



Standard Practice for Determining Relative Spectral Correction Factors for Emission Signal of Fluorescence Spectrometers¹

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1. Scope

1.1 This practice (1)² describes three methods for determining the relative spectral correction factors for grating-based fluorescence spectrometers in the ultraviolet-visible spectral range. These methods are intended for instruments with a 0°/90° transmitting sample geometry. Each method uses different types of transfer standards, including 1) a calibrated light source (CS), 2) a calibrated detector (CD) and a calibrated diffuse reflector (CR), and 3) certified reference materials (CRMs). The wavelength region covered by the different methods ranges from 250 to 830 nm with some methods having a broader range than others. Extending these methods to the near infrared (NIR) beyond 830 nm will be discussed briefly, where appropriate. These methods were designed for scanning fluorescence spectrometers with a single channel detector, but can also be used with a multichannel detector, such as a diode array or a CCD.

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

1.3 *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:*³

[E131 Terminology Relating to Molecular Spectroscopy](#)

[E388 Test Method for Wavelength Accuracy and Spectral Bandwidth of Fluorescence Spectrometers](#)

¹ This practice is under the jurisdiction of ASTM Committee E13 on Molecular Spectroscopy and Separation Science and is the direct responsibility of Subcommittee E13.01 on Ultra-Violet, Visible, and Luminescence Spectroscopy.

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

³ 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.

[E578 Test Method for Linearity of Fluorescence Measuring Systems](#)

[E2719 Guide for Fluorescence—Instrument Calibration and Qualification](#)

3. Significance and Use (Intro)

3.1 Calibration of the responsivity of the detection system for emission (EM) as a function of EM wavelength (λ_{EM}), 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 1](#). This type of calibration is necessary because the spectral responsivity of a detection system can change significantly over its useful wavelength range (see [Fig. 1](#)). It is highly recommended that the wavelength accuracy (see [Test Method E388](#)) and the linear range of the detection system (see [Guide E2719](#) and [Test Method E578](#)) be determined before spectral calibration is performed and that appropriate steps are taken to insure that all measured intensities during this calibration are within the linear range. For example, when using wide slit widths in the monochromators, attenuators may be needed to attenuate the excitation beam or emission, thereby, decreasing the fluorescence intensity at the detector. Also note that when using an EM polarizer, the spectral correction for emission is dependent on the polarizer setting. (2) It is important to use the same instrument settings for all of the calibration procedures mentioned here, as well as for subsequent sample measurements.

3.2 When using CCD or diode array detectors with a spectrometer for λ_{EM} selection, the spectral correction factors are dependent on the grating position of the spectrometer. Therefore, the spectral correction profile versus λ_{EM} must be determined separately for each grating position used. (3)

3.3 Instrument manufacturers often provide an automated procedure and calculation for a spectral correction function for emission, or they may supply a correction that was determined at the factory. This correction can often be applied during spectral collection or as a post-collection correction. The user

TABLE 1 Summary of Methods for Determining Spectral Correction of Detection System Responsivity

NOTE 1—“Drop-In” refers to whether or not the material/hardware can be put in the sample holder and used like a conventional sample; “Off-Shelf” refers to whether or not the material/hardware can be purchased in an immediately-usable format; “Uncertainty” is the estimated expanded (k=2) total uncertainty; “Caveats” refer to important information that a user should know about the method before attempting to use it; “Certified Values” refers to whether or not the material/hardware is supplied with appropriate values as a function of emission wavelength and their corresponding total uncertainties; the references (Ref.) give examples and more in-depth information for each method.

Method	λ_{EM}	Drop-In	Off-Shelf	Uncertainty	Caveats	Certified Values	Ref.
CS	UV-NIR	N	Y	$< \pm 5 \%$	difficult setup	Y	E578, (3-6)
CD+CR	UV-NIR	N	Maybe	$\pm 10 \%$	difficult setup	Y	E578, (4, 5, 7)
CRMs	UV-NIR	Y	Y	$\pm 5 \%$		Y	E131, (8-13)

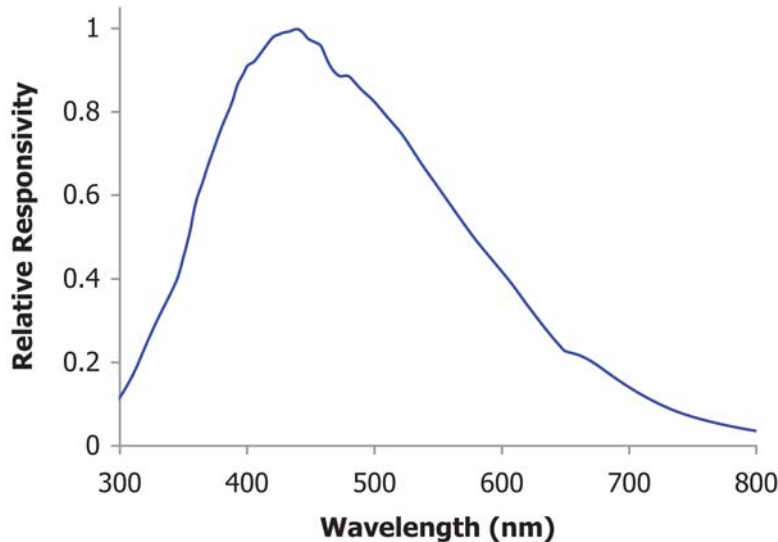


FIG. 1 Example of Relative Spectral Responsivity of Emission Detection System (Grating Monochromator-PMT Based), (see Test Method E578) for which a Correction Needs to be Applied to a Measured Instrument-Specific Emission Spectrum to Obtain its True Spectral Shape (Relative Intensities).

should be advised to verify that the automated vendor procedure and calculation or supplied correction are performed and determined according to the guidelines given within this standard.

4. Calibrated Optical Radiation Source (CS) Method (see Test Method E578, (4-6, 14))

4.1 *Materials:*

4.1.1 A calibrated tungsten lamp is most commonly used as a CS in the visible region due to its high intensity and broad, featureless spectral profile. Its intensity falls off quickly in the ultraviolet (UV) region, but it can typically be used down to 350 nm or so. It also displays a high intensity in the near infrared, peaking at about 1000 nm. Its intensity gradually decreases beyond 1000 nm, but continues to have significant intensity out to about 2500 nm. A calibrated deuterium lamp can be used to extend farther into the UV with an effective range from about 200 to 380 nm. The effective range of a CS is dependent on the intensity of the CS and the sensitivity of the detection system. This range can be determined by measuring the low-signal regions where the signal profile of the light from the CS becomes flat or indistinguishable from the background signal, implying that the signal afforded by the CS is not measurable in these λ_{EM} regions.

4.1.2 A calibrated reflector (CR) is often used to reflect the light from the CS into the emission detection system. A diffuse reflector made of compressed or sintered polytetrafluoroethylene (PTFE) is most commonly used as a CR, due to its nearly Lambertian reflectance, which prevents both polarization and spatial dependence of the reflectance. In addition, PTFE possesses a reflectance profile that is nearly flat, changing by less than 10 % from 250 to 2500 nm. For a CS and a CR, “calibrated” implies that the spectral radiance and the spectral reflectance, respectively, are known (calibrated wavelength dependence of the spectral radiant factor including measurement uncertainty) and traceable to the SI (International System of Units). This is commonly done through certification of these values by a national metrology institute (NMI). (15, 16, 7)

4.2 *Procedure:*

4.2.1 Direct the optical radiation from a CS 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, place a CR at the sample position to reflect the optical radiation from the CS into the EM detection system. Ensure that the CS is aligned such that its light is centered on the entrance slit of the λ_{EM} selector, and on all optics it encounters before the entrance slit. Ideally, the light should fully and uniformly fill the

entrance slit. Make sure that the detection system is still operated within its linear range (see 3.1).

NOTE 1—Correction factors, supplied by the manufacturer and automatically applied by the software to the collected spectrum, must be switched off for the signal channel during this procedure.

4.2.2 Scan the λ_{EM} -selector over the EM region of interest, using the same instrument settings as employed with the subsequent measurement of the fluorescence of the sample, and collect the signal channel output (S'').

4.2.3 Use the known radiance of the CS incident on the detection system (L) to calculate the relative correction factor (C_{CS}), such that $C_{CS} = L/S''$. Note that L may be replaced by the spectral irradiance or the spectral radiant flux, since the correction factors determined herein are relative, not absolute. The corrected EM intensity is equal to the product of the signal output of the sample (S) and C_{CS} . Since C_{CS} values are relative correction factors, they can be scaled by any constant. For instance, it is often useful to scale them with a constant that gives a C_{CS} value of one at a particular λ_{EM} .

4.2.4 Note that L is given in power units, not photon units, whereas, the units for S and S'' are either in power or photon units depending on whether your detector measures an analog or a digital (photon counting) signal, respectively. In either case, the corrected signal will be in power units, so a conversion, that is, dividing the corrected signal by λ_{EM} , is necessary if photon units are needed.

5. Calibrated Detector (CD) with Calibrated Reflector

Method (see Test Method E578, (4, 5, 17))

5.1 Materials:

5.1.1 A calibrated photodiode, by itself, as part of a trap detector or mounted in an integrating sphere, is most commonly used as a calibrated detector (CD). Using a trap detector or photodiode with integrating sphere is typically more accurate than using a photodiode alone. A Si photodiode covers the range from 200 to 1100 nm. An InGaAs or Ge photodiode can be used in the NIR from 800 to 1700 nm. For a CD, “calibrated” implies that the wavelength dependence of the spectral responsivity is known, its associated uncertainties have been determined and the measurements are traceable to the SI. This is typically done through values certified by an NMI. (18, 19) A photodiode usually outputs a current that is proportional to the power of the light incident on it.

5.1.2 Alternatively, a quantum counter solution can be used instead of a CD. (6, 20, 8) This is a dye solution at a sufficiently high concentration such that all photons incident on it are absorbed. In addition, it must have an emission (EM) spectrum whose shape and intensity do not change with excitation (EX) wavelength, that is, its fluorescence quantum yield is independent of excitation wavelength. Note that there are several drawbacks to using a quantum counter (QC) instead of a CD. Firstly, QCs tend to have a more limited range than CDs and uncertainties that are not certified or even well known. In addition, a QC is prone to polarization and geometry effects that are concentration and solvent dependent, thus requiring that the ideal concentration for proper functioning be determined for the measurement geometry to be used. It should also be noted that the output measured from the QC will be

proportional to the quantum flux (number of photons per second) at the sample, not the flux in power units. This can result in enhanced measurement uncertainties compared to the use of a calibrated detector.

5.2 Procedure:

5.2.1 Unlike the CS method, this is a two-step method. The first step uses a CD (or a QC) placed at the sample position, which measures the excitation intensity incident on the sample as a function of EX wavelength by scanning the EX wavelength selector over the desired range. 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. Follow the procedures in either 5.2.1.1 or 5.2.1.2 depending upon whether you are using a CD or a QC, respectively.

NOTE 2—Correction factors, supplied by the manufacturer and automatically applied by the software to the collected spectrum, must be switched off for signal and reference channels during this procedure.

5.2.1.1 *Step 1 with Calibrated Detector*—Place the CD at the sample position and scan the λ_{EX} -selector over the EX region of interest while collecting the signal from the CD (S_{CD}) as a function of λ_{EX} . Be sure to use the same instrument settings as those employed with the sample. Calculate the flux of the EX beam (ϕ_x), using $\phi_x = S_{CD}/R_{CD}$, where R_{CD} is the known responsivity of the CD. Note that if the instrument has its own reference detector with output (Rf) for monitoring the excitation intensity, then the correction factor for the responsivity of the reference detector $C_R = \phi_x/Rf$ can be calculated. Multiplying an Rf value by C_R at a particular λ_{EX} will give a corrected Rf value in the same units as ϕ_x , typically Watts.

5.2.1.2 *Step 1 with Quantum Counter*—Place the QC solution at the sample position in a cuvette (typically fused silica) that transmits the excitation and emission wavelengths of interest. If front face detection is possible, then use a standard cuvette with the EX beam at normal incidence. If 90° detection is chosen, then use a right-triangular cuvette with the excitation beam at 45° incidence to the hypotenuse side and one of the other sides facing the detector. Scan the λ_{EX} -selector over the EX region of interest with the λ_{EM} fixed at a position corresponding to the long-wavelength tail of the emission band and collect the signal intensity (S_{QC}) as a function of λ_{EX} . Be sure to use the same instrument settings for the excitation beam as those employed with the sample. S_{QC} is the relative quantum flux of the excitation beam at the sample. Note that if the instrument has its own reference detector with output (Rf) for monitoring the excitation intensity, then the correction factor for the responsivity of the reference detector $C_R = S_{QC}/Rf$ can be calculated. Multiplying an Rf value by C_R at a particular λ_{EX} will give a corrected Rf value in units of relative quantum flux.

5.2.1.3 *Step 2 Using a Calibrated Reflector*—Place the CR at the sample position at a 45° angle relative to the excitation beam, assuming a right-angle-detection geometry relative to the excitation beam. Use the same instrument settings as those employed with the sample. Synchronously scan 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'). Calculate the relative correction factor (C_{CD}) using the equation $C_{CD} = (C_R R_{CR} Rf')/S'$. If the instrument does not have a reference detector, then use $C_{CD} = (\phi_x R_{CR})/S'$ or $C_{CD} =$

$(S_{QC}R_{CR})/S'$, depending upon whether you are using a CD or a QC, respectively, in Step 1. Since C_{CD} are relative correction factors, they can be scaled by any constant. For instance, it is often useful to scale them with a constant that gives a C_{CD} value of one at a particular λ_{EM} .

6. Certified Reference Material Method

6.1 Materials:

6.1.1 Certified reference materials (see Terminology E131) (CRMs) have been released by NMIs for the relative spectral correction of fluorescence emission. (9-13, 21, 22) They are presently sold by the NMIs and other secondary manufacturers. These CRMs are supplied with certified relative intensity and uncertainty values as a function of λ_{EM} at a fixed λ_{EX} . Instructions for use are also supplied in the accompanying certificate(s). Unlike the other materials and methods explained to this point, these can be used easily by non-experts, since they are designed to be measured in the same way as typical samples, that is, with the same instrument settings and without requiring sophisticated attenuation procedures or a different measurement geometry. As an alternative to CRMs, there are other materials with corrected relative intensity emission spectra that have been published in the literature. (23, 24) These should be used with caution, since the uncertainties in the published values are not specified in most cases, and the values themselves have not been tested using a variety of calibrated instruments. (25, 26) Moreover, sometimes even the reference quantity used, that is, the spectral radiance or the spectral photon radiance, is not clearly stated, in contrast to CRMs.

6.2 Procedure:

6.2.1 Place the CRM at the sample position and scan its emission spectrum using the excitation wavelength, emission range and other specifications given in the certificate. Collect the measured emission signal (S_m) and compare it to the

certified spectrum (S_c) according to the instructions given on the certificate. If the instrument has a reference channel to monitor the intensity of the excitation beam, then simultaneously collect the reference channel output (Rf).

6.2.2 The easiest way to calculate the relative correction factor (C_{CRM}) as a function of λ_{EM} in the emission range of a single CRM is to use the equation $C_{CRM} = (S_c Rf)/S_m$. If the instrument does not have a reference detector, then the equation becomes $C_{CRM} = S_c/S_m$. Note again that because these are relative correction factors, they can be scaled by any constant. For instance, it is often useful to scale them with a constant that gives a C_{CRM} value of one at a particular λ_{EM} . More complex procedures for determining correction factors may be given in the certificate or may be included in software available with the CRM.

7. Documentation and Reporting

7.1 Spectral correction factors should be reported as a function of emission wavelength along with the instrument settings under which they were measured; for example, spectral bandwidth of emission, emission polarizer setting, emission filter setting, gain on the detector, emission grating position (for diode array and CCD based instruments); the date of measurement, the calibration standards used and their reference values and uncertainties. Recommended instrument settings and basic advice on documenting results may be given in CRM certificates. (See Terminology E131, (9-13).) Guide E2719 and many of the references it cites discusses sources of uncertainty, Terminology E131 therein being particularly useful as it also discusses how uncertainties should be combined to calculate a total uncertainty.

8. Keywords

8.1 calibration; fluorescence; fluorometer; luminescence; qualification; reference materials; spectral correction; spectrometer; spectroscopy; validation

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⁴ The term "Standard Reference Material" is a registered trademark of The National Institute of Standards and Technology (NIST).

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