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

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January 2013

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1. **Scope**

   1.1 This practice 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 range covered by the different methods is from 250 nm 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, when appropriate. These methods were designed for scanning fluorescence spectrometers with a single channel detector, but can also be used with a multiplex detector, such as a diode array or a CCD.

2. **Significance and Use (Intro)**

   2.1 Calibration of the relative 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 relative 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 ASTM **E388**) [1] and the linear range of the detection

system (see ASTM **E2719** and ASTM **E578**) [2, 3] be determined before spectral calibration is performed and appropriate steps are taken to insure that all measured intensities during this calibration are within the linear range. For example, the use of attenuators before the sample may be needed to attenuate the excitation beam, thereby, decreasing the fluorescence intensity. Also note that when using an EM polarizer, the spectral correction for emission is dependent on the polarizer setting. [4]

Table 1 : Summary of Methods for Determining Spectral Correction of Detection System Responsivity

Method	λ_{EM}	Drop-In	Off-Shelf	Uncertainty	Caveats	Certified Values	Ref.
CS	UV-NIR	N	Y	$< \pm 5\ \%$	difficult setup	Y	4, 6-9
CD+CR	UV-NIR	N	Maybe	$\pm 10\ \%$	difficult setup	Y	4, 7-8, 13
CRMs	UV-NIR	Y	Y	$\pm 5\ \%$		Y	18-24

Note: In Table 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.

2.2 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. [5]
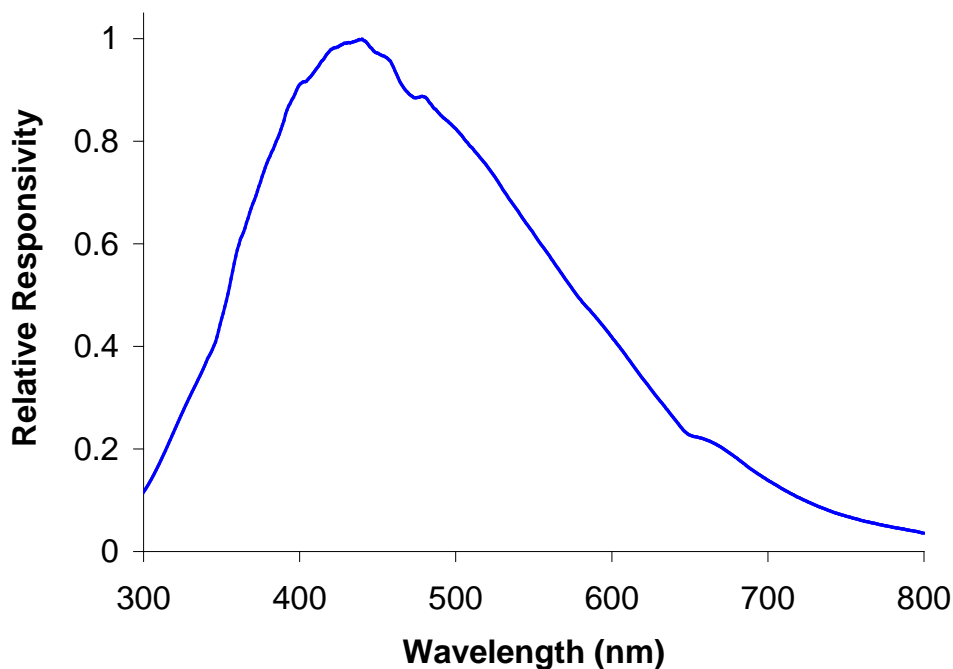
Fig. 1: Example of the relative spectral responsivity of an emission detection system (grating monochromator-PMT based), [4] for which a correction needs to be applied to a measured emission spectrum to obtain its true spectral shape (relative intensities).

3. Calibrated Optical Radiation Source (CS) Method [4, 6-9]

3.1 Materials

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 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. It 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 nm 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 CS is not able to measure the signal in these λ_{EM} regions.

A calibrated reflector (CR) is often used to reflect the light from the CS into the emission detection system. A diffuse reflector made out of compressed or sintered polytetrafluoroethylene (PTFE) is most commonly used as a CR, due to its 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 nm to 2500 nm. For a CS and a CR, "calibrated" implies that the spectral radiance and the spectral reflectance, respectively, are known and traceable to the SI. This is commonly done through certification of these values by a national metrology institute (NMI). [10-12]

3.2 Procedure

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 all optics it encounters and the entrance slit, before and while entering the λ_{EM} selector.

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

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 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} 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}.

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, i.e. dividing the corrected signal by λ_{EM}, is necessary if photon units are needed.

4. **Calibrated Detector (CD) with Calibrated Reflector Method** [4, 7, 8, 13]

   4.1 Materials

   A calibrated photodiode, either by itself or mounted inside an integrating sphere, is most commonly used as a calibrated detector (CD). The CD with integrating sphere is typically the more accurate of the two, because it insures spatially uniform illumination of the photodiode. A Si photodiode covers the range from 200 nm to 1100 nm. An InGaAs photodiode can be used in the NIR from 800 nm to 1700 nm. For a CD, "calibrated" implies that the spectral responsivity is known and traceable to the SI. This is typically done through values certified by an NMI. [14, 15] A photodiode usually outputs a current or voltage that is proportional to the power of the light incident on it.

Alternatively, a quantum counter solution can be used instead of a CD.[9, 16, 17] This is a dye solution at a sufficiently high concentration such that all of the photons incident on it are absorbed. In addition, it has an emission (EM) spectrum whose shape and intensity do not change with excitation (EX) 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 with uncertainties that are not certified or well known. In addition, a QC is prone to polarization and geometry effects that are concentration dependent. 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.

4.2 Procedure

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 section 5.2.1 or 5.2.2 depending upon whether you are using a CD or a QC, respectively.

4.2.1 *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 that used 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.

4.2.2   *Step 1 with Quantum Counter*- Place the QC solution at the sample position in a quartz cuvette. 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 $\lambda_{EX.}$ Be sure to use the same instrument settings for the excitation beam as that used 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.

4.2.3   *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 that used 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}.

5. Certified Reference Material Method

5.1 Materials

Certified reference materials [18] (CRMs) have been released by national metrology institutes (NMIs) for the relative spectral correction of fluorescence emission. [19- 25] 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. 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. As an alternative to CRMs, there are other materials with corrected relative intensity emission spectra that have been published in the literature. [26, 27] These should be used with caution, since the uncertainties in the published values are not specified in most cases.

5.2 Procedure

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).

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. [28]

6. Documentation and Reporting

Spectral correction factors should be reported as a function of emission wavelength along with the instrument settings under which it was measured; e.g., spectral bandwidth of emission, emission polarizer setting, emission filter setting, gain on the detector, emission grating position (for diode array and CCD based instruments); and the date of measurement. Recommended instrument settings and basic advice on documenting results may be given in CRM certificates. [19-24]

1. ASTM E 388-04, "Standard Test Method for Spectral Bandwidth and Wavelength Accuracy of Fluorescence Spectrometers," In: Annual book of ASTM standards, vol 03.06 (2004, original version 1972).

2. ASTM E 2719 -09, "Standard Guide for Fluorescence—Instrument Calibration and Qualification," In: Annual book of ASTM standards, vol 03.06 (2009).

3. ASTM E 578 -07, "Standard Test Method for Linearity of Fluorescence Measuring Systems," In: Annual book of ASTM standards, vol 03.06 (2007, original version 1983).

4. DeRose, P.C., Early, E.A., Kramer, G.W., "Qualification of a Fluorescence Spectrometer for Measuring True Fluorescence Spectra," *Rev.Sci.Instru.*, **78**, 033107 (2007).

5. Gaigalas, A.K., Wang, L., He, H.-J., DeRose, P.C., "Procedures for Wavelength Calibration and Spectral Response Correction of CCD Array Spectrometers," *J.Res.NIST*, **114**, 215-228 (2009).

6. a) Hollandt, J., Taubert,R.D., Seidel, J., Resch-Genger, U., Gugg-Helminger, A., Pfeifer, D., Monte, C. and Pilz, W., "Traceability in Fluorometry-Part I: Physical Standards," *J.Fluoresc.*, **15**, 301 (2005). b) Resch-Genger, U., Pfeifer, D., Monte, C., Pilz, W., Hoffmann, A., Spieles, M., Rurack, K., Hollandt, J., Taubert,D., Schonenberger, B. and Nording, P., "Traceability in Fluorometry-Part II: Spectral Fluorescence Standards," *J.Fluoresc.*, **15**, 315 (2005).

7. Roberts, G.C.K., Chapter 7, "Correction of Excitation and Emission Spectra," in *Techniques in Visible and Ultraviolet Spectrometry*, Vol. 2, *Standards in Fluorescence Spectrometry*, J.N.Miller, Ed. (Chapman and Hall, New York, 1981), pp. 54-61.

8. Costa, L.F., Mielenz, K.D., Grum, F., Chapter 4, "Correction of Emission Spectra," in *Optical Radiation Measurements*, Vol. 3, *Measurement of Photoluminescence*, K.D.Mielenz, Ed., Academic Press, New York (1982), pp. 139-174.

9. Hofstraat, J.W., Latuhihin, M.J., "Correction of Fluorescence Spectra,"*Applied Spec.*, **48**, 436 (1994).

10. Yoon, H.W., Gibson, C.E., NIST (U.S.) Spec. Publ. 250-89, (U.S. GPO, Washington, D.C., 2011).

11. Walker, J.H., Saunders, R.D., Hattenburg, A.T., Natl. Bur. Stand. (U.S.) Spec. Publ. 250-1, (U.S. GPO, Washington, D.C., 1987).

12. Barnes, P.Y., Early, E.A., Parr, A.C., NIST (U.S.) Spec. Publ. 250-48, (U.S. GPO, Washington, D.C., 1998).

13. Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, 2nd edition, Kluwer Academic/ Plenum Publishers, New York, 1999, pp.51–52.

14. Larason, T.C., Bruce, S.S., Cromer, C.L., *J. Res. Natl. Inst. Stand. Technol.*, **101**, 133 (1996).

15. Larason, T.C., Houston, J.M., NIST (U.S.) Spec. Publ. 250-41, (U.S. GPO, Washington, D.C., 2008).

16. Demas, J.N. "Measurement of Photon Yields," in *Optical Radiation Measurements*, Volume 3, *Measurement of Photoluminescence*, K.D.Mielenz, Ed., Academic Press, New York (1982) pp 228-233.

17. Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, 2nd edition, Kluwer Academic/ Plenum Publishers, New York, 1999, p.50.

18. ASTM E 131-10, "Terminology Related to Molecular Spectroscopy," In: Annual book of ASTM standards, vol 03.06 (2010, original version 1957).

19. Certificate of Analysis, Standard Reference Material® 2944, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Red Emission. National Institute of Standards and Technology (2011). (https://www-s.nist.gov/srmors/view_detail.cfm?srm=2944)

20. Certificate of Analysis, Standard Reference Material® 2943, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Blue Emission. National Institute of Standards and Technology (2009). (https://www-s.nist.gov/srmors/view_detail.cfm?srm=2943)

21. Certificate of Analysis, Standard Reference Material® 2942, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Ultraviolet Emission. National Institute of Standards and Technology (2009). (https://www-s.nist.gov/srmors/view_detail.cfm?srm=2942)

22. Certificate of Analysis, Standard Reference Material® 2941, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Green Emission. National Institute of Standards and Technology (2007). (https://www-s.nist.gov/srmors/view_detail.cfm?srm=2941)

23. Certificate of Analysis, Standard Reference Material® 2940, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Orange Emission. National Institute of Standards and Technology (2007). (https://www-s.nist.gov/srmors/view_detail.cfm?srm=2940)

24. Certificate of Analysis, Certified Reference Materials BAM-F001-BAM-F005, Calibration Kit, Spectral Fluorescence Standards. Federal Institute for Materials Research and Testing: Berlin (2006).

25. Certificate of Analysis, Standard Reference Material 936a, quinine sulfate dihydrate. National Institute of Standards and Technology (1994).

26. Gardecki, J.A., Maroncelli, M., "Set of Secondary Emission Standards for Calibration of the Spectral Responsivity in Emission Spectroscopy," *Appl. Spectr.,* **52**, 1179 (1998).

27. Velapoldi, R.A., Tonnesen, H.H., "Corrected Emission Spectra and Quantum Yields for a Series of Fluorescent Compounds in the Visible Spectral Region, *J. Fluoresc.,* **14,** 465 (2004).

28. Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.