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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[1] This guide is under the jurisdiction of ASTM Committee E13 on Molecular Spectroscopy and Separation Science and is the direct responsibility of Subcommittee E13.01 on UV/VIS Task Group.

Current edition approved May 1, 2014. Published June 2014. Originally approved in 2009. Last previous edition approved in 2009 as E2719–09. DOI: 10.1520/E2719-09R14.

[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.

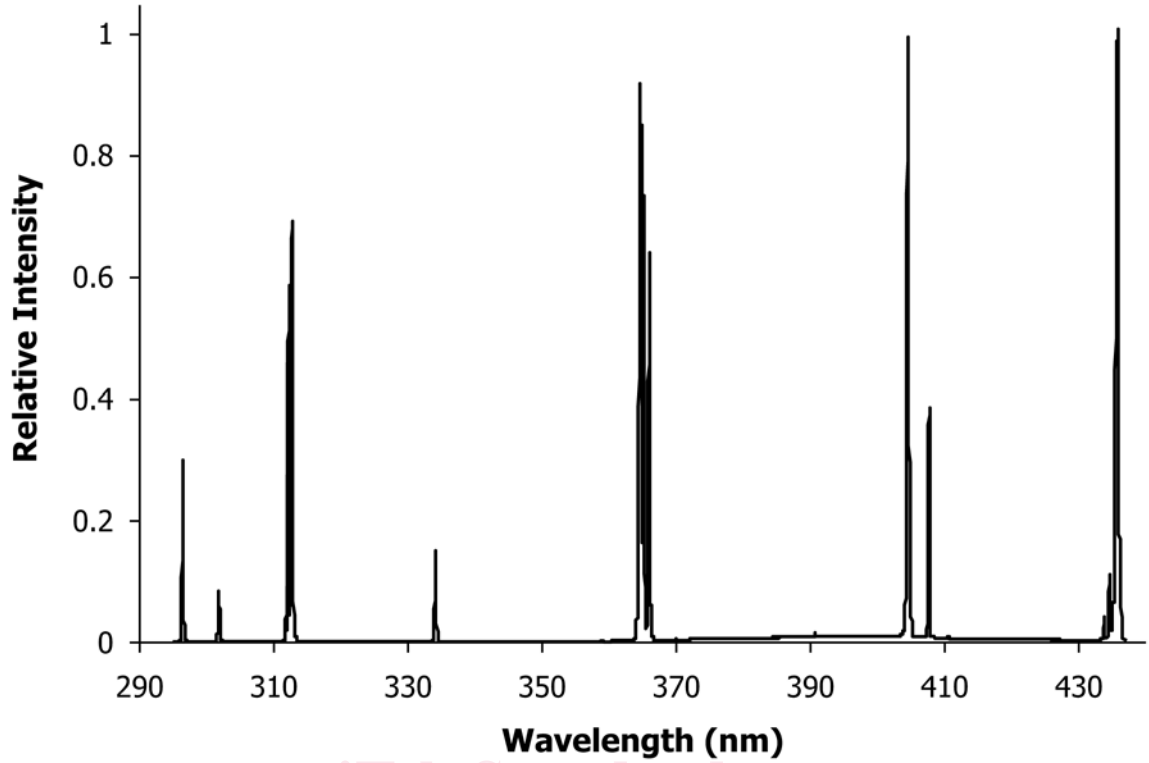

FIG. 1 Hg Pen Lamp Spectrum

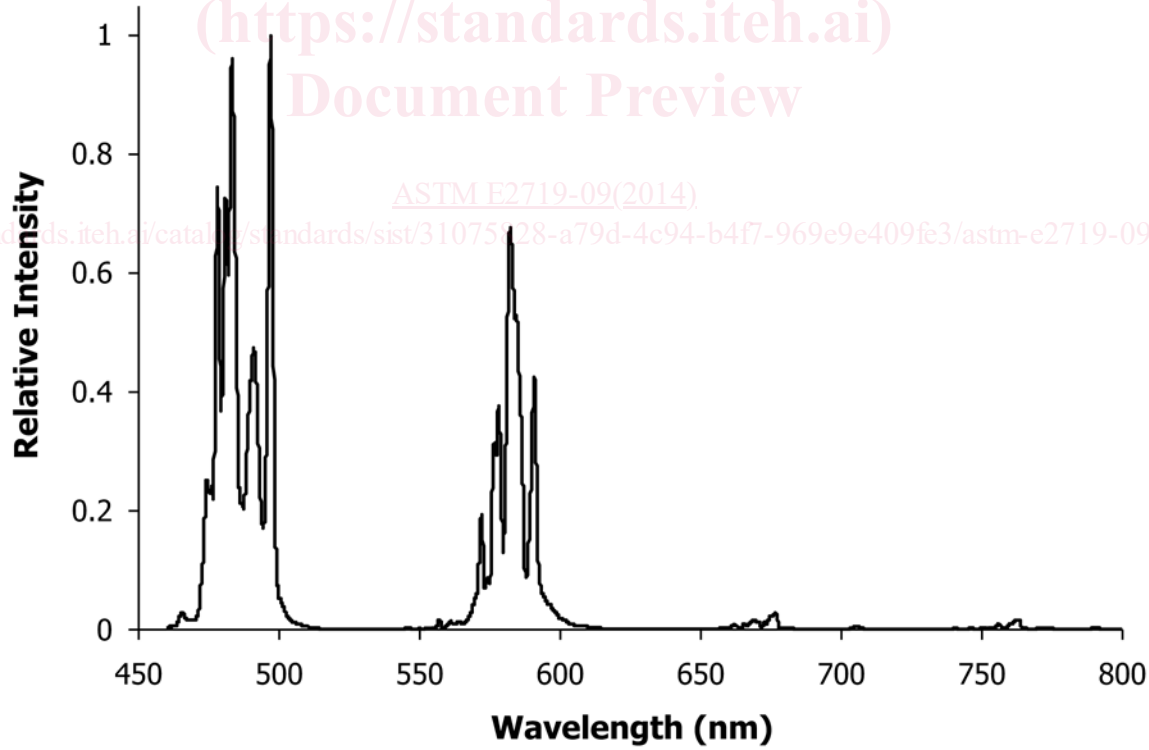

FIG. 2 EM Spectrum of a Dy-YAG Crystal Excited at 352.7 nm

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

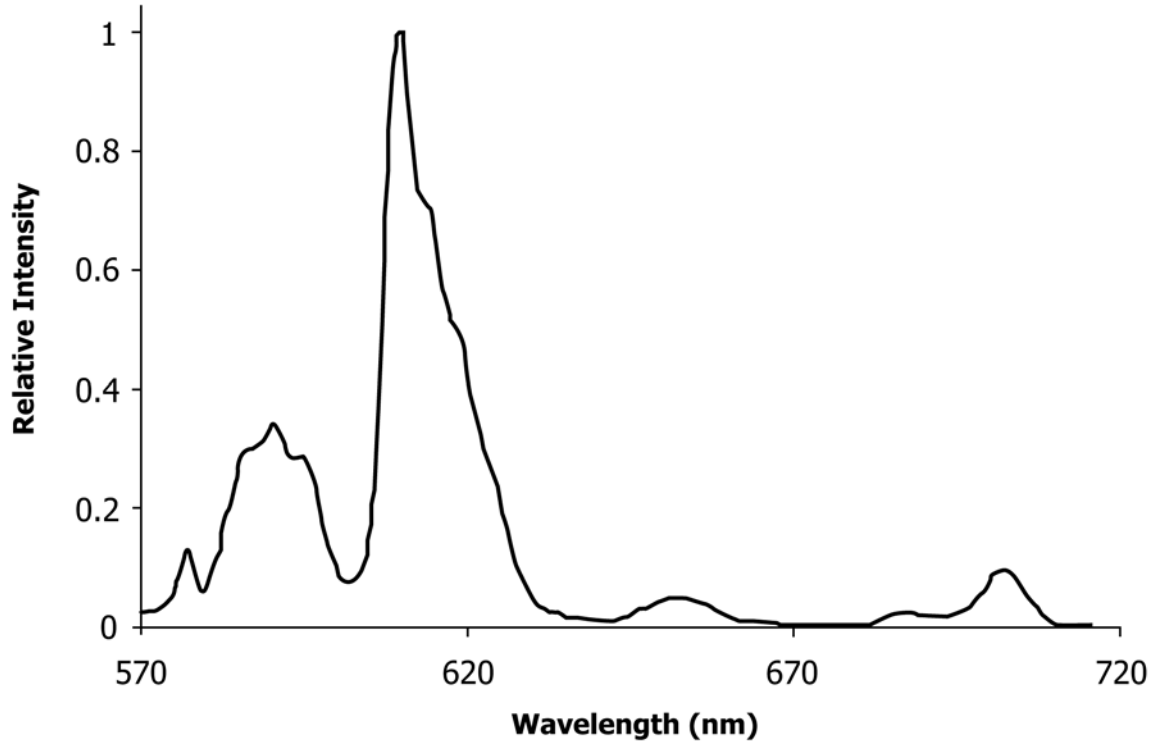

FIG. 3 EM Spectrum of a Eu-Ion-Doped Glass Excited at 392 nm

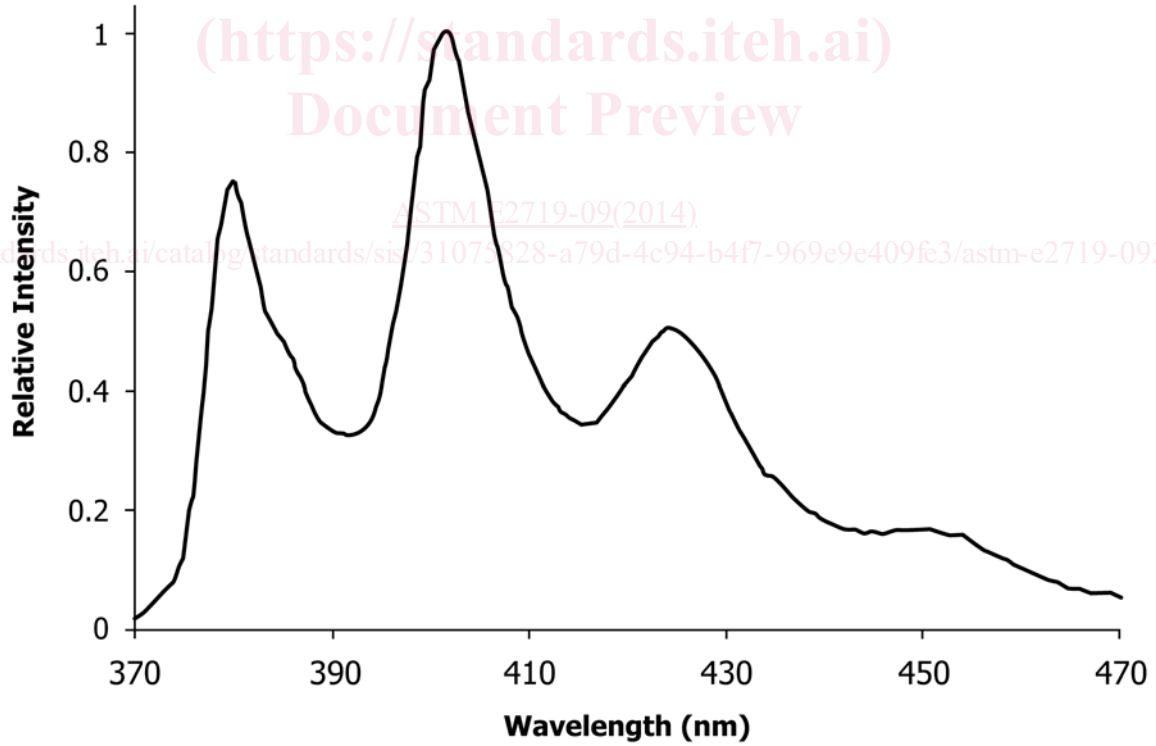

FIG. 4 EM Spectrum of Anthracene-Doped PMMA Excited at 360 nm

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