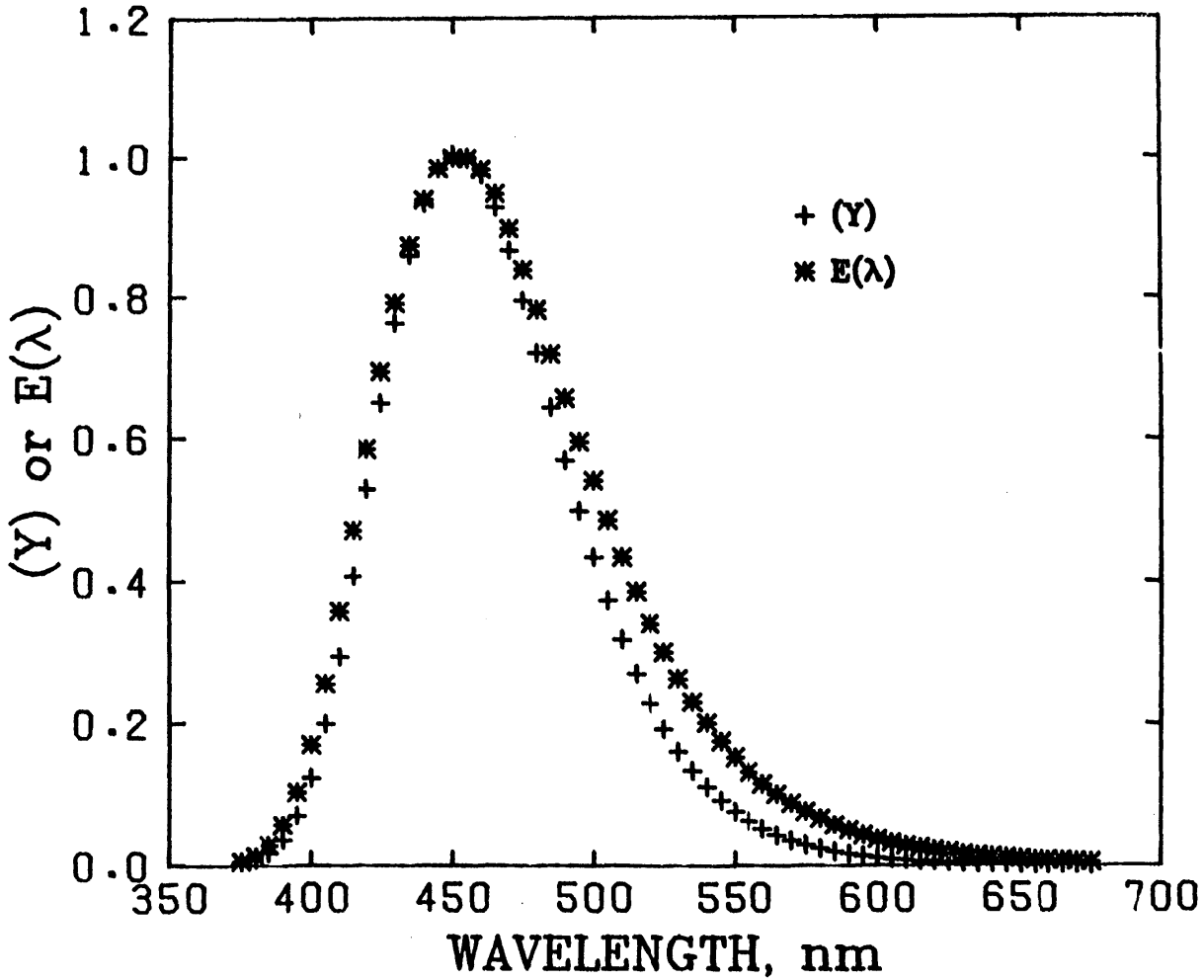


Figure 31. The compensated fluorescence emission spectrum (Y) and the corrected emission spectrum E(λ) for quinine sulfate. Solvent = 0.1 mol/L $HClO_4$; λ_{ex} = 347.5 nm, T = 25.0 °C.

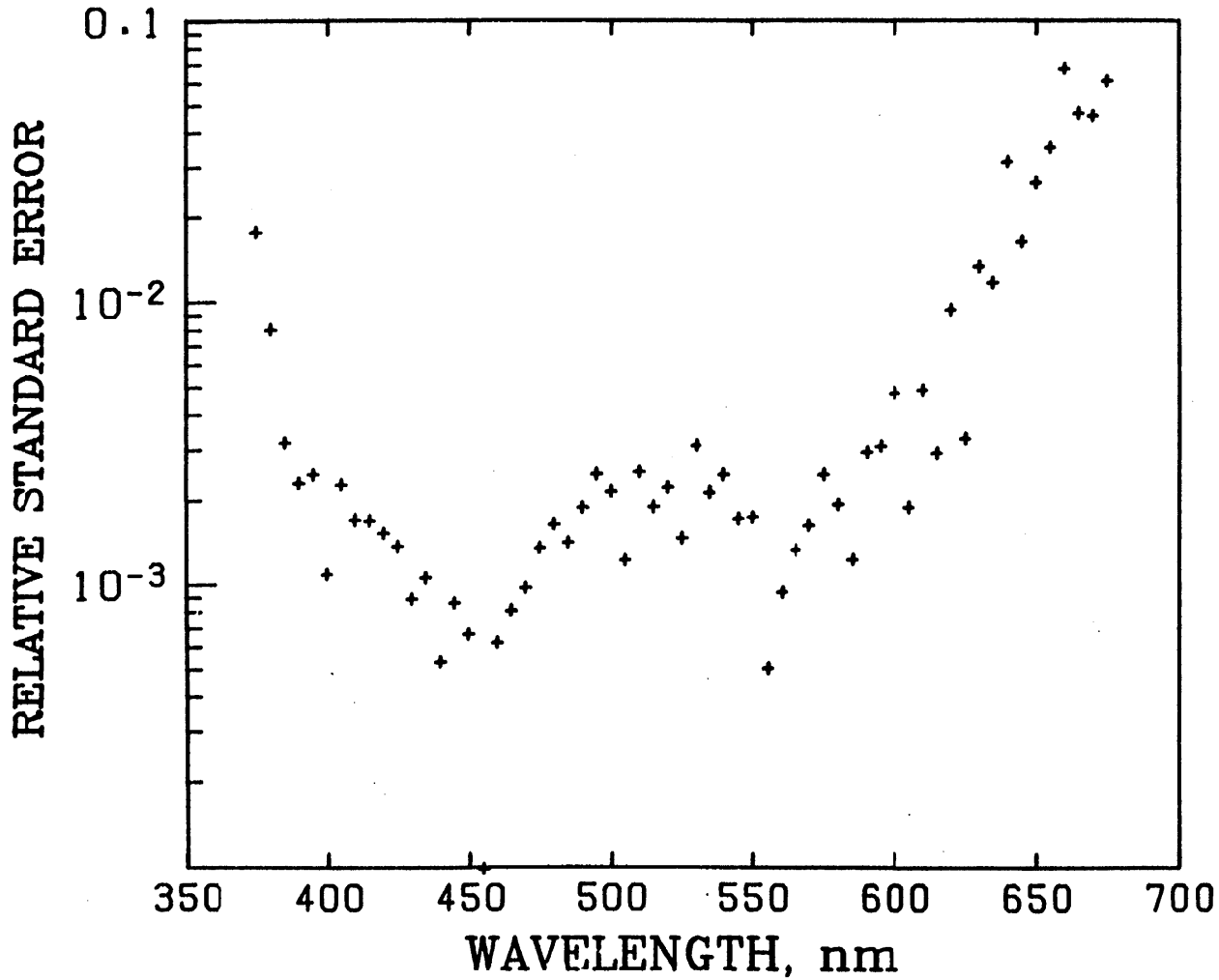


Figure 32. The relative standard errors in the values for the corrected emission spectrum $E(\lambda)$ of quinine sulfate. Solvent = 0.1 mol/L $HClO_4$; λ_{ex} = 347.5 nm.

Table 19. The Average Relative Standard Errors (RSE) in $E(\lambda)$ at Selected Signal Levels.

λ, nm	Signal Level as ~Percent of Maximum	RSE[a]
375.0	0.5	0.0194
380.0	1.0	.0097
395.0	10.0	.0022
420.0	50.0	.0030
455.0	100.0	.0008
505.0	50.0	.0019
565.0	10.0	.0018
645.0	1.0	.0266
670.0	0.5	.0549

[a] Average of RSE values for λ, λ+5 nm, and λ-5 nm except for 375 nm data which is the average of λ and λ+5 nm data.

Table 20. Interpolated $E_p(\tilde{\nu})$ Values at Even Wavenumber Intervals.

Wavenumber $cm^{-1}(10^{-4})$	$E_p(\tilde{\nu})$	Wavenumber $cm^{-1}(10^{-4})$	$E_p(\tilde{\nu})$
1.70	0.111	2.15	0.995
1.75	.164	2.20	.980
1.80	.233	2.25	.899
1.85	.327	2.30	.748
1.90	.444	2.35	.568
1.95	.572	2.40	.385
2.00	.707	2.45	.228
2.05	.830	2.50	.114
2.10	.931		

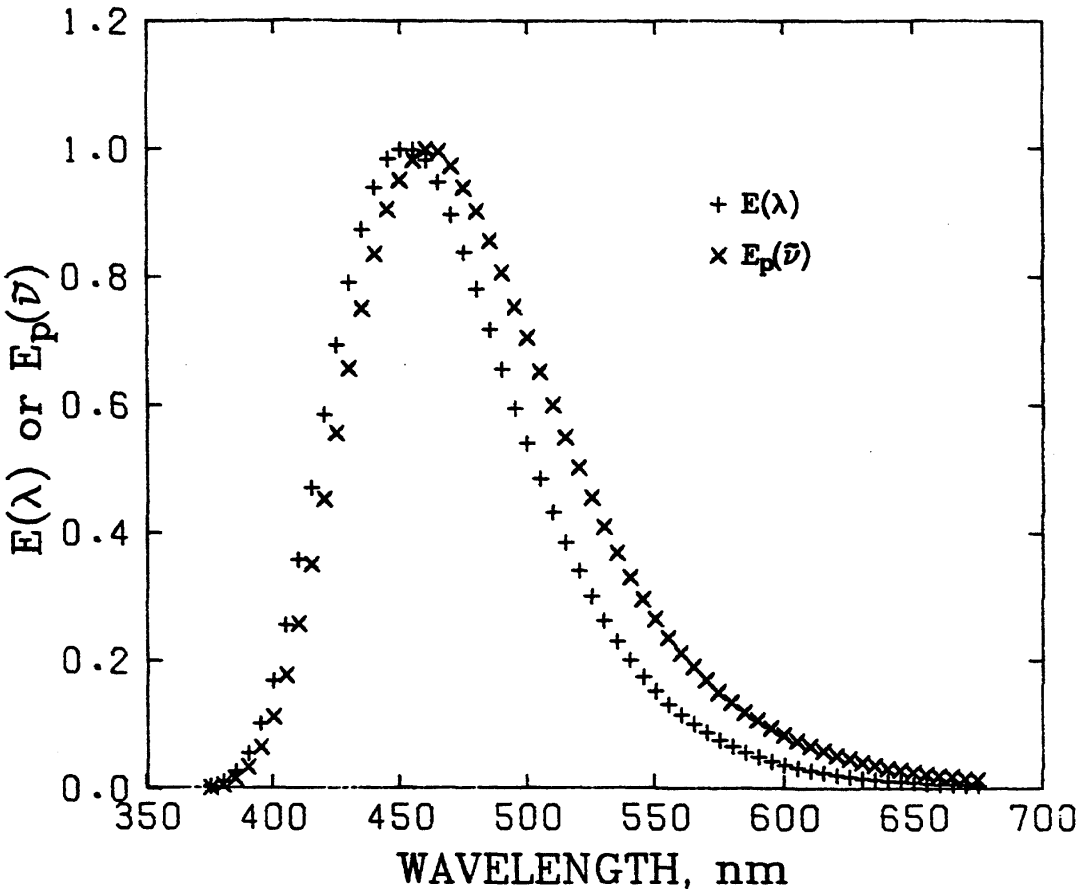


Figure 33. The corrected emission spectra of quinine sulfate in $E(\lambda)$ and $E_p(\tilde{\nu})$ units. Solvent = 0.1 mol/L $HClO_4$; λ_{ex} = 347.5 nm, T = 25.0 °C.

IX. Composite Errors and Precisions

A. Errors

Three basic sources of error have to be considered when estimates of the total errors in the $E^T(\lambda)$ and $E(\lambda)$ values are determined. These are: (1) the errors in the values determined for the spectral responsivity of the detection system; (2) the error in the wavelength position of the peak maximum; and (3) systematic errors such as those presented in Sections VII-A-F for which corrections can be made.

1. Errors in the Spectral Responsivity of the Detection System

The relative errors in the spectral responsivity for the detection system are summarized in table 20 and consist of (1) the quadrature addition of individual errors in the various components in the calibration procedure of the standard lamp (e.g., uncertainties in black body quality, temperature determination of the black body, current measurement, etc.) (2) spectral radiance changes as a function of operating time and current; and (3) laboratory lamp transfer (e.g., lamp alignment, quartz envelope reflections, convection air currents, soldered socket contacts, etc.).

In the calculation of the spectral responsivity of the detection system, the spectral radiance values reported for the standard lamp were normalized at 500 nm, equation (22). Thus, the error associated with the spectral radiance value at 500 nm was propagated through all the spectral radiance values. This additional error component in each normalized value was added in quadrature to the error for the individual L value to give the normalized errors NE:

$$NE = \sqrt{\left(\frac{\Delta L}{L}\right)^2 + \left(\frac{\Delta L_n}{L_n}\right)^2} \qquad (25)$$

The NE values for the spectral responsivity are given in Table 21 and depicted graphically in figure 34. [Note: In order to calculate the relative errors in $E^T(\lambda)$ and $E(\lambda)$, Table 17, the estimated percent error in the spectral radiance values were determined at 5 nm wavelength intervals by use of an Omnitab interpolation routine [56].]

Table 21. Percent Error Limits (PEL) at the 95 Percent Confidence Level for the Spectral Responsivity of the Detection System.

Wavelength nm	PEL	Normalized PEL
350	2.28	2.70
400	2.70[a]	3.06
450	1.80	2.32
550	1.30	1.96
654.6	1.10	1.84
750.0	1.08	1.82
850.0	1.56[a]	2.14

[a] Value includes the averages of the errors for the 8.803 and 6.000 A data.

2. Error in the Emission Peak Maximum

Due to the broad character of the quinine sulfate emission peak, the computed peak maxima for $E^T(\lambda)$ and $E(\lambda)$ have an uncertainty of ±0.2 nm in wavelength position. This uncertainty in wavelength gives rise to possible errors in the $E^T(\lambda)$ and $E(\lambda)$ values which were calculated at selected wavelengths by determining $\Delta E(\lambda)/\Delta\lambda$ at intervals of 0.02 nm.

These estimated errors are summarized in Table 22 and plotted in figure 34. They vary from a high of 2.44 percent at 375 nm to a low of ∿0.01 percent near the peak maximum.

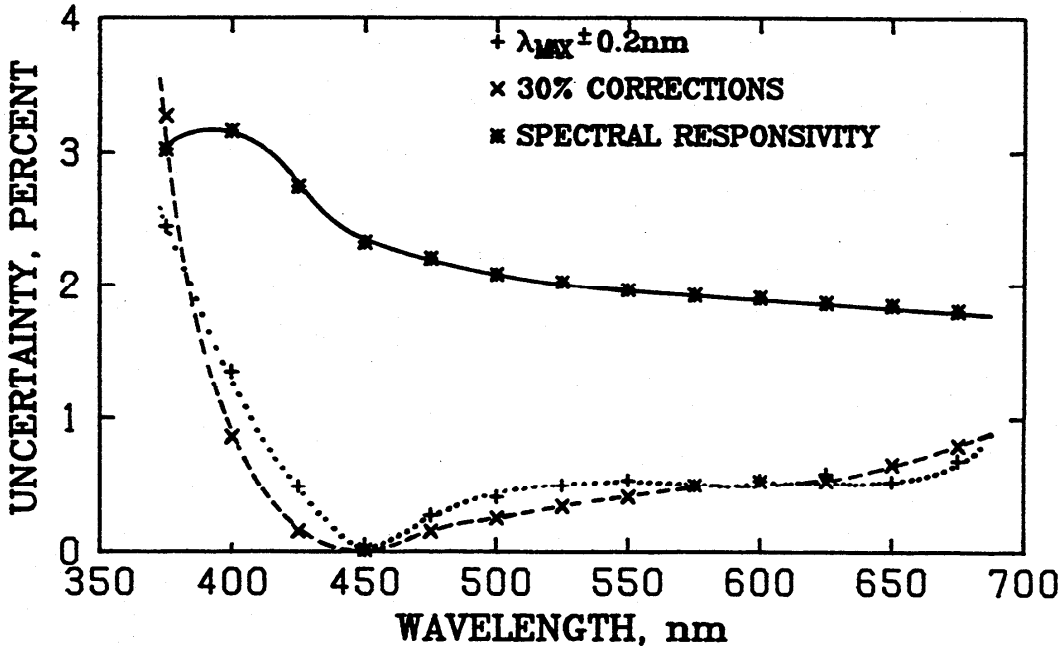


Figure 34. Percent estimated errors in the reported values at the 95 percent confidence level due to the calibration of the standard lamp, the 0.2 nm uncertainty in the emission peak position, and the errors estimated at 30 percent of the individual corrections for systematic error.

Table 22. Summary of Estimated Component Errors and Percent Systematic Error Limits in $E^T(\lambda)$ and $E(\lambda)$ Values.

	Estimated Errors, Percent								
λ, nm	SL[a]	PM[b] ±0.2 nm	PMT[c]	B.W.[d]	λ_E[e]	PSEL $E^T(\lambda)$[f]	R.I.[g]	τ[h]	PSEL $E(\lambda)$[i]
375	3.02	2.44	0.008	2.772	0.158	8.40	0.305	0.030	8.73
400	3.16	1.35	.008	0.576	.076	5.17	.185	.018	5.37
425	2.74	0.49	.004	.030	.026	3.29	.087	.007	3.38
450	2.32	.01	.000	.000	.000	2.33	.006	.000	2.34
475	2.20	.27	.002	.060	.014	2.55	.061	.007	2.62
500	2.08	.41	.004	.102	.014	2.61	.119	.013	2.74
525	2.02	.50	.006	.135	.008	2.67	.168	.021	2.86
550	1.96	.54	.008	.168	.001	2.68	.212	.022	2.91
575	1.92	.50	.008	.201	.009	2.64	.250	.026	2.92
600	1.90	.54	.008	.189	.020	2.66	.283	.029	2.97
625	1.86	.59	.008	.147	.027	2.63	.313	.032	2.98
650	1.84	.53	.008	.237	.047	2.66	.340	.034	3.03
675	1.80	.69	.008	.353	.043	2.89	.364	.038	3.29

[a] Spectral responsivity of detection system Section VIII C.
[b] Peak maximum Section VIII-A-2.
[c] Photomultiplier nonlinearity, Section VII-A-3.
[d] Bandwidth, Section VII-B.
[e] Monochromator wavelength error, Section VII C.
[f] Determined from equation (26).
[g] Refractive Index, Section VII-E.
[h] Cuvette window transmittance, Section VII-F.
[i] Determined from equation (27).

3. Errors in the Applied Correction Factors

The corrections calculated from theoretical considerations for the systematic errors attributable to experimental and instrumental parameters (Sections VII-A-F) involve assumptions that introduce errors into the corrections and thus into the $E^T(\lambda)$ and $E(\lambda)$ values. These errors are estimated to be ∿30 percent of the correction and are listed in Table 22 at 25 nm intervals. The error for all the corrections were obtained by addition of the absolute values of the individual errors and are plotted in figure 35. (See Section IX-A-5 below).

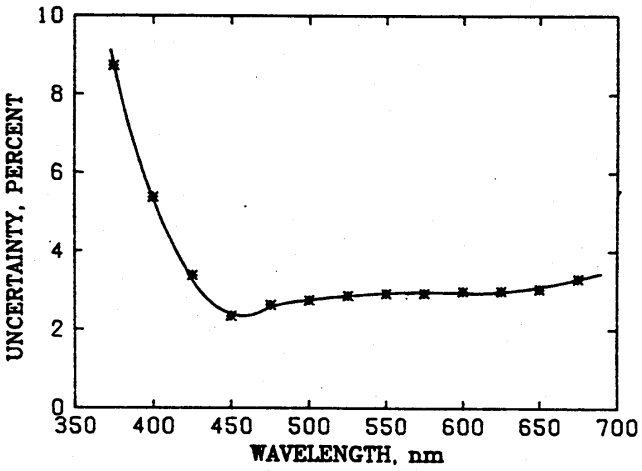


Figure 35. The percent systematic error limits in the values for the corrected emission spectrum of quinine sulfate in 0.105 mol/L $HClO_4$ at the 95 percent confidence level.

4. Percent Systematic Error Limits in the $E^T(\lambda)$ Values, PSEL $E^T(\lambda)$

The uncertainties in the $E^T(\lambda)$ values, PSEL $E^T(\lambda)$, were calculated by adding the absolute values of the uncertainties in the spectral responsivity, the peak maximum, and the applied corrections, for the instrumental factors only, equation (26):

$$\text{PSEL } E^T(\lambda) = \frac{\Delta SL}{SL} + \frac{\Delta PM}{PM} + \frac{\Delta PMT}{PMT} + \frac{\Delta BW}{BW} + \frac{\Delta \lambda_E}{\lambda_E} \quad (26)$$

The PSEL $E^T(\lambda)$ values are summarized in column 7 of Table 22 at 25 nm intervals and in Table 17 at 5 nm intervals.

5. Percent Systematic Error Limits in the $E(\lambda)$ Values, PSEL $E(\lambda)$

The percent systematic error limits in the $E(\lambda)$ values were determined by the addition of the errors used in determining PSEL $E^T(\lambda)$ plus the additional error estimates in the applied correction factors for the sample parameters of refractive index and cuvet window transmittance:

$$\text{PSEL}[E(\lambda)] = \text{PSEL } E^T(\lambda) + \frac{\Delta N}{N} + \frac{\Delta \tau}{\tau} \quad (27)$$

These values are summarized in the last column of Table 22 at 25 nm intervals and in Table 17 at 5 nm intervals and also are plotted in figure 35b.

B. Precision

The relative standard errors calculated for the $E(\lambda)$ values are experimental precision values at the 67 percent confidence level and are given in Table 17 and plotted in figure 24. They are compatible with the usual propagation of error and precision treatments (see discussion in Section VIII-C and D).

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[46] (a) The semi-micro balance was accurate to ±0.00001 g as determined by using NBS #6273D calibrated weights. (b) The top loader balance was accurate to ±0.01 g as determined by using NBS #3617 calibrated weights.

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[59] A Tungsten lamp similar to the calibrated lamp was used.

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[61] In this paper, weight absorptivity is replaced by specific weight absorbance. The specific weight absorbance, WA in $g_{solv}(g_{sol})^{-1}cm^{-1}$ is calculated by:

$$WA = \frac{A}{cb}$$

where A is the absorbance
b = cell pathlength in cm
c = sample concentration in $g_{sol}(g_{solv})^{-1}$.

[62] Under typical conditions the emission spectrum of quinine is measured at the 1 ppm (1×10^{-6} mol/L) level. Assuming the impurity is homogeneously distributed in the original quinine sulfate material, is present at the 0.1 percent level, and has a molecular weight similar to quinine sulfate (~783 g/mol), then the impurity concentration is:

$$C = (10^{-9}g/mL)(10^{3}g/L)(1\ mL/g)(783\ g/mol)^{-1}$$

$$C = 1.3 \times 10^{-9} mol/L$$

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[111] The normalization wavelengths were: quinine sulfate data ∿450 nm, fitting interval 435 to 470 nm; 6.0000 amp standard lamp data - ∿500 nm, fitting interval 475 to 525 nm; 8.8030 amp standard lamp data - 420 nm, fitting interval 400 to 425 nm.

[112] The data were "fitted" using polynomials of the 3rd to 6th order. The 3rd order polynomial was adequate to smooth the data at λ_{max} such that the standard deviation of the precision for the predicted value of λ_n (which would be used to normalize the spectrum) was better than the standard deviation in the data.

[113] In general, precisions of $\sim$0.2 percent were obtained for a single point and since four spectra were combined, the precision should be $0.2/\sqrt{4}$ or $\sim$0.1 percent.

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U.S. Department of Commerce
Juanita M. Kreps
Secretary

National Bureau of Standards
Ernest Ambler, Director

National Bureau of Standards

Certificate

Standard Reference Material 936

Quinine Sulfate Dihydrate

R. A. Velapoldi and K. D. Mielenz

This Standard Reference Material is intended for use in the evaluation of methods and the calibration of fluorescence spectrometers. It is certified for the relative molecular emission spectrum, E(λ), in radiometric units for a solution of 1.28×10^{-6} mol/L quinine sulfate dihydrate in 0.105 mol/L perchloric acid using an excitation wavelength of 347.5 nm. The certified values of the molecular emission spectrum at 5 nm wavelength intervals from 375 to 675 nm are given in table 1. These values have been corrected for instrument and sample parameters, including the spectral responsivity of the detection system, monochromator bandwidth, photomultiplier tube nonlinearity, monochromator wavelength error, solvent refractive index, and cell window transmittance. The relative standard error in E(λ), RSE [E(λ)], is given in table 1. The estimate of the relative systematic error limits in the molecular emission spectrum, RSEL [E(λ)], is also given in table 1 and was determined by the addition of the absolute values of the estimated systematic errors. These relative error limits include uncertainties in the calibration values for the spectral responsivity, the wavelength position of the emission peak maximum, and in the corrections applied for instrument and sample parameters.

From the certified values of E(λ), values may be calculated for the molecular emission spectrum in the various photon, radiometric, wavelength, and wavenumber units using the following equation: [1,2]

$$E(\lambda) = \frac{E_p(\lambda)}{\lambda} = \frac{E(\tilde{\nu})}{\lambda^2} = \frac{E_p(\tilde{\nu})}{\lambda^3}$$

These values have been calculated and are given in NBS Special Publication 260-64.

The technical emission spectrum, $E^T(\lambda)$, i.e., the emission spectrum corrected for instrument parameters only, is also given in SP 260-64. The quinine sulfate dihydrate used for SRM 936 was a special lot of material obtained from the J. T. Baker Chemical Co., Phillipsburg, N.J.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by T. W. Mears and R. W. Seward.

Washington, D.C. 20234
April 1, 1979

George A. Uriano, Chief
Office of Standard Reference Materials

(over)

Table 1. The Molecular Emission Spectrum, E(λ), of Quinine Sulfate Dihydrate in 0.105 mol/L $HClO_4$, the Relative Standard Error, RSE, and the Estimated Relative Systematic Error Limits, RSEL, in the E(λ) Values.

λ,nm	E(λ)	RSE [E(λ)]	RSEL [E(λ)]	λ,nm	E(λ)	RSE [E(λ)]	RSEL [E(λ)]
375.0	0.005	0.019	0.087	525.0	0.302	0.001	0.029
380.0	.012	.006	.078	530.0	.264	.003	.029
385.0	.028	.003	.071	535.0	.231	.003	.029
390.0	.057	.003	.064	540.0	.201	.002	.029
395.0	.103	.002	.059	545.0	.175	.002	.029
400.0	.170	.002	.054	550.0	.153	.001	.029
405.0	.257	.003	.049	555.0	.132	.001	.029
410.0	.359	.003	.045	560.0	.116	.001	.029
415.0	.471	.003	.041	565.0	.101	.002	.029
420.0	.586	.003	.037	570.0	.088	.002	.029
425.0	.694	.003	.034	575.0	.076	.003	.029
430.0	.792	.002	.031	580.0	.065	.003	.029
435.0	.874	.002	.028	585.0	.057	.001	.029
440.0	.940	.001	.026	590.0	.050	.003	.030
445.0	.984	.001	.024	595.0	.043	.004	.030
450.0	.999	.001	.023	600.0	.037	.006	.030
455.0	.997	.001	.023	605.0	.032	.002	.030
460.0	.982	.001	.024	610.0	.028	.006	.030
465.0	.947	.001	.024	615.0	.024	.003	.030
470.0	.897	.001	.025	620.0	.021	.011	.030
475.0	.838	.002	.026	625.0	.018	.003	.030
480.0	.782	.002	.027	630.0	.016	.015	.030
485.0	.719	.002	.027	635.0	.014	.014	.030
490.0	.657	.002	.027	640.0	.011	.037	.030
495.0	.595	.003	.027	645.0	.010	.015	.030
500.0	.541	.002	.027	650.0	.009	.027	.030
505.0	.486	.001	.028	655.0	.008	.035	.031
510.0	.434	.003	.028	660.0	.007	.073	.031
515.0	.386	.003	.028	665.0	.006	.046	.032
520.0	.342	.002	.028	670.0	.005	.053	.032
				675.0	.004	.065	.033

SUPPLEMENTARY DATA

The following data for the specific molar absorbances, water content, photon yields, and fluorescence lifetimes are considered to be supplementary and are not to be considered certified values.

The quinine sulfate dihydrate (QSD) used for SRM 936 was found to be homogeneous to better than 0.5% by thin-layer chromatography with development by two solvent systems and the determination of specific molar absorbances, ϵ, at three different wavelengths. The SRM contains approximately 1.7% of an impurity as determined by high performance liquid chromatography using absorbance and fluorescence detection. This impurity is believed to be dihydroquinine sulfate dihydrate, which has optical characteristics that are similar to those of the quinine sulfate dihydrate. The ultraviolet absorption spectrum of SRM 936 in 0.105 mol/L $HClO_4$ exhibits the following absorption maxima:

$$250.0 \text{ nm}, \epsilon_{max} = 56{,}990 \pm 90 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$$
$$347.5 \text{ nm}, \epsilon_{max} = 10{,}810 \pm 20 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$$

and, on the side of a peak:

$$365.0 \text{ nm}, \epsilon_{obs} = 6{,}920 \pm 10 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$$

The water content of this material was measured by two methods. The average of six determinations by the Karl-Fischer method gave a value of (4.74 ± 0.05%), while the average of four determinations by a weight loss procedure gave a value of (4.57 ± 0.04%). The theoretical value for water in quinine sulfate dihydrate is 4.60%.

The photon yield, Q, and the fluorescence lifetime, τ, of SRM 936 were compared to values obtained for a sample of purified quinine sulfate dihydrate and are summarized below:

	Q 0.5 mol/L H_2SO_4	τ, ns 0.5 mol/L H_2SO_4
SRM 936, QSD	0.544 ± 0.03	19.1 ± 0.1
Purified QSD	0.546[a]	19.2 ± 0.1

[a]Melhuish, W. H., J. Phys. Chem. 65, 229 (1961); *ibid,* New Zealand J. Sci. Tech. 37, 142 (1955).

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PREPARATION AND USE OF SRM 936

This Standard Reference Material is for "in vitro" diagnostic use as a clinical laboratory standard. A "stock" standard solution containing 0.1 mg/mL of quinine sulfate may be prepared as follows: Weigh 0.100 g of SRM 936 to the nearest one-tenth milligram and quantitatively transfer it to a 1000-mL volumetric flask. Dilute to the calibrated volume with 0.105 mol/L $HClO_4$, to give a solution that is 1.28×10^{-4} mol/L (0.1 mg/mL) in quinine sulfate. Store this solution in the dark in a well-stoppered, glass bottle. A "working" standard solution containing 1 μg/mL may be prepared by transferring 10 mL of the above "stock" standard solution to a 1000-mL volumetric flask and diluting to the calibrated volume with 0.105 mol/L $HClO_4$ to give a solution that is 1.28×10^{-6} mol/L (1 μg/mL) in quinine sulfate. Store this solution in the same manner as the above "stock" standard solution.

Several opinions regarding the stability of quinine sulfate solutions have appeared in the literature [3]. NBS considers the 0.1 mg/mL "stock" standard solution prepared from SRM 936 to be stable for 3 months when stored as specified; and the 1 μg/mL "working" standard solution to be stable for 1 month when so stored.

SRM 936 should be kept in its original bottle and stored in the dark at room temperature (30 °C or less). It should not be subjected to heat or direct sunlight during storage. Experience at NBS indicates that under proper storage this material is stable for at least 3 years. If this material degrades beyond the limits certified, purchasers will be notified by NBS. It is recommended that the material not be used after 3 years from the date of purchase.

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This Standard Reference Material has been measured and certified at the laboratories of the National Bureau of Standards, Gaithersburg, Maryland. All inquiries should be addressed to:

Office of Standard Reference Materials
Room B311, Chemistry Building
National Bureau of Standards
Washington, D.C. 20234

The date of issuance and certification of SRM 936 was April 1, 1979

Appendix B

Supplemental Analytical Data[a] for SRM Grade Quinine Sulfate Dihydrate

Product No. 4891
Lot Number: 517677
Date of Analysis: 6-23-75

Formula: $(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4 \cdot 2H_2O$
FW 782.95

	ACTUAL ANALYSIS
Assay, Anhydrous Basis ($(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4$) (by non-aqueous acid-base titration)	100.2%[a]
Elemental Analysis (anhydrous basis)[c]	
Carbon (C) (by combustion)	64.50%
Hydrogen (H) (by combustion)	6.74%
Nitrogen (N) (by Kjeldahl)	7.36%
Sulfur (S) (as sulfate by barium sulfate gravimetry)	12.83%
Absence of Other Cinchona Alkaloids	passes test
Fluorescence Excitation and Emission Curves in 0.1 N Sulfuric Acid	authentic
Infrared Spectrum	authentic
Particulate Matter (after solution in hydrochloric acid)	0.001%
Residue after Ignition	0.001%
Specific Rotation[d]	-242°
Thin-Layer Chromatography[e]	no extraneous fluorescent spot
Ultraviolet-Visible Absorption Spectrum in 0.1 N Sulfuric Acid	authentic; smooth curve
Water (H_2O)[f]	4.5%
Weight Absorptivity at 25°C in 0.1 N Sulfuric Acid (kg per g-cm)	
at 250.0 nm	76.3
at 347.5 nm	14.2

	ACTUAL ANALYSIS
NON-METALLIC IMPURITIES in parts per million (ppm)	
Boron (B)[g]	0.6
Halide (as Cl)	12
Silicon (Si)[g]	0.3
METALLIC IMPURITIES[g] in parts per million (ppm)	
Aluminum (Al)	0.4
Bismuth (Bi)	< 0.1
Cadmium (Cd)	< 2
Calcium (Ca)	< 1
Chromium (Cr)	0.1
Cobalt (Co)	< 0.1
Copper (Cu)	0.03
Iron (Fe)	0.6
Lead (Pb)	0.2
Magnesium (Mg)	0.007
Manganese (Mn)	0.03
Molybdenum (Mo)	< 0.02
Nickel (Ni)	0.2
Silver (Ag)	0.04
Sodium (Na)	5
Tin (Sn)	< 0.1
Titanium (Ti)	0.03
Vanadium (V)	< 0.02
Zinc (Zn)	< 2

[a]Analytical data supplied by J. T. Baker Chemical Company, Phillipsburg, New Jersey 08865.

[b]Average value for 2 determinations: 100.2% and 100.2%.

[c]Average value for duplicate determinations: carbon, 64.58% and 64.42%; hydrogen, 6.72% and 6.75%; nitrogen, 7.32% and 7.39%; and sulfur (as sulfate), 12.83% and 12.83%.

[d]$[a]_D^{20}$, anhydrous basis, c=2 in 0.1 N hydrochloric acid.

[e]No extraneous fluorescent spot on Baker-flex^R Silica Gel IB2 TLC flexible sheets on separate development with 1-propanol-88% formic acid (80:20 v/v) and chloroform-methanol-acetic acid (75:20:5 v/v) and ultraviolet illumination.

[f]By Karl Fischer titration; average value for 2 determinations: 4.48% and 4.59%.

[g]Average value for duplicate samples analyzed by dc-arc spectrography (after 50-fold concentration by heating with sulfuric acid followed by ashing with addition of lithium carbonate; against commercial standards in lithium carbonate, reading of lines in 2450-3875 A region); key elements found absent are reported as < (less than) the detection limit.

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