Designation: E2719 – 09 (Reapproved 2014)

Standard Guide for Fluorescence—Instrument Calibration and Qualification[1]

This standard is issued under the fixed designation E2719; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide (1)[2] lists the available materials and methods for each type of calibration or correction for fluorescence instruments (spectral emission correction, wavelength accuracy, and so forth) with a general description, the level of quality, precision and accuracy attainable, limitations, and useful references given for each entry.

1.2 The listed materials and methods are intended for the qualification of fluorometers as part of complying with regulatory and other quality assurance/quality control (QA/QC) requirements.

1.3 Precision and accuracy or uncertainty are given at a 1 σ confidence level and are approximated in cases where these values have not been well established.[3]

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[4]

E131 Terminology Relating to Molecular Spectroscopy

E388 Test Method for Wavelength Accuracy and Spectral Bandwidth of Fluorescence Spectrometers

E578 Test Method for Linearity of Fluorescence Measuring Systems

E579 Test Method for Limit of Detection of Fluorescence of Quinine Sulfate in Solution

3. Terminology

3.1 *Definitions*(2)*:*

3.1.1 *absorption coefficient (α), n*—a measure of absorption of radiant energy from an incident beam as it traverses an absorbing medium according to Bouguer's law, $I/I_o = e^{-\alpha b}$, where I and I_o are the transmitted and incident intensities, respectively, and b is the path length of the beam through the sample. **E131**

3.1.1.1 *Discussion*—Note that transmittance $T = I/I_o$ and absorbance $A = -\log T$.

3.1.2 *absorptivity (a), n*—the absorbance divided by the product of the concentration of the substance and the sample pathlength, $a = A/bc$. **E131**

3.1.3 *Beer-Lambert law, n*—relates the dependence of the absorbance (A) of a sample on its path length (see *absorption coefficient*, α) and concentration (c), such that $A = a\,bc$.

3.1.3.1 *Discussion*—Also called Beer's law or Beer-Lambert-Bouquer law. **E131**

3.1.4 *calibrated detector (CD), n*—optical radiation detector whose responsivity as a function of wavelength has been determined along with corresponding uncertainties (**3**).

3.1.5 *calibrated diffuse reflector (CR), n*—Lambertian reflector whose reflectance as a function of wavelength has been determined along with corresponding uncertainties (**4**).

3.1.6 *calibrated optical radiation source (CS), n*—optical radiation source whose radiance as a function of wavelength has been determined along with corresponding uncertainties (**5, 6**).

3.1.7 *calibration, n*—set of procedures that establishes the relationship between quantities measured on an instrument and the corresponding values realized by standards.

3.1.8 *certified reference material (CRM), n*—material with properties of interest whose values and corresponding uncertainties have been certified by a standardizing group or organization. **E131**

3.1.9 *certified value, n*—value for which the certifying body has the highest confidence in its accuracy in that all known or

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] Certain commercial equipment, instruments, or materials are identified in this guide to foster understanding. Such identification does not imply recommendation or endorsement by ASTM International nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

[4] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

suspected sources of bias have been investigated or accounted for by the certifying body (**7**).

3.1.10 *diffuse scatterer, n*—material that scatters optical radiation in multiple directions; this includes diffuse reflectors, which are often Lambertian, and scattering solutions, which are not Lambertian.

3.1.11 *fluorescence anisotropy (r), n*—measure of the degree of polarization of fluorescence, defined as $r = (I_{II} - I_{\perp})/(I_{II} + 2I_{\perp})$, where I_{II} and $I_{\perp}$ are the observed fluorescence intensities when the fluorometer's emission polarizer is oriented parallel and perpendicular, respectively, to the direction of the polarized excitation.

3.1.12 *fluorescence band, n*—region of a fluorescence spectrum in which the intensity passes through a maximum, usually corresponding to a discrete electronic transition.

3.1.13 *fluorescence lifetime, n*—parameter describing the time decay of the fluorescence intensity of a sample component; if a sample decays by first-order kinetics, this is the time required for its fluorescence intensity and corresponding excited state population to decrease to 1/*e* of its initial value.

3.1.14 *fluorescence quantum efficiency, n*—ratio of the number of fluorescence photons leaving an emitter to the number of photons absorbed.

3.1.15 *fluorescence quantum yield (Φ), n*—probability that a molecule or species will fluoresce once it has absorbed a photon.

3.1.15.1 *Discussion*—This quantity is an innate property of the species and is typically calculated for a sample as the ratio of the number of molecules that fluoresce to the number of molecules that absorbed.

3.1.16 *flux (or radiant flux or radiant power), n*—rate of propagation of radiant energy typically expressed in Watts.

3.1.17 *grating equation, n*—relationship between the angle of diffraction and wavelength of radiation incident on a grating, that is, $m\lambda = d(\sin\alpha + \sin\beta)$, where *d* is the groove spacing on the grating; α and β are the angles of the incident and diffracted wavefronts, respectively, relative to the grating normal; and *m* is the diffraction order, which is an integer (**8**).

3.1.18 *inner filter effects, n*—decrease in the measured quantum efficiency of a sample as a result of significant absorption of the excitation beam, reabsorption of the emission of the sample by itself, or both, and this causes the measured quantum efficiency to be dependent on the absorbance, concentration, and excitation and emission path lengths of the sample (**9, 10**).

3.1.19 *Lambertian reflector, n*—surface that reflects optical radiation according to Lambert's law, that is, the optical radiation is unpolarized and has a radiance that is isotropic or independent of viewing angle.

3.1.20 *limit of detection, n*—estimate of the lowest concentration of an analyte that can be measured with a given technique, often taken to be the analyte concentration with a measured signal-to-noise ratio of three.

3.1.21 *noise level, n*—peak-to-peak noise of a blank.

3.1.22 *photobleaching, n*—loss of emission or absorption intensity by a sample as a result of exposure to optical radiation.

3.1.22.1 *Discussion*—This loss can be reversible or irreversible with the latter typically referred to as photodegradation or photodecomposition.

3.1.23 *qualification, n*—process producing evidence that an instrument consistently yields measurements meeting required specifications and quality characteristics.

3.1.24 *quantum counter, n*—photoluminescent emitter with a quantum efficiency that is independent of excitation wavelength over a defined spectral range.

3.1.24.1 *Discussion*—When a quantum counter is combined with a detector to give a response proportional to the number of incident photons, the pair is called a quantum counter detector.

3.1.25 *quasi-absolute fluorescence intensity scale, n*—fluorescence intensity scale that has been normalized to the intensity of a fluorescent reference sample or artifact under a fixed set of instrumental and experimental conditions.

3.1.25.1 *Discussion*—This artifact should be known to yield a fluorescence intensity that is reproducible with time and between instruments under the fixed set of conditions.

3.1.26 *Raman scattering, n*—inelastic scattering of radiation (the wavelengths of the scattered and incident radiation are not equal) by a sample that occurs because of changes in the polarizability of the relevant bonds of a sample during a molecular vibration. (See Terminology E131, *Raman spectrum*.)

3.1.26.1 *Discussion*—The radiation being scattered does not have to be in resonance with electronic transitions in the sample, unlike fluorescence (**11**).

3.1.27 *Rayleigh scattering, n*—elastic scattering of radiation by a sample, that is, the scattered radiation has the same energy (same wavelength) as the incident radiation.

3.1.28 *responsivity, n*—ratio of the photocurrent output and the radiant power collected by an optical radiation detection system.

3.1.29 *sensitivity, n*—measure of an instrument's ability to detect an analyte under a particular set of conditions.

3.1.30 *spectral bandwidth (or spectral bandpass or resolution), n*—measure of the capability of a spectrometer to separate radiation or resolve spectral peaks of similar wavelengths. (See Terminology E131, *resolution*.)

3.1.31 *spectral flux (or spectral radiant flux or spectral radiant power), n*—flux per unit spectral bandwidth typically expressed in W/nm.

3.1.32 *spectral responsivity, n*—responsivity per unit spectral bandwidth.

3.1.33 *spectral slit width, n*—mechanical width of the exit slit of a spectrometer divided by the linear dispersion in the exit slit plane. **E131**

3.1.34 *traceability, n*—linking of the value and uncertainty of a measurement to the highest reference standard or value through an unbroken chain of comparisons, where *highest*

refers to the reference standard whose value and uncertainty are not dependent on those of any other reference standards, and *unbroken chain of comparisons* refers to the requirement that any intermediate reference standards used to trace the measurement to the highest reference standard must have their values and uncertainties linked to the measurement as well (**12**).

3.1.35 *transfer standard, n*—reference standard used to transfer the value of one reference standard to a measurement or to another reference standard.

3.1.36 *transition dipole moment, n*—oscillating dipole moment induced in a molecular species by an electromagnetic wave that is resonant with an energy transition of the species, for example, an electronic transition.

3.1.36.1 *Discussion*—Its direction defines that of the transition polarization and its square determines the intensity of the transition.

4. Significance and Use

4.1 By following the general guidelines (Section 5) and instrument calibration methods (Sections 6 – 16) in this guide, users should be able to more easily conform to good laboratory and manufacturing practices (GXP) and comply with regulatory and QA/QC requirements, related to fluorescence measurements.

4.2 Each instrument parameter needing calibration (for example, wavelength, spectral responsivity) is treated in a separate section. A list of different calibration methods is given for each instrument parameter with a brief usage procedure. Precautions, achievable precision and accuracy, and other useful information are also given for each method to allow users to make a more informed decision as to which method is the best choice for their calibration needs. Additional details for each method can be found in the references given.

5. General Guidelines

5.1 General areas of concern and precautions to minimize errors for fluorescence measurements are given by topic. All topics applicable to a user's samples, measurements and analysis should be considered.

5.2 *Cuvettes*—Various types of cuvettes or optical "cells" are available. They vary in material composition and in size. The former will determine the effective spectral range of the cuvette. To check the spectral transmission characteristics, measure a cuvette's transmittance in a UV/Vis spectrophotometer, after filling it with a solvent of interest. Check to insure that the cuvettes being used transmit energy through the entire analytical wavelength range. Many organic solvents dissolve plastic, so plastic cuvettes should not be used in these cases. Standard cuvettes have inner dimensions of 10 mm × 10 mm × 45 mm. If only a small amount of sample is available, then microcuvettes can be used. Black self-masking quartz microcuvettes are recommended since they require no masking of the optical beam. Cuvette caps or stoppers should be used with volatile or corrosive solvents.

5.2.1 *Handling and Cleaning*—For highest quality work, windows should never be touched with bare hands. Clean, powder-free, disposable gloves are recommended. Cuvettes should be rinsed several times with solvent after use and stored wet in the normal solvent system being used. For prolonged storage, cuvettes should be stored dry, wrapped in lens tissue and sealed in a container. To clean a cuvette more thoroughly, it should be filled with an acid, such as 50 % concentrated nitric acid, and allowed to sit for several hours. It should then be rinsed with deionized water several times to remove all traces of acid.

TABLE 1 Spectral Transmission Characteristics of Cuvette Materials

	Wavelength Range (nm)
Glass	350 to 2500
Near Infrared Quartz	220 to 3800
Far UV Quartz	170 to 2700
Polystyrene	400 to 1000
Acrylic	280 to 1000

5.3 *Selection of Solvent*—Solvents can change the spectral shape, cause peak broadening, and alter the wavelength position of a fluorophore (**13**). Check to insure that the solvent does not itself absorb or contain impurities at the analytical wavelength(s). As standard practice, when optimizing a procedure, one should first scan the solvent using the analytical parameters to see if the solvent absorbs or fluoresces in the analytical wavelength range. This will also identify the position of the Raman band of the solvent and any second order bands from the grating. It is essential to examine the quality of solvents periodically since small traces of contaminants may be enough to produce high blank values.

5.3.1 Water is the most common solvent and deionized-distilled water should always be employed. All other reagents used in the assay should be carefully controlled and high quality or spectrophotometric grades are recommended.

5.3.2 Solvents should not be stored in plastic containers since leaching of organic additives and plasticizers can produce high blank values.

5.3.3 Reagent blanks should be measured during the analytical procedure and the actual value of the blank determined before the instrument is zeroed.

5.4 *Other Contaminants:*

5.4.1 Soaking glassware in detergent solutions is a general method of cleaning. Some commercial preparations are strongly fluorescent. Before use, the fluorescence characteristics of a dilute solution of the detergent should be measured, so that the user knows if detergent contamination is a cause for concern.

5.4.2 Stopcock grease is another common contaminant with strong native fluorescence.

5.4.3 The growth of micro-organisms in buffer or reagent solutions will affect blank values by both their fluorescence and light scattering properties.

5.4.4 Filter paper and lab wipes can be sources of contamination due to fluorescent residues. These should be checked before use.

5.5 *Working with Dilute Solutions*—It is common practice to store concentrated stock solutions and make dilutions to produce working standards. It is always better to confirm the

TABLE 2 Summary of Methods for Determining Wavelength Accuracy

Sample	λ Region	Drop-In	Off-Shelf	Precision, Accuracy	Limitations	Established Values	Refs.
Pen Lamp	UV-NIR (EM)	Maybe	Y	± 0.1 nm or better	alignment	Y	Test Method E388
Dy-YAG crystal	470nm-760nm (EM) 255nm-480nm (EX)	Y	Y	± 0.1 nm		Y	14
Eu glass	570nm-700nm (EM) 360nm-540nm (EX)	Y	Y	± 0.2 nm		N	15
Anthracene in PMMA	380nm-450nm (EM) 310nm-380nm (EX)	Y	Y	± 0.2 nm	limited range	N	
Ho_2O_3 + DR	330nm-800nm (EM or EX)	Maybe	Y	± 0.4 nm	need blank	Y	16-18
Xe Source	400nm-500nm (EX)	Y	Y	± 0.2 nm	limited range, calibration	N	19
Xe Source + DR	UV-NIR	Maybe	Y	± 0.2 nm	one mono must be calibrated	Y	19
Water Raman	UV-blue	Y	Y	± 0.2 nm	one mono must be calibrated	N	20

concentration of the stock solution spectrophotometrically before the calibration curve is prepared. Final solutions are always very dilute and should never be stored for long periods. Standards should be measured in duplicate or triplicate to insure accuracy.

5.5.1 *Adsorption*—Loss of fluorophore by adsorption onto the walls of the container can occur at low concentration levels. Glass surfaces should be thoroughly cleaned in acid before use.

5.5.2 *Photo-Decomposition and Oxidation*—Since fluorescence intensity is directly proportional to the intensity of incident light, fluorescence instruments employ intense light sources to produce high sensitivity. In some cases the level of incident light may be sufficient to decompose the sample under investigation. This should be checked and samples should be measured as quickly as possible. The presence of trace oxidizing agents, for example, dissolved oxygen or traces of peroxides, can reduce fluorescence intensity.

5.6 *Selection of Optimal Wavelength*—To choose an appropriate analyte excitation band, scan the analyte with a UV/Vis spectrophotometer to determine the absorbance maxima and to see if there is any interfering compound or scattering at the analytical wavelength. The optimal wavelength is usually that which shows the strongest absorbance and is free from interference by other components including solvent. In some cases, a lesser absorbing wavelength is selected to eliminate interferences from other compounds that absorb at the same wavelength or to avoid photobleaching.

5.7 *Selection of Spectral Bandwidth*—Ideally, one would like to select the widest slit possible to give the greatest signal to noise ratio while maintaining spectral selectivity.

6. Wavelength Accuracy

6.1 Methods for determining the accuracy of the emission (EM) or excitation (EX) wavelength for a fluorescence instrument are given here and summarized in Table 2 with an emphasis on monochromator (mono) based wavelength selection.

6.2 *Low-Pressure Atomic Lamps (see Test Method E388)*—These low-pressure atomic lamps, often referred to as pen lamps because of their size and shape, should be placed at the sample position and pointed toward the detection system for EM wavelength accuracy determination. The EM wavelength selector (λ_{EM}-selector) is then scanned over the wavelength range of interest (see Fig. 1). High accuracy is only achieved when the light from the lamp is aligned properly into the wavelength selector, for example, the optical radiation must fill the entrance slit of the monochromator. Atomic lines that are too close to each other to be resolved by the instrument should not be used. Although these lamps can be placed at the EX source position for EX wavelength accuracy determination, weaker signals are typically observed, for example, by a reference detector, and alignment is more difficult than for the EM wavelength accuracy determination.

6.3 *Dysprosium-Yttrium Aluminum Garnet (Dy-YAG) Crystal* (**14**)—This sample is available in standard cuvette format, so it can simply be inserted into a cuvette holder, referred to as "drop in" in the tables. An EX or EM spectrum is then collected for an EX or EM wavelength accuracy determination, respectively (see Fig. 2). Peaks that are too close to each other to be resolved by the instrument should not be used.

6.4 *Europium (Eu)-Doped Glass*[5] (**15**) *or Polymethylmethacrylate (PMMA)*—This sample is available in standard cuvette format, so it can simply be inserted into a cuvette holder. An EX or EM spectrum is then collected for an EX or EM wavelength accuracy determination, respectively (see Fig. 3). Accurate peak positions for this glass have not been well established, and the positions of peaks can change somewhat depending on the particular glass matrix used and sample temperature. For these reasons, a one time per sample determination of these peak positions using another wavelength calibration method is recommended.

6.5 *Anthracene-Doped PMMA*[6]—This sample is available in standard cuvette format, so it can simply be inserted into a cuvette holder. An EX or EM spectrum is then collected for an EX or EM wavelength accuracy determination, respectively (see Fig. 4).

6.6 *Holmium Oxide (Ho_2O_3) Solution or Doped Glass with Diffuse Reflector, Scatterer, or Fluorescent Dye* (**16-18**)—This

[5] Other rare earth doped glasses have narrow EX and EM transitions, but Eu-doped glass is the only one listed because it is one of the most commonly used and most readily available.

[6] Other polyaromatic hydrocarbon-doped PMMAs have narrow EX and EM transitions, including those with ovalene, *p*-terphenyl, and naphthalene.

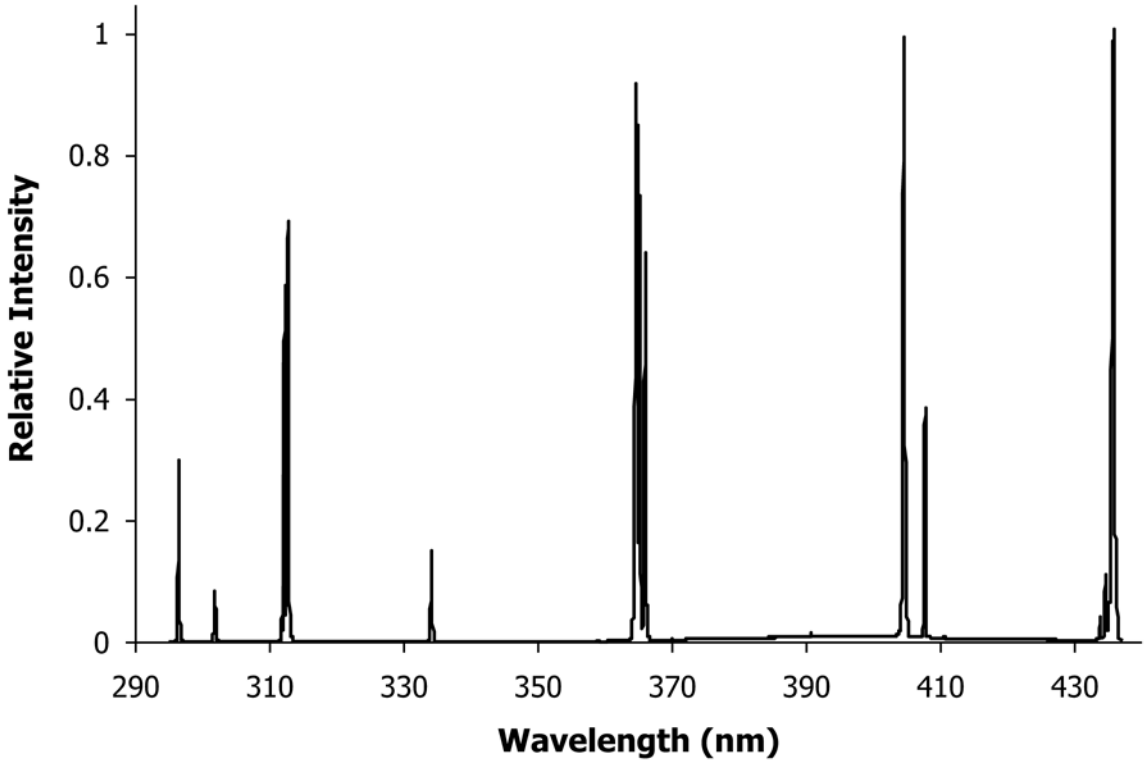

FIG. 1 Hg Pen Lamp Spectrum

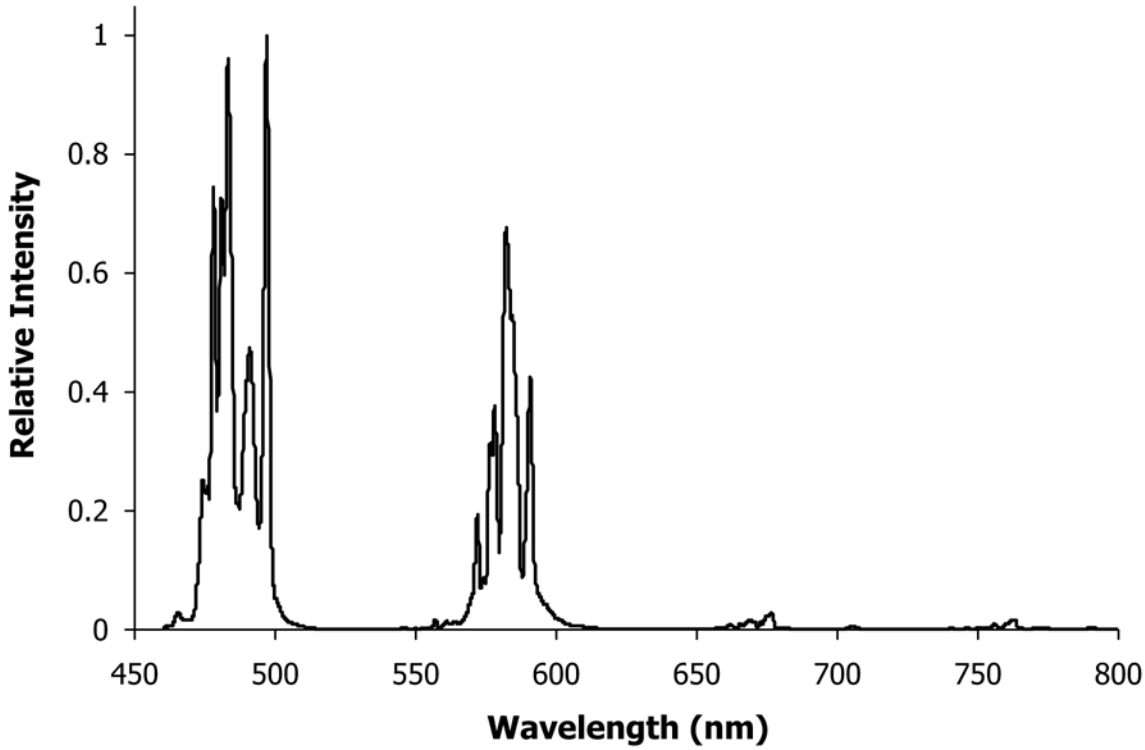

FIG. 2 EM Spectrum of a Dy-YAG Crystal Excited at 352.7 nm

sample is available in standard cuvette format, so it can simply be inserted into a cuvette holder. An EX or EM spectrum is then collected for an EX or EM wavelength accuracy determination, respectively. The wavelength selector not being scanned shall be removed or set to zero order, that is, in this position a grating behaves like a mirror reflecting all wavelengths. The diffuse reflector, scatterer, or fluorescent dye is scanned with and without the Ho_2O_3 sample in place, and the

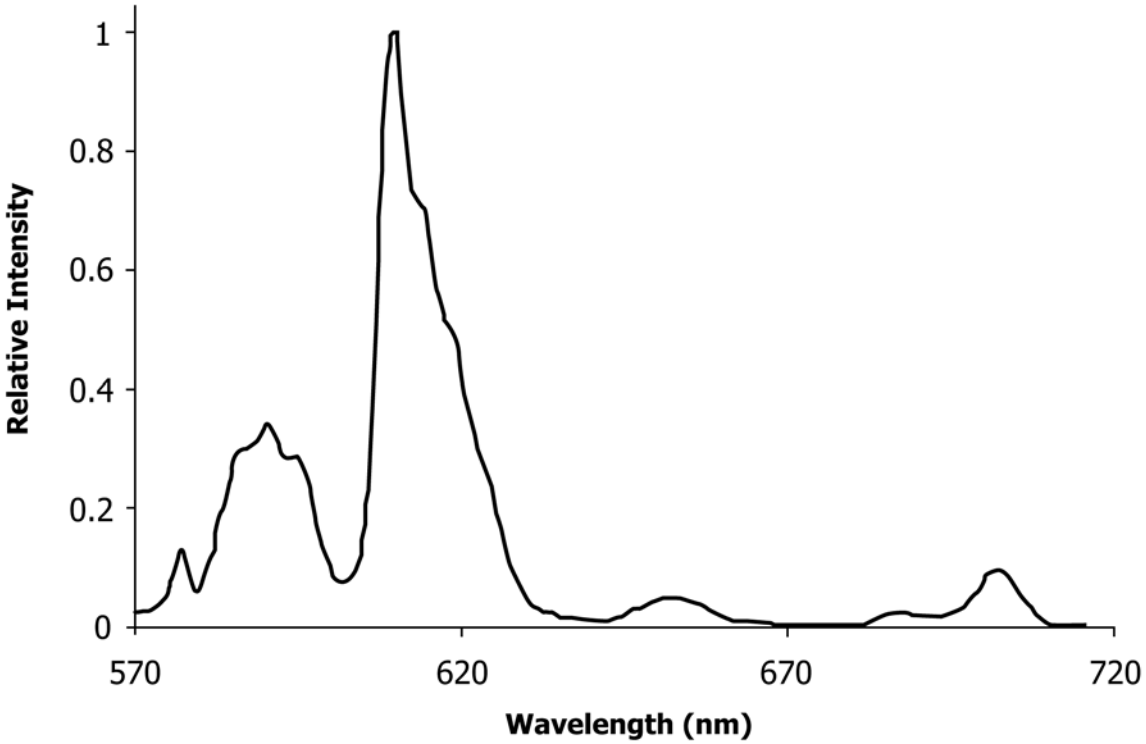

FIG. 3 EM Spectrum of a Eu-Ion-Doped Glass Excited at 392 nm

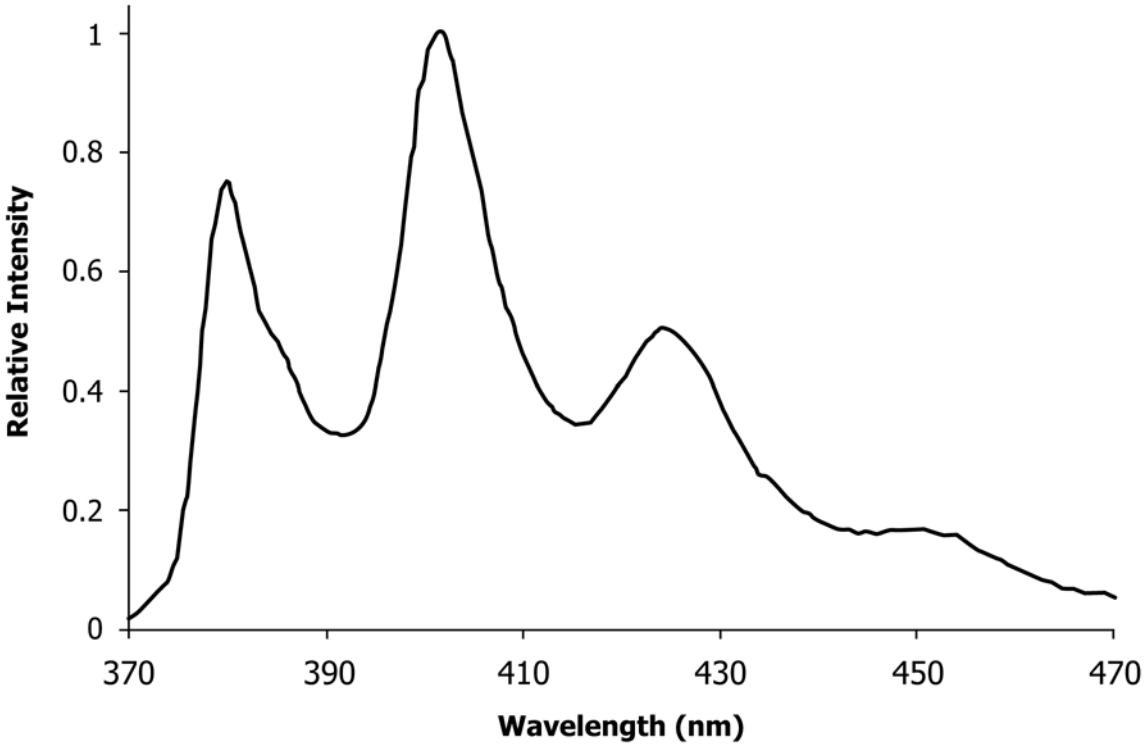

FIG. 4 EM Spectrum of Anthracene-Doped PMMA Excited at 360 nm

ratio of the two intensities is calculated to obtain an effective transmittance spectrum with dips in the intensity ratio corresponding to absorption peaks of the sample (see Fig. 5).

6.7 *Xenon (Xe) Source Lamp* (**19**)—This method is for fluorometers that use a high-pressure Xe arc lamp as an EX source. A few peaks between 400 and 500 nm can be used, but

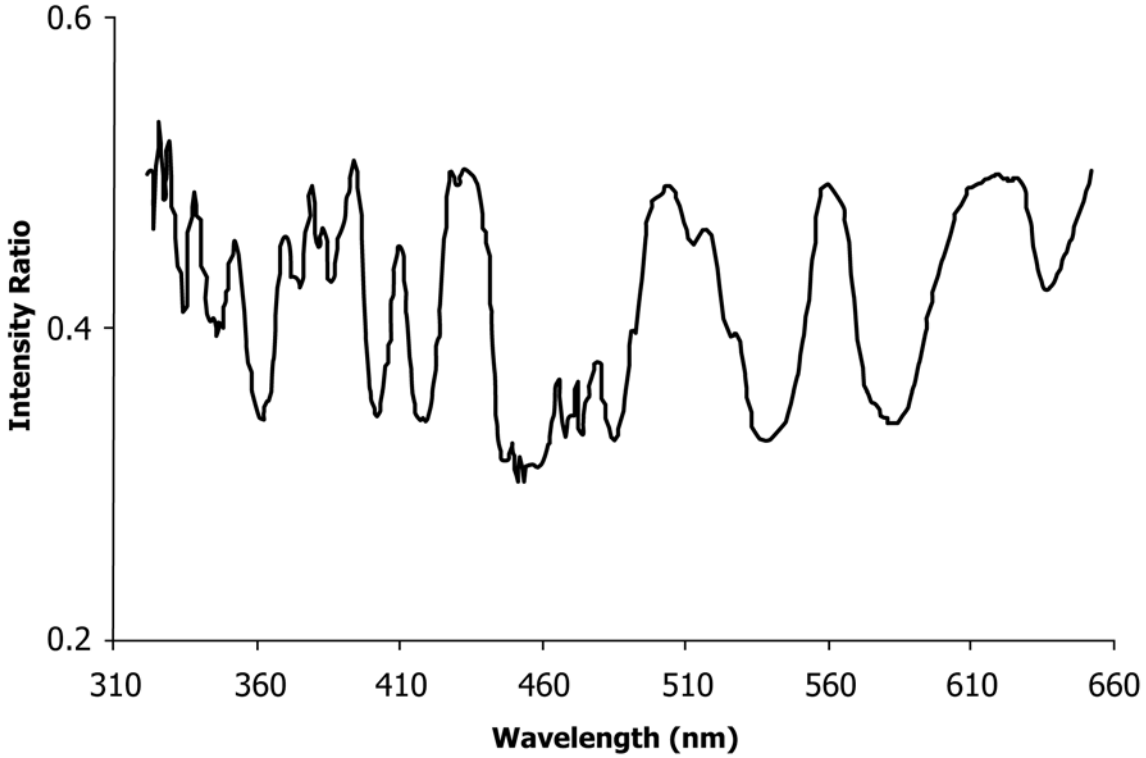

FIG. 5 Effective Transmittance Spectrum of a Ho_2O_3-Doped Glass with Diffuse Reflector

most of these are a result of multiple lines, so their positions are not well established (see Fig. 6). For this reason, a determination of these peak positions (one time per lamp) using another wavelength calibration method is recommended. For EX wavelength calibration, the EX wavelength selector

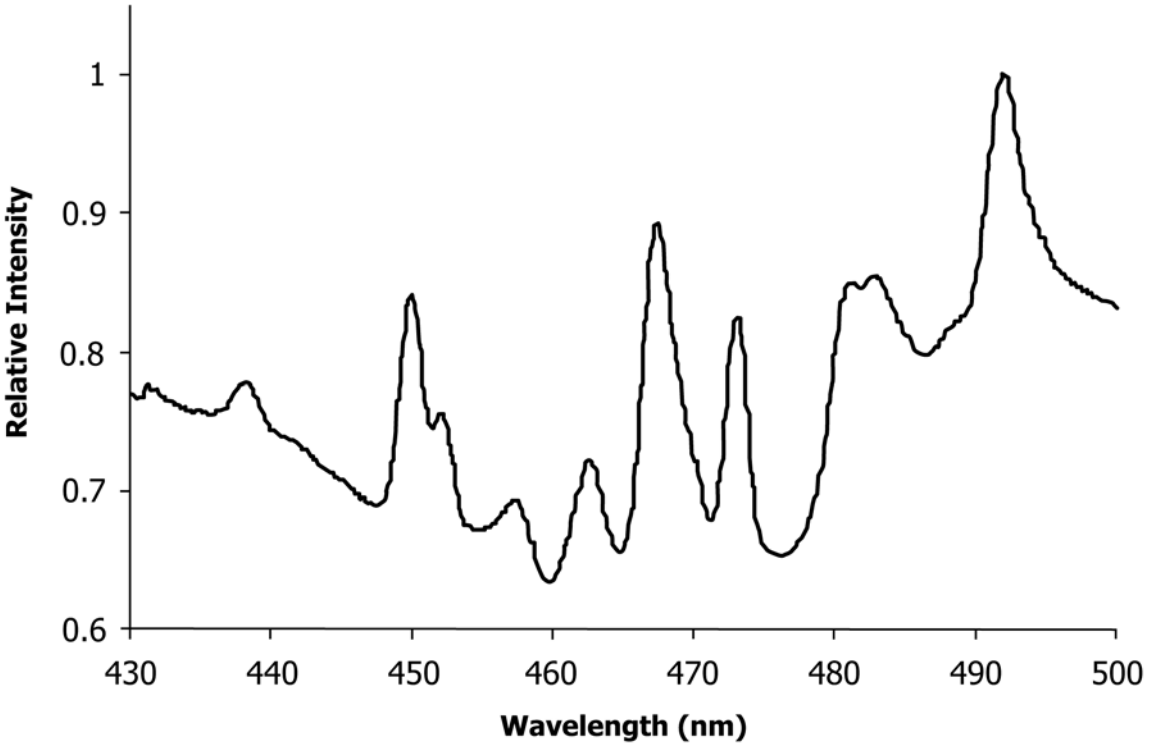

FIG. 6 Xe Source Lamp (High-Pressure, 450-W) Spectrum in a Spectral Region Containing Peak Structure

(λ_{EX}-selector) is scanned while collecting the reference detector signal. If this is used for EM wavelength calibration, a diffuse reflector or scatterer shall be placed at the sample position and the λ_{EX}-selector shall be removed or set to zero order.

6.8 *Instrument Source with Diffuse Reflector or Scatterer* (**19**)—A dilute scattering solution in a standard cuvette or a solid diffuse reflector set at 45° relative to the EX beam can be used to scatter the EX beam into the detection system. One wavelength selector is fixed at a wavelength of interest and the other scans over the fixed wavelength (see Fig. 7). The difference between the fixed wavelength and the observed peak position is the wavelength bias between the two wavelength selectors at that wavelength. Either the EX or the EM wavelength selector shall have a known accuracy at the desired wavelengths to use this method to calibrate the unknown side.

6.9 *Water Raman* (**20**)—Deionized water is used. One wavelength selector is fixed at a wavelength of interest and the other is scanned (see Fig. 8). The water Raman peak appears at a wavelength that is about 3400 cm^{-1} lower in energy than the EX wavelength (**21**). The Raman scattering intensity is proportional to λ^{-4}, so the Raman intensity quickly becomes too weak to use this method when going into the visible region. Either the EX beam or the EM wavelength selector shall have a known accuracy at the desired wavelengths to use this method to calibrate the unknown side.

7. Spectral Slit Width Accuracy

7.1 Spectral slit width accuracy of the EM or EX wavelength selector can be determined by measuring the spectral bandwidth, taken to be the full width at half the peak maximum (FWHM), of a single line of a pen lamp, using the same setup and with the same precautions described in 6.2 (see Test Method E388). For fluorescence spectrometers with both EX and EM monochromators, an alternative method may be used in which one monochromator is scanned over the position of the other using the setup described in 6.8 (**19**). The uncertainties involved in either method have not been well established, but a ±0.5 nm uncertainty or better is estimated here based on what has been reported.

8. Linearity of the Detection System

8.1 Several methods can be used to determine the linear intensity range of the detection system. They can be separated into three types based on the tools used to vary the intensity of optical radiation reaching the detector: *(1)* double aperture, *(2)* optical filters, polarizers or both, and *(3)* fluorophore concentrations. The double-aperture method is the most well established and probably the most accurate when done correctly, but it is also the most difficult to perform (**22, 23**). A variety of methods using optical filters, polarizers, or a combination of the two have been reported (**19, 24**). These methods require high-quality, often costly, components, and some user expertise. The third method is the most popular and easiest to implement. It uses a set of solutions obtained by serial dilution of a fluorescent stock solution, similar to that used for obtaining calibration curves for analyte concentration, as described in 11.3. In this case, solutions with a low concentration ($A < 0.05$ at 1-cm path length) should be used and fluorophore adsorption to cuvette walls may affect measurements at very low concentrations (see Test Method E578). In

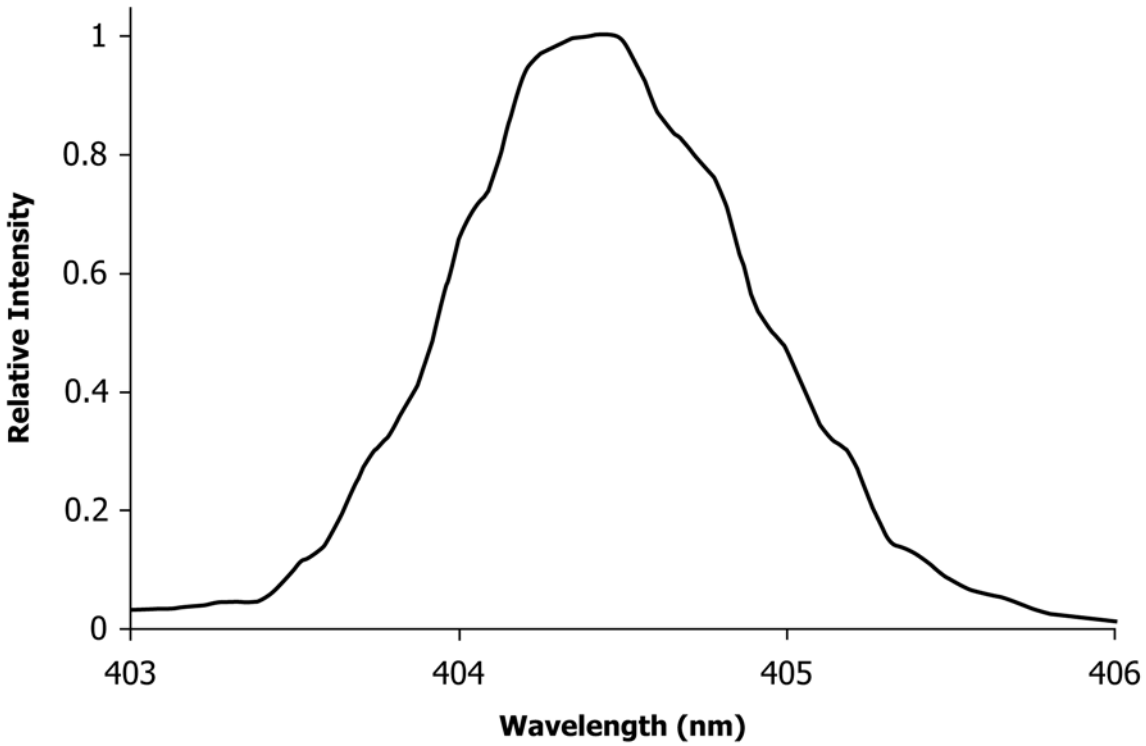

FIG. 7 EX Source Profile with EX Wavelength Fixed at 404.3 nm (EX Bandwidth of 1.0 nm) and EM Monochromator Scanned (EM Bandwidth of 0.1 nm)

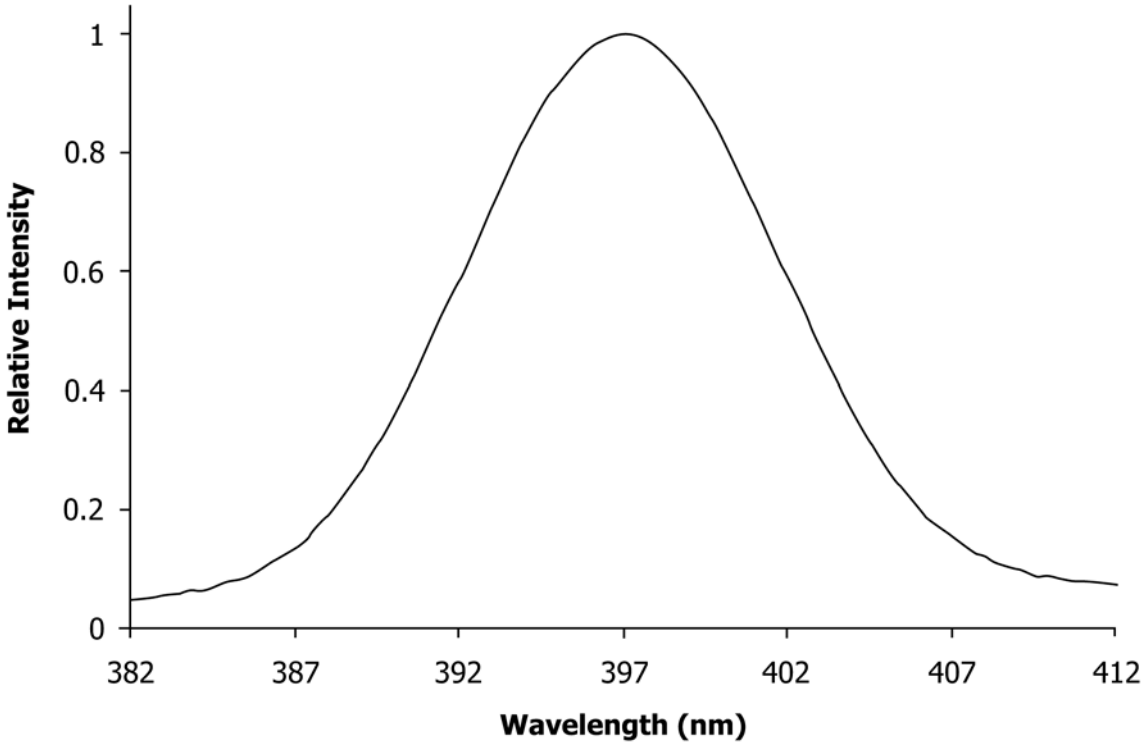

FIG. 8 Water Raman Spectrum with EX Wavelength Set at 350 nm and EX and EM Bandwidths at 5 nm

addition, fluorophores are needed that are not prone to reabsorption effects and that do reveal concentration-independent emission spectra. Users shall insure that the fluorescence signal intensities of samples are reproducible and do not decrease over the time period they are being excited and measured because the organic dyes typically used can be prone to photobleaching and other degradation over time.

9. Spectral Correction of Detection System Responsivity

9.1 Calibration of the relative responsivity of the EM detection system with EM wavelength, also referred to as spectral correction of emission, is necessary for successful quantification when intensity ratios at different EM wavelengths are being compared or when the true shape or peak maximum position of an EM spectrum needs to be known. Such calibration methods are given here and summarized in Table 3. This type of calibration is necessary because the relative spectral responsivity of a detection system can change significantly over its useful wavelength range (see Fig. 9). It is highly recommended that the linear range of the detection system be determined (see Section 8) before spectral calibration is performed and appropriate steps are taken (for example, the use of attenuators) to insure that all measured intensities during this calibration are within the linear range. Also note that when using an EM polarizer, the spectral correction for emission is dependent on the polarizer setting.

9.2 *Calibrated Optical Radiation Source (CS)–Tungsten*[7] Lamp (**19, 24-27**)—The optical radiation from a CS is directed into the EM detection system by placing the CS at the sample position. If the CS is too large to be placed at the sample position, a calibrated diffuse reflector (CR) may be placed at the sample position to reflect the optical radiation from the CS into the EM detection system. The λ_{EM}-selector is scanned over the EM region of interest, using the same instrument settings as that used with the sample, and the signal channel output (S'') is collected. The known radiance of the CS incident on the detection system (L) can be used to calculate the relative correction factor (C_{CS}), such that $C_{CS} = L/S''$. The corrected EM intensity is equal to the product of the signal output of the sample (S) and C_{CS}.

[7] Other types of calibrated lamps can be used, but tungsten is ideal in the visible range due to its broad, featureless spectral profile and high intensity.

TABLE 3 Summary of Methods for Determining Spectral Correction of Detection System Responsivity

Sample	λ Region	Drop-In	Off-Shelf	Precision, Accuracy	Limitations	Certified Values	Refs.
CS	UV-NIR	N	Y	<± 5 %	difficult setup	Y	**19, 24-27**
CD + CR	UV-NIR	N	Maybe	± 10 %	difficult setup	Y	**19, 25, 26, 28**
CRMs	UV-NIR	Y	Y	± 5 %		Y	**29-31**

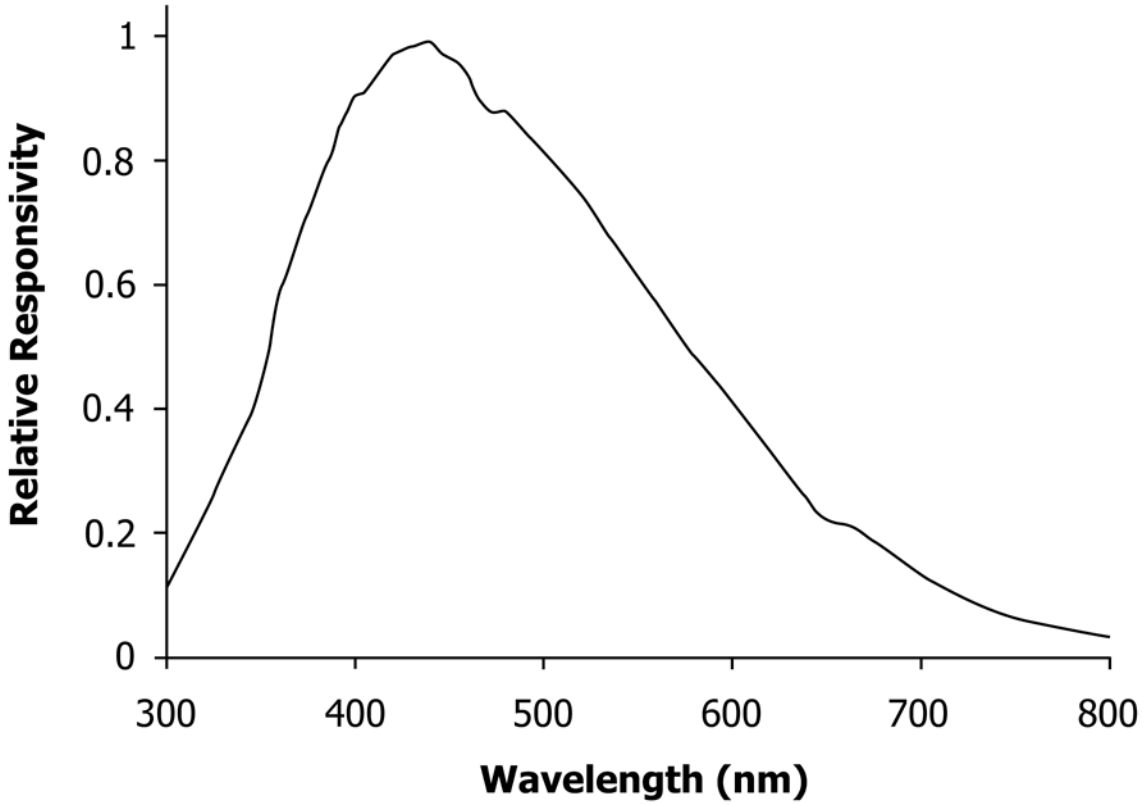

FIG. 9 Example of the Relative Spectral Responsivity of an EM Detection System (Grating Monochromator-PMT Based) (19) for Which a Correction Needs to be Applied to a Measured EM Spectrum to Obtain Its True Spectral Shape (Relative Intensities)

9.3 *Calibrated Detector (CD)*[8] with CR (**19, 25, 26, 28**)—This is a two-step method. The first step uses a CD to measure the flux of the EX beam as a function of EX wavelength, as described in 10.2. Alternatively, a quantum counter solution can be used instead of a CD, as described in 10.3. The second step uses a CR with reflectance R_{CR} to reflect a known fraction of the flux of the EX beam into the detection system. This is done by placing the CD at the sample position at a 45° angle relative to the excitation beam, assuming a right-angle detection geometry relative to the excitation beam, and synchronously scanning both the λ_{EX}- and λ_{EM}-selectors over the EM region of interest while collecting both the signal output (S') and the reference output (Rf'). This method enables the relative correction factor (C_{CD}) to be calculated using the equation $C_{CD} = (C_R R_{CR}$ Rf')/S'. See Section 3 for definitions of terms.

9.4 *Certified Reference Materials (CRMs)* (**29-31**)—The CRMs presently available are either organic dye solutions or solid, inorganic glasses released by national metrology institutes (NMIs) with certified relative fluorescence spectra, that is, relative intensity and uncertainty values are given as a function of EM wavelength at a fixed EX wavelength. They have been designed to resemble closely typical samples. A CRM is placed at the sample position and its spectrum is collected and compared to the certified spectrum according to the instructions given on the accompanying certificate, yielding spectral correction factors for the instrument. The corrected EM spectra of some commonly used dyes have also been reported recently in the literature (**32, 33**).

10. Spectral Correction of Excitation Beam Intensity

10.1 Calibration of the EX intensity with EX wavelength is necessary for successful quantification when intensity ratios at different EX wavelengths are being compared or the true shape or peak maximum position of an EX spectrum needs to be

[8] It is assumed in what follows that a calibrated detector is either a photodiode mounted inside an integrating sphere or a photodiode alone, whose spectral responsivity is known. The former is typically the more accurate of the two, because the integrating sphere insures spatially uniform illumination of the photodiode.

TABLE 4 Summary of Methods for Determining Spectral Correction of EX Beam Intensity

Sample	λ Region	Drop-In	Off-Shelf	Precision, Accuracy	Limitations	Certified Values	Refs.
CD - Si	UV-NIR	N	Y	± 2 %	difficult setup	Y	19, 24
Quantum Counter	UV-NIR	Y	Y	± 5 %	limited range	N	27, 35
Photodiode - Si	UV-NIR	N	Y	≤± 50 %		N	19

known. Such calibration methods are given here and summarized in Table 4. This type of calibration is necessary because the relative spectral flux of an EX beam at the sample can change significantly over its wavelength range (see Fig. 10). The neglect of EX intensity correction factors can often cause greater errors than that of EM correction factors (**19**, **34**). Fortunately, many fluorescence instruments have a built-in reference detection system to monitor the intensity of the EX beam. This is commonly done using a photodiode or a photomultiplier tube (PMT) or a quantum counter detector to measure a fraction of the EX beam that is split off from the rest of the beam. The collected reference signal can be used to correct the fluorescence signal for fluctuations caused by changes in the EX beam intensity. Reference detectors are often not calibrated with EX wavelength, introducing errors, which can be particularly large over longer EX wavelength ranges (for example, greater than 50 nm) or in a wavelength region in which the EX intensity changes rapidly with EX wavelength, such as the ultraviolet (UV). Also note that, when using an EX polarizer, the spectral correction for EX intensity is dependent on the polarizer setting.

10.2 *Calibrated Detector-Si Photodiode (CD-Si)* (**19**, **24**)—A CD is put at the sample position with the excitation beam incident on it. The output of the CD (S_{CD}) is measured as a function of EM wavelength by scanning the λ_{EX}-selector over the EX region of interest using the same instrument settings as that used with the sample. The known responsivity of the CD (R_{CD}) is used to calculate the flux of the EX beam (φ_x), such that $\varphi_x = S_{CD}/R_{CD}$. The instrument's reference detector can also be used to measure the intensity of the EX beam by measuring its output (Rf_{CD}) simultaneously with S_{CD}. Then, the correction factor for the responsivity of the reference detector $C_R = \varphi_x/Rf_{CD}$.

10.3 *Quantum Counters* (**27**, **35**, **36**)—A quantum counter solution is a concentrated dye solution that absorbs all of the photons incident on it and has an EM spectrum whose shape and intensity do not change with EX wavelength. The quantum counter solution is placed at the sample position in a quartz cuvette. If front face detection is possible, then a standard cuvette can be used with the EX beam at normal incidence. If 90° detection only is possible, then a right-triangular cuvette can be used with the excitation beam at 45° incidence to the hypotenuse side and one of the other sides facing the detector. Scan the EX wavelength over the region of interest with the EM wavelength fixed at a position corresponding to the long-wavelength tail of the EM band and collect the signal intensity (S_{QC}). The instrument's reference detector can also be used to measure the intensity of the EX beam by measuring its output (Rf_{QC}) simultaneously with S_{QC}. Then, the correction factor for the responsivity of the reference detector $C_R = S_{QC}/Rf_{CD}$ is calculated. Note that each quantum counter has a limited range. For instance, Rhodamine B can achieve the specified uncertainty from 250 to 600 nm. Beyond this range, the intensity falls off and uncertainties increase. Also note that S_{QC} will be proportional to the quantum flux at the sample, not the flux in power units. In addition, a quantum counter is prone

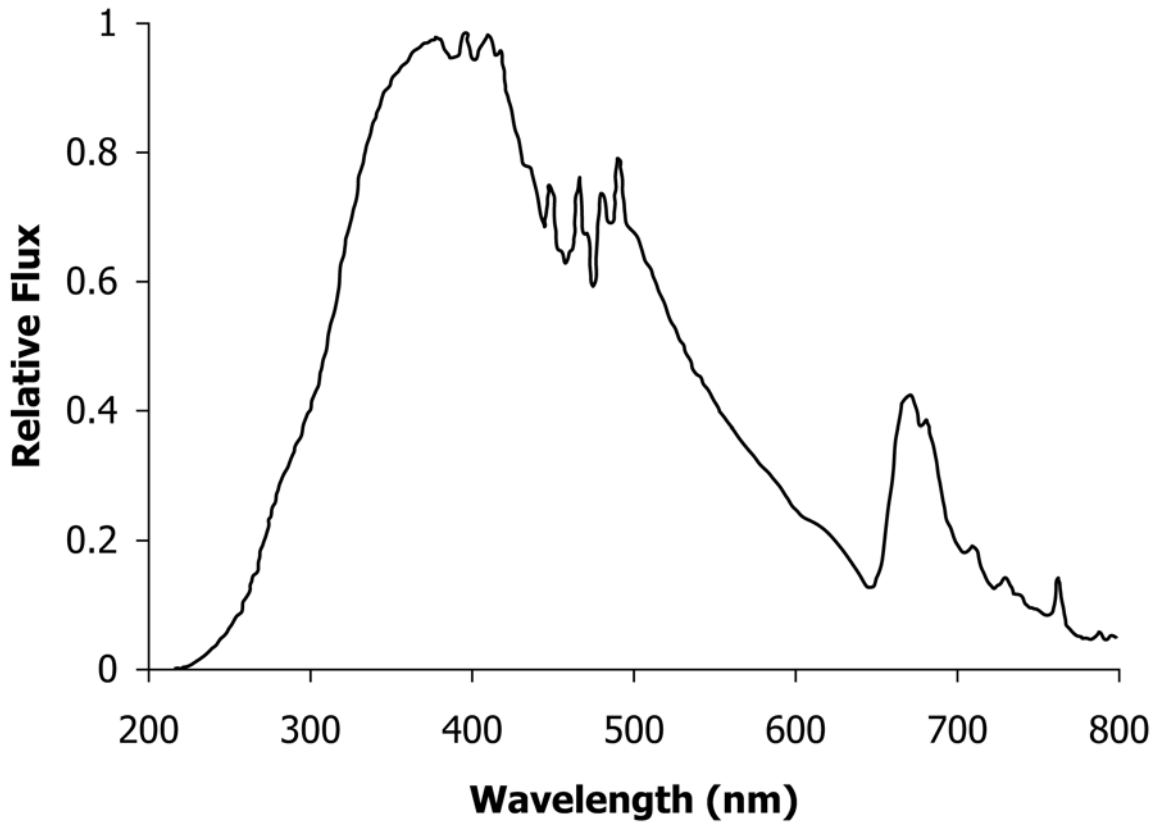

FIG. 10 Example of the Relative Flux of an EX Beam (Xe Lamp-Grating Monochromator Based) (19) for Which a Correction Needs to be Applied to a Measured EX Spectrum to Obtain Its True Spectral Shape (Relative Intensities)

to polarization and geometry effects that are concentration dependent. The spectral range and corresponding uncertainty of a quantum counter should be known and not assumed.

10.4 *Si Photodiode (Uncalibrated)* (**19**)—This is used in the same way as a calibrated Si photodiode (see 9.2), except its spectral responsivity is not known. A Si photodiode is sometimes erroneously assumed to have a responsivity that is qualitatively flat over its effective range. In fact, using its output to correct an EX spectrum can lead to quantitatively significant errors, particularly over a large EX range and in the UV region. That said, using an uncalibrated Si photodiode for correction will in most cases yield a more accurate spectrum than using no correction.

11. Calibration Curves for Concentration

11.1 *Guidelines*—Calibration curves of fluorescence intensity, that is, instrument responsivity, as a function of fluorophore concentration can be determined for a particular instrument and fluorophore. Reference materials composed of the fluorophore of interest shall be used. The highest accuracy is obtained when the fluorophore in both the standard and the sample experience the same microenvironment. For example, they are dissolved in the same solvent or attached to the same biomolecules. This type of calibration enables concentrations and amounts of fluorophores to be compared over time and between instruments without determining the absolute responsivity of the instrument (see Section 9).

11.1.1 *Concentration Range of the Standards*—The concentration of the highest and lowest standards should bracket the concentrations of the unknowns that are being measured. For best precision and accuracy, the concentrations of the analyte should be low enough that the absorbance at the excitation wavelength is less than 0.05 to prevent inner filter effects. A test for concentration quenching is to dilute the sample in half. If the resultant signal is not half the previous value, then concentration quenching is occurring and one needs to work at a lower initial sample concentration.

11.1.2 *Measurements:*

11.1.2.1 Make up a stock standard solution. Verify the concentration of the solution, if possible, using a UV/Vis spectrophotometer.

11.1.2.2 Use concentrations covering the range of interest for the unknown samples and which produce acceptable fluorescence values. When making standards, individual aliquots can be used to make the standards, instead of a serial dilution, as a check of accuracy.

11.1.2.3 A calibration curve using at least three standards, that is, three data points, should be used.

11.1.2.4 The highest and lowest standard should bracket the concentration level of the analytical assay.

11.1.2.5 Measure the fluorescence of standard at the analytical wavelength.

11.1.2.6 Make a plot of the fluorescence signal as the ordinate and the concentration as the abscissa.

11.1.2.7 *Handling of Standards*—Always insure that the samples are handled in the same way as the standards, particularly for extraction procedures and filtration because of errors due to partition coefficients.

11.2 *Fluorophores with Specified Purity and Uncertainty* (**37**)—If the purity of a fluorophore (for example, a high-purity, organic dye powder) is known, then it can be put in the same microenvironment (for example, solvent) as an unknown sample to produce a standard sample. Several standard samples should be produced to cover the concentration range of interest. These standard samples are measured under the same conditions as that of any unknowns and the fluorescence intensities are recorded. Fluorescence intensity versus standard sample concentration is plotted and the points are fitted to a polynomial, typically a straight line. The concentration of an unknown is determined by using the fitted polynomial along with the measured intensity of the unknown to find the corresponding concentration.

11.3 *Fluorophore Solutions with Specified Concentration and Uncertainty* (**38**) *(see Test Method 578)*—Standard solutions with known concentrations can be used in the same way as a standard fluorophore (see 10.2). In this case, the fluorophores are in solution, so they are ready to use or they can be diluted to produce standard solutions of lower concentration. In both cases, the solvent used in the standard and unknown solutions should be the same.

11.4 *Molecules of Equivalent Soluble Fluorophore (MESF)* (**39-42**)—The matching of microenvironments between sample and standard solutions, as emphasized in 10.2 and 10.3, cannot always be achieved. This is of particular concern when the sample contains immobilized fluorophores, for example, those attached to a cell. In many such cases, it is very difficult or impossible to determine the concentration of fluorophores in a candidate standard solution. MESF units are used, particularly in flow cytometry, to quantify such complex systems. These units express the fluorescence intensity of a fluorescent analyte, for example several immobilized fluorophores bound to a microbead or cell, as the corresponding number of free fluorophores of the same type in a standard solution with the same intensity. The MESF scale for a particular fluorophore is determined using the same procedure as that given in 10.3. This scale is transferred from a conventional fluorometer to a flow cytometer using fluorophore-labeled microbead suspensions with predetermined MESF values.

11.5 *Errors*—A number of sources of error can be introduced into the system from sample preparation, instrumental limitations and chemical interferences, causing deviations from the Beer-Lambert law. An awareness of these potential problems is important.

11.5.1 *Weighing Error*—Gravimetric and volumetric errors associated with weighing and diluting of the sample.

11.5.2 *Non-Linearity*—The proportional relationship between light absorption and fluorescence emission is only valid for cases where the absorption is small. As the concentration of fluorophore increases, deviations occur and the plot of emission versus concentration becomes non-linear. This is due to inner filter effects. In cases where it is necessary to work at high concentrations, it is possible to increase the linear concentration range by the use of microcuvettes.

11.5.3 *Temperature Effects*—Increases in temperature affect the viscosity of the medium and hence the number of collisions

of the molecules of the fluorophore with solvent molecules. This increases the probability of a return to the ground state without the emission of fluorescence. In such cases, the use of thermostatted or thermoelectric sample holders is recommended. Sufficient time for the solution to reach equilibrium before measurement is important.

11.5.4 *pH Effects*—Relatively small changes in pH can sometimes affect the intensity and spectral characteristics of fluorophores. Accurate pH control is essential particularly when buffer solutions are recommended in an assay.

11.5.5 *Inner-Filter Effects*—Fluorescence intensity can be reduced by the presence of any compound which is capable of absorbing a portion of either the excitation or emission energy. High concentrations of the fluorophore can cause non-uniform absorption of the excitation energy. If the excitation or emission light is absorbed by another compound in the solution then the linearity will be affected.

11.5.6 *Overlapping Bands*—Problems can occur if fluorescing compounds have overlapping excitation or emission bands. Incomplete spectral resolution of overlapping components can add error due to the interfering compound. Narrowing the spectral bandwidth to obtain better selectivity is suggested as a remedy.

11.5.7 *Solvents*—Solvent can exert a considerable influence on fluorescence spectra, especially where strong interaction occurs between the solvent molecules and the compound of interest. Solvent absorption can prevent accurate analysis. It is advisable to use a solvent that does not absorb significantly in the excitation region of the analyte.

11.5.8 *Aggregation*—If aggregation occurs, select a solvent to minimize aggregation of fluorophores.

11.5.9 *Scattering*—Filter the sample to minimize particulates.

11.5.10 *Adsorption*—Measure the sample as quickly as possible after preparation and thoroughly clean all glassware and cuvettes (see 5.5.1).

11.5.11 *Instrumental Noise*—Work at concentrations and use instrumental parameters (for example, integration time and spectral bandwidth) that provide an acceptable signal to noise ratio.

12. Day-to-Day and Instrument-to-Instrument Intensity

12.1 The determination of the stability of an instrument over time and comparability between instruments of fluorescence intensity is made possible by performance validation standards. The fluorescence intensity of such standards can be monitored over time and between instruments, enabling an absolute intensity scale to be established without performing absolute fluorescence measurements. These standards shall emit a fluorescence intensity that does not change with time or irradiation. Even though such standards do not need to be certified, their long term stability and related uncertainties need to be known. Another possibility is that they be single-use standards that can be made with a highly reproducible fluorescence intensity. In this case, the uncertainties introduced by the reproduction of the standard need to be known. It is not necessary for these standards to reproduce the exact spectrum of analyte samples, but they should be measurable with routine instrument settings, for example, typical EX intensity, bandwidths, and EM wavelengths.

12.2 *Cuvette Format* (**15, 29-31**)—This is the most commonly used format in conventional, benchtop fluorometers as well as in many portable instruments. Both solid and liquid standards are available for this format and most can be used in both 0/90° and front-face geometries. Standards of this type have been released by NMIs and industry, but the most well-known of these is high-purity water in which its Raman line is used as a pseudo-fluorescence signal (**24, 43, 44**). Unfortunately, the "water Raman" method is effectively limited to the UV-to-violet region of the spectrum. Inorganic solid standards are the most robust, most photostable, longest lasting, and easiest to use of fluorescent samples available in a cuvette format, although organic dyes may more closely resemble the behavior of fluorescent probes.

12.3 *Microwell Plate Format* (**45, 46**)—Some solid materials, similar to those used to make some of the cuvette standards mentioned in 11.2, have been used to make microwell plate reference materials. These have typically been made by taking a microplate-sized piece of the material and putting a mask over it that mimics the well boundaries. Organic dye solutions, such as those used in 11.2, can be put into the empty microwells of any plate and used as a standard. Of course, a fresh dye solution of known concentration has to be dispensed each time such a standard is used. The reference materials described here can be used as day-to-day intensity standards for filter-based instruments if the same filter is always used. For instrument-to-instrument comparisons or when filters are changed, the spectral differences between filters shall be considered. As yet, no microwell plate standards have been recommended by NMIs or generally accepted by the community at large.

12.4 *Microarray Format* (**47-50**)—Standard slides containing arrays of fluorescent dye samples with a morphology and intensity that is more consistent than that of a typical microarray sample are commercially available. No microarray standards have been recommended by NMIs or generally accepted by the community at large to this point.

13. Limit of Detection and Sensitivity (24) (Test Method E579)

13.1 The limit of detection of an instrument for a particular analyte often needs to be known to establish the lowest concentration of analyte that can be detected on that instrument or for comparing the sensitivity of one instrument to another. Methods and reference materials used in Sections 11 and 12 can also be used for determining limits of detection and sensitivity by using samples that approach the limit of detection of the instrument, that is, within two orders of magnitude of the background intensity of a blank or the noise of the detection system, and comparing the sample intensity to the background intensity or noise.

14. Lifetimes (51, 52)

14.1 Time-domain and frequency-domain measurements are the two types of fluorescence measurements used to determine fluorescence lifetimes. Conventional instruments include those based on time-correlated single-photon counting (time-domain) and multifrequency phase and modulation (frequency-domain) techniques, which are typically used to measure lifetimes from picoseconds to microseconds. More simple, time-domain instruments are commonly used to measure lifetimes on the order of milliseconds or longer. Criteria for fluorescence lifetime standards include: *(1)* high purity, *(2)* a single exponential decay component, *(3)* a lifetime close to the value of a sample(s) of interest, and *(4)* a lifetime independent of EX and EM wavelengths. Possibly, the most thorough comparison of fluorescence lifetime candidates was recently performed by nine expert laboratories (**51**). Almost all of the candidates mentioned in the literature for use as lifetime standards have been liquid, organic dye solutions, probably because of the more complex, excited-state kinetics that exist in most solid fluorescent samples. Lifetime standards are measured in the same way as typical unknown samples. A bias in the measured lifetime or an observed multiexponential decay of the standard indicates the presence of systematic errors in the instrument.

15. Fluorescence Quantum Yield (53)

15.1 Measured as the ratio of the number of molecules that fluoresce a photon to the number of molecules that absorb a photon from the EX source, this quantity is an intrinsic property of a particular molecular species in a particular environment. Sample effects, such as inner filter effects, often introduce errors into measured quantum yield values. Care has to be taken to control the temperature and oxygen concentration of samples as both can also affect measured values. In addition, the quantum yield of a fluorophore can be highly dependent on its microenvironment, for example, polarity, viscosity, proticity, pH, presence of potential quenchers such as heavy metal ions, and whether or not the fluorophore is bound to another species (**54**).

15.1.1 Absolute quantum yields can be particularly difficult to measure accurately because of instrumental errors that need to be avoided or corrected and experimental setups requiring some expertise on the part of the investigator. Because of this, relative quantum yields are much more commonly measured than absolute by using a species with a known quantum yield as a reference. This makes the accuracy of the unknown, relative quantum yield dependent on the accuracy of the known, reference value. Unfortunately, the fluorescence quantum yields of very few species have been well established. Absolute spectroscopic methods also use a reference, for example, a diffuse scatterer, but no reference value needs to be known in advance as the reference has an effective quantum yield of one. Only methods that can use a conventional fluorescence spectrometer as a detector are summarized in the following paragraphs. All of these methods are capable of yielding quantum yield values with an accuracy of ±10 % or better for simple cases, such as small organic dyes, when systematic uncertainties are minimized.

15.2 *Absolute Methods:*

15.2.1 *Optically Dilute Samples (A < 0.05):*

15.2.1.1 This most commonly used absolute method was developed by Weber and Teale (**55**). The variation of this method described here, using a spectrometer as a detector, was first done by Eastman (**56**). An optically dilute scattering solution is used as a reference, such as colloidal silica or glycogen. A right-angle geometry and identical instrument settings (for example, EX wavelength, EM range, bandwidths, and so forth) should be used for both sample and reference measurements. In addition, the response of the detector should not be polarization dependent and the EX optical radiation should be unpolarized, or correction factors should be determined to compensate for these. If all of these conditions are not met, then large systematic errors may result.

15.2.1.2 Solutions at several optical densities, preferable all with $A < 0.05$, of both the fluorescent sample (f subscript) and the scatterer (s subscript) are made and their absorbances (A) and EM spectra with fluorescence signals (S) are measured at the EX wavelength. The values of S_f/A_f and S_s/A_s extrapolated to zero optical density, m_f and m_s, respectively, are calculated (**57**). The response of the detection system (R_d) as a function of EM wavelength is determined. The polarization (p) and the refractive index (n) of the sample and the reference are measured. The quantum yield (φ) is equal to a summation over the EM wavelength range of the product $R_d\,(m_f/m_s)\,(n_f/n_s)^2\,(3 + p_f)/(3 + p_s)$. Alternatively, the EM monochromator can be set to zero order, so all wavelengths reach the detector at once, thereby removing the summation from the equation. This is less tedious and closer to Weber and Teale's original method in which no EM monochromator was used, although stray light is more likely to introduce error in this case.

15.2.2 *Optically Dense Samples*—This method, first described by Vavilov (**58**), is similar to the Weber and Teale method, but a solid, diffuse scatterer, for example, barium sulfate or sintered polytetrafluoroethylene, with a known diffuse reflectance is used as a reference. Front-face detection with the sample parallel to the detector is the best optical geometry (**59**). This method requires a detailed knowledge of the optical geometry of the instrument used, among other complications. Therefore, this method is only recommended when optically dense samples must be used.

15.2.3 *Integrating Sphere at Sample:*

15.2.3.1 An integrating sphere can be placed at the sample position of a fluorescence spectrometer with the sample placed inside the sphere (**60-63**). This eliminates the need for refractive index, polarization, and spatial anisotropy corrections. EX and EM ports, facing the EX beam and detection system (right-angle geometry), respectively, are open on the integrating sphere. The optical radiation coming out of the exit port is collected by the detection system. If the sample holder in the sphere is able to rotate the sample in and out of the direct path of the EX beam, this enables secondary EX and EM, resulting from EX light that has been reflected back to the sample by the sphere, to be subtracted out, and the absorptance (α) of a sample to be measured accurately with the same system. The response of the sample-integrating sphere-detection system

shall first be determined as a function of wavelength. The quantum yield and absorptance can then be calculated using the following equations:

$$\varphi_f = \frac{E_{in} - (1 - \alpha)E_{out}}{X_{empty}\alpha} \quad (1)$$

$$\alpha = \frac{X_{out} - X_{in}}{X_{out}}$$

where:

E_{in} and E_{out} = integrated emission spectra of the sample after direct (sample in the beam path) and secondary (sample out of the beam path) excitation, respectively, and

X = integrated profile of the excitation obtained with the integrating sphere-detection system.

15.2.3.2 Both the EM spectra and the EX profile are collected with the EX beam set at a fixed wavelength and the appropriate spectral range being chosen or scanned by the detection system. The subscripts empty, in and out refer to an empty sphere (no sample), the sample directly in the path of the beam, and the sample in the sphere but out of the direct path of the beam, respectively.

15.2.3.3 Eq 1 and the corresponding method are effective for determining the quantum yield of samples that scatter light diffusely. However, for a sample with polished surfaces, such as a solution in a cuvette, the specular reflection of the excitation beam off the sample's surface can introduce significant errors. In this case, a blank, that is, the solvent in the same cuvette, must also be measured in the sphere (**63**). The quantum yield can then be calculated using the following equation:

$$\varphi_f = \frac{E_{in} - E_{bk}}{X_{bk} - X_{in}} \quad (2)$$

where:

E_{bk} = integrated emission spectra of the blank after direct (sample in the beam path) excitation, and

X_{bk} = integrated profile of the excitation obtained with direct excitation of the blank.

15.3 *Relative Methods:*

15.3.1 A relative method using an optically dilute quantum yield standard (reference) with a quantum yield that is known with high accuracy is the most commonly used method for quantum yield determination. Absorbance values (A) and EM spectra for the unknown sample and standard reference are measured at their corresponding EX wavelengths. A reference detector is used to measure the relative intensity of the EX beam simultaneously with the collection of the EM spectra. The response of the detection system (R_d) as a function of EM wavelength is determined and used to correct the EM spectra. The following equation is used to calculate the quantum yield:

$$\varphi_f = \varphi_r \left[\frac{A_r(\lambda_r)}{A_f(\lambda_f)}\right]\left[\frac{I_r(\lambda_r)}{I_f(\lambda_f)}\right]\left[\frac{n_f}{n_r}\right]\left[\frac{E_f}{E_r}\right] \quad (3)$$

where:

n = refractive index at the peak EM wavelength,

$I(\lambda)$ = relative EX intensity at EX wavelength λ,

E = integrated area under the corrected EM spectrum, and

subscripts f and r = unknown and reference samples, respectively.

15.3.2 The fluorescence quantum yields of only a short list of compounds have been characterized to the extent necessary to be used as standards (**64**) with quinine sulfate being one of the most thoroughly established (**37**).

15.3.3 This same procedure can be used to measure the relative quantum yield of optically dense samples. In this case, the absorbance ratio in Eq 3 is equal to one, since the EX beam is absorbed completely, thereby simplifying the equation. Optically dense samples are used for both the unknown and the reference. Unfortunately, there are many complications associated with measuring optically dense samples, making this method less accurate than its optically dilute counterpart, in most cases.

16. Fluorescence Anisotropy (65)

16.1 Fluorescence anisotropy standards are used to calibrate or verify the performance of instruments that measure polarization or anisotropy of fluorescence. Such standards should have a known anisotropy at a set or range of specified excitation and emission wavelengths (**65, 66**). In addition, they should cover the anisotropy (r) range from 0.0 to 0.4. Isotropic emitters (r = 0) are also useful for measuring G-factors (**19**).

17. Keywords

17.1 anisotropy; calibration; fluorescence; fluorometer; lifetime; limit of detection; luminescence; qualification; quantum yield; reference materials; spectral correction; spectrometer; spectroscopy; validation; verification; wavelength

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