NRC Publications Archive Archives des publications du CNRC

State-of-the art comparability of corrected emission spectra.1. Spectral correction with physical transfer standards and spectral fluorescence standards by expert laboratories

Resch-Genger, Ute; Bremser, Wolfram; Pfeifer, Dietmar; Spieles, Monika; Hoffmann, Angelika; DeRose, Paul C.; Zwinkels, Joanne C.; Gauthier, François; Ebert, Bernd; Taubert, R. Dieter; Monte, Christian; Voigt, Jan; Hollandt, Jörg; Macdonald, Rainer

This publication could be one of several versions: author's original, accepted manuscript or the publisher's version. / La version de cette publication peut être l'une des suivantes : la version prépublication de l'auteur, la version acceptée du manuscrit ou la version de l'éditeur.

For the publisher's version, please access the DOI link below. / Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

Publisher's version / Version de l'éditeur:

<https://doi.org/10.1021/ac2034503>

Analytical Chemistry, 84, 9, pp. 3889-3898, 2012-02-28

NRC Publications Record / Notice d'Archives des publications de CNRC:

<https://nrc-publications.canada.ca/eng/view/object/?id=e5a79d00-3eb4-4582-93ef-7b3733c89ffb>

<https://publications-cnrc.canada.ca/fra/voir/objet/?id=e5a79d00-3eb4-4582-93ef-7b3733c89ffb>

Access and use of this website and the material on it are subject to the Terms and Conditions set forth at

<https://nrc-publications.canada.ca/eng/copyright>

READ THESE TERMS AND CONDITIONS CAREFULLY BEFORE USING THIS WEBSITE.

L'accès à ce site Web et l'utilisation de son contenu sont assujettis aux conditions présentées dans le site

<https://publications-cnrc.canada.ca/fra/droits>

LISEZ CES CONDITIONS ATTENTIVEMENT AVANT D'UTILISER CE SITE WEB.

Questions? Contact the NRC Publications Archive team at

PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

State-of-the Art Comparability of Corrected Emission Spectra.1. Spectral Correction with Physical Transfer Standards and Spectral Fluorescence Standards by Expert Laboratories

Ute Resch-Genger,* Wolfram Bremser, Dietmar Pfeifer, Monika Spieles, and Angelika Hoffmann

Division I.5, BAM, Federal Institute for Materials Research and Testing, Richard-Willstaetter-Strasse 11, D-12489 Berlin, Germany

Paul C. DeRose*

National Institute of Standards and Technology (NIST), Gaithersburg, Maryland 20899-8312, United States

Joanne C. Zwinkels and François Gauthier

National Research Council (NRC), Canada, Ottawa, Ontario K1A 0R6, Canada

Bernd Ebert, R. Dieter Taubert, Christian Monte, Jan Voigt, Jörg Hollandt, and Rainer Macdonald

Physikalisch-Technische Bundesanstalt (PTB), Abbestraße 2-12, 10587 Berlin, Germany

Supporting Information

ABSTRACT: The development of fluorescence applications in the life and material sciences has proceeded largely without sufficient concern for the measurement uncertainties related to the characterization of fluorescence instruments. In this first part of a two-part series on the state-of-the-art comparability of corrected emission spectra, four National Metrology Institutes active in high-precision steady-state fluorometry performed a first comparison of fluorescence measurement capabilities by evaluating physical transfer standard (PTS)-based and reference material (RM)-based calibration methods. To identify achievable comparability and sources of error in instrument calibration, the emission spectra of three test dyes in the wavelength region from 300 to 770 nm were corrected and compared using both calibration methods. The results, obtained for typical spectrofluorometric ($0^\circ/90^\circ$ transmitting) and colorimetric ($45^\circ/0^\circ$ front-face) measurement geometries, demonstrated a comparability of corrected emission spectra within a relative standard uncertainty of 4.2% for PTS- and 2.4% for RM-based spectral correction when measurements and calibrations were performed under identical conditions. Moreover, the emission spectra of RMs F001 to F005, certified by BAM, Federal Institute for Materials Research and Testing, were confirmed. These RMs were subsequently used for the assessment of the comparability of RM-based corrected emission spectra of field laboratories using common commercial spectrofluorometers and routine measurement conditions in part 2 of this series (subsequent paper in this issue).

Over the past two decades, fluorescence techniques have developed into quantitative analytical tools widely used in the life and material sciences for yielding information on target-specific quantities such as emission and excitation spectra and intensities, fluorescence quantum yields, fluorescence lifetimes, and emission anisotropies.^{1–4} The growing interest in these techniques is due to their relative ease of use, non-invasive nature, very high sensitivity, potential for combining spectrally, temporally, and spatially resolved measurements, and suitability for multiplexing and remote sensing.⁵ However, fluorescence-based methods suffer from drawbacks, such as instrument-specific contributions to the fluorophore- or analyte-

specific signals of interest, which are wavelength-, polarization-, and time-dependent (aging of instrument components).^{6–8} Moreover, the fluorescence properties of most chromophores depend on their local environment. At the same time, general difficulties exist in measuring absolute fluorescence intensities accurately.⁹ These factors hinder quantification from relative fluorescence intensities and limit the comparability of fluorescence data across instruments and for the same instrument over time,

Received: December 23, 2011

Accepted: February 28, 2012

Published: February 28, 2012

especially if the data have not been properly corrected for these instrument-specific effects.

The increase in biological and medical applications of fluorescence, engendered by the expanding suite of commercial fluorescent labels and probes,^{5,10–12} along with the increasing diversity of fluorescence instrumentation, is making standardization more and more important. In turn, this is inspiring a renaissance of research activities dedicated to the development of fluorescence standards and quality assurance in fluorometry,^{3,4,13} which has not been seen in more than a generation.^{14–22} This process is supported by the globalization-induced trends of harmonization of measurements, traceability, and accreditation.²³

At present, the only fully standardized photoluminescence measuring techniques are colorimetry or surface fluorescence^{24,25} and, in part, flow cytometry,^{4,26} but new initiatives to improve the comparability and measurement uncertainties of photoluminescence measurements are in progress. Aside from the increasing number of publications and workshops dedicated to these topics,^{3,4,7,8,13,27–37} ongoing activities include efforts by the International Union of Pure and Applied Chemistry (IUPAC; e.g., Project 2004-021-1-300) to develop technical notes for the characterization and validation of instruments and methods and a new guide from ASTM International, formerly known as the American Society for Testing and Materials, for fluorescence instrument qualification.³⁸ Moreover, only recently, a first multinational, multilaboratory comparison was performed evaluating fluorescence lifetime standards for time and frequency domain fluorescence spectroscopy²⁹ as well as a multinational, multilaboratory comparison on fluorescence immunoassays.³⁹ In addition, optical methods for the determination of fluorescence quantum yield were recently assessed,⁴⁰ and new commercially available integrating sphere setups for the absolute measurement of emission spectra and absolute fluorescence quantum yields were evaluated.^{36,37} Concomitantly, first requirements and quality criteria for fluorescence standards were established.^{33,41} The overall goal of these research activities^{38,42,43} is the eventual replacement of the few existing guidelines and recommendations, developed more than 20 years ago in most cases, for the qualification of fluorescence instruments and the measurement of key physical properties of fluorescence.

The development of fluorescence standardization methods involves suitable instrument calibration, validation procedures, and standards for all instrument quantities and parameters that can affect the analyte-specific spectral position, spectral shape, and intensity of measured fluorescence signals. Relevant quantities include the (relative) spectral responsivity of the emission channel, the (relative) spectral irradiance of the excitation beam at the sample, and the accuracy of excitation and emission wavelengths and bandwidths.^{6,38,44} For this purpose, new spectral fluorescence standards were recently developed by the National Institute of Standards and Technology (NIST; glass-based reference materials (RMs))^{45–53} and the Federal Institute for Materials Research and Testing (BAM, Germany; liquid RMs).^{6,54,55} The importance of the reliable determination of the spectral responsivity of the detection channel for the comparability of emission spectra and the determination of reliable fluorescence quantum yields encouraged four National Metrology Institutes (NMIs) currently active in the area of high-precision steady-state fluorometry to evaluate the state-of-the-art comparability of corrected fluorescence emission spectra, reported here. For this purpose, different instrument calibration procedures using calibrated physical transfer standards (PTs), such as standard (calibrated) lamps, detectors, and diffuse reflectors, and easy-to-

use, certified, commercially available RMs⁵⁵ were assessed. This assessment was based upon the relative uncertainties of the resulting corrected emission spectra of three test dyes emitting in the wavelength region from 300 to 770 nm.

■ INSTRUMENTATION AND MATERIALS

Participants. The Physikalisch-Technische Bundesanstalt (PTB, Germany),⁵⁶ BAM, NIST (Gaithersburg, MD), and the National Research Council (NRC, Canada) participated in this study organized and coordinated by BAM and NIST. The participants chose to keep data ownership anonymous, yet the symbols used here throughout the figures and tables and in the Supporting Information always refer to data from the same laboratory.

Instrumentation. PTB, NIST, and BAM employed commercial spectrofluorometers using a 0°/90° measurement geometry and NRC a custom-designed goniometer-type instrument for spectrally resolved measurements in a 45°/0° geometry; see also the Supporting Information (Table 1S).

PTB used a SPEX Fluorolog 2 (Horiba Jobin Yvon, Edison, NJ) spectrofluorometer of Czerney–Turner design (two double monochromators with 1200 grooves/mm gratings blazed at 330 and 500 nm for excitation and emission, respectively), Peltier-cooled photomultiplier tube (PMT) R928 from Hamamatsu as the emission detector, and noncooled R928 PMT as the reference detector.

NIST employed a SPEX Fluorolog 3 (Horiba Jobin Yvon)⁵⁷ spectrofluorometer (double monochromators of Czerney–Turner design with 1200 grooves/mm gratings blazed at 330 and 500 nm for excitation and emission selection, respectively), a Hamamatsu R928-P photon-counting PMT for emission detection, and a silicon photodiode as the reference detector. Vertical masks with heights of 6.00 and 5.00 mm placed between the exit slit of the excitation monochromator and the entrance slit of the emission monochromator, respectively, served to better define the size of the excitation beam and emission slit image on the sample.^{7,58}

BAM used a Spectronics Instruments 8100 spectrofluorometer of T-type design (UV/vis emission channel, double monochromator (Seya Namioka type with holographic gratings) and Peltier-cooled PMT R928 from Hamamatsu operated in the photon counting (PC) mode; vis/NIR emission channel, single monochromator (Czerney–Turner, 500 mm) and a Si avalanche photodiode (operated in the analog or current mode); reference channel, Peltier-cooled R928 PMT).^{6,34,59} This fluorometer underwent two custom-built modifications: (i) the excitation polarizer was placed behind the excitation monochromator and in front of the beam splitter to control the polarization of the light incident on the beam splitter, and (ii) the original lenses were exchanged for custom-made achromatic lenses to minimize achromatic aberrations and, thus, the wavelength dependence of the excited and radiating volume. Typical fluorescence measurements by BAM were performed with Glan–Thompson polarizers placed in the excitation channel and the two emission channels. Polarizers were not used by the other three laboratories (see the Supporting Information, Table 3S).

At NRC, measurements of the reflected and total spectral radiance factors of the fluorescent dye solutions were performed on the well-described NRC reference spectrofluorometer based on the two-monochromator method using a 45° annular illumination and normal viewing (45a:0) in accordance with the International Commission on Illumination (CIE)⁶⁰ and ASTM International⁶¹ colorimetric standards.^{62,63}

To enhance the instrument's sensitivity over its operation range (200–1040 nm) for the measurement of dye solutions, two different holographic gratings of 1200 grooves/mm, optimized over different wavelength regions (grating A, 200–700 nm; grating B, 450–1100 nm), were used.^{24,64}

Calibration Procedures and Physical Standards. Each NMI performed an instrument qualification following its own “routine”, PTS-based calibration procedure for its fluorescence spectrometer.^{6,7,65} These qualifications included the determination of (i) the wavelength accuracy and spectral resolution of both the excitation and emission monochromators, (ii) the linear range of the emission detection system, and (iii) the relative spectral responsivity of the detection system.^{6,7,34} On the basis of these instrument qualifications detailed in the Supporting Information (Table 2S), each NMI provided complete wavelength-dependent uncertainty budgets for the PTS-based corrected emission spectra.

Materials. For the RM-based emission correction, F001 to F005 (solvent ethanol), covering the wavelength region between 300 and 770 nm, were provided by BAM as solutions. Design criteria for the development of these spectral emission standards included (i) broad and unstructured emission bands to minimize the dependence of the shape of the spectra on the instrument spectral band pass/instrument resolution, (ii) a large Stokes shift as a prerequisite for minimum dependence of the emission spectra on the dye concentration and the measurement geometry on the spectral shape, (iii) moderate to strong fluorescence quantum yields to increase the signal-to-noise ratios and strongly reduce the influence of stray light, solvent emission, and fluorescent impurities on the emission spectra, and (iv) a small fluorescence anisotropy of $r \leq 0.05$ within the analytically relevant room temperature region to circumvent additional polarization effects. This minimizes uncertainties for the use of the fluorescence standards under measurement conditions that can dispense with or for instruments that lack polarizers. In addition, for the design of the standard set, the fluorescence spectra of spectrally neighboring chromophores had to cross at points of sufficient fluorescence intensity, e.g., at least at 20% of the relative maximum fluorescence intensity.

The test dyes included BAM dye X (solvent ethanol), NIST Standard Reference Material (SRM) 936a, quinine sulfate dihydrate (dye QS; solvent 0.1 mol/L perchloric acid), and BAM dye Y (solvent acetonitrile). These dyes were chosen to cover the spectral region used for calibration and to display a comparatively large Stokes shift to minimize inner filter effects, concentration dependencies, and influences of measurement geometry. Dye X was selected for its slightly structured emission spectrum to check on effects of spectral resolution. For the three commercial spectrofluorometers, dye solutions with absorbances of both 0.04 and 0.08 at the longest wavelength absorption maximum were used, and for the less sensitive NRC reference spectrofluorometer, only solutions with an absorbance of 0.08 were used. These dyes were measured using instrument settings given in the Supporting Information (Table 3S). The resulting data were preprocessed by each NMI according to detailed standard operating procedures (SOPs) evaluated and provided by BAM and NIST.

Safety Considerations. Material Safety Data Sheets for dyes and organic solvents should be consulted to ensure that proper safety procedures for their handling, storage, and disposal are followed. Generally, organic solvents should be handled in hoods to prevent inhalation, and safety glasses,

gloves, and lab coats should be worn to prevent eye and skin contact by both organic solvents and dyes.

DATA ANALYSIS

Data Assessment. Preliminary statistical data evaluation including the calculation of the S/N ratio was performed by NIST and BAM. The wavelength-dependent uncertainties of the RM-based corrected emission spectra were calculated by BAM and NIST on the basis of the data provided, i.e., from the respective wavelength-dependent uncertainties of the fluorescence standards F001 to F005, determined by BAM,⁵⁵ and the standard deviations of the emission spectra measured by each participant.

Data Pretreatment. The emission maximum of each spectrum was determined by fitting seven data points around the maximum to a second-order polynomial. All emission intensities were then divided by the fitted estimate of the peak maximum, yielding a normalized emission spectrum. In addition, the spectral position of the half-maximum at the leading (high-energy) and the tailing (low-energy) wings of the spectrum were determined. A straight line was regressed over five data points in the vicinity of half of the value of the emission maximum, and the exact value of the position of the half-maximum was obtained from the regressed line. From these data, the full width at half the maximum height (fwhm) of each spectrum was calculated.

Determination of the Intercomparison Reference Function (ICRF). The ICRF of each dye represents the best estimate of its true emission spectrum derived from all spectral data measured by the participants. Because of the systematic uncertainties inherent in each measurement, both the emission intensity values y_{jk} and the corresponding emission wavelength settings x_{jk} were assumed to be distorted from their “true” values, where y_{jk} is the value measured by laboratory k at setting j of the independent variable x_{jk} . Constant correction factors f_k were assumed to correct for the spectral responsivities of the fluorometers used and correction functions $\varphi(x_k)$ to account for deviations related to uncertainties in the wavelength scale. $\varphi(x_k)$ can be any reasonable transformation of the independent variable, i.e., additive, multiplicative, combined, or even nonlinear. Both f_k and $\varphi(x_k)$ are characteristic for each participant. An ICRF can be determined using the data from all four laboratories such that the sum of the squared deviations (SSD), eq 1, is minimized. This was done using an iterative algorithm; see the next section (2D averaging) and the Supporting Information (section S4) for further details.

$$\text{SSD} = \sum_{j,k} [f_k y_{jk}(\varphi(x_{jk})) - \text{ICRF}(\varphi(x_{jk}))]^2 = \min \quad (1)$$

Two adjustments for each laboratory were made in the ICRF determination: (1) a scaling factor in $y(f_k)$ and (2) an additive shift in $x(\delta_k)$. Higher order adjustments were considered such as a linear distortion in the wavelength scale, but were found to be unnecessary. The ICRF was then fitted against adjusted data of the form

$$\begin{aligned} \varphi(x_{jk}) &= x_{jk} + \delta_k \\ y'_{jk} &= f_k y_{jk} \end{aligned} \quad (2)$$

The adjustment vector thus consisted of four factors f_k and four individual shift values δ_k , i.e., a pair for each participant.

2D Averaging. A 2D averaging procedure was used to determine the ICRF and the values of f_k and δ_k . The wavelength position and value of the ICRF at each point j were calculated as the corresponding mean over k in both the x and the y directions according to the following equation:

$$\overline{f_k y_{jk}} = \text{ICRF}_j(\overline{x_{jk}} + \delta_k) \quad (3)$$

The total SSD was minimized by adjusting f_k and δ_k in an iterative procedure. After convergence was reached, i.e., best-fit estimates for f_k and δ_k were found, the joint confidence region (JCR) for each of the points which make up the ICRF was determined. Upper and lower confidence intervals of the points on the ICRF were then estimated as the points where the bisecting line of the ICRF frequency polygon passed through the JCR.

RESULTS AND DISCUSSION

PTS-Based Spectral Correction: Data Treatment. When all dyes F001 to F005 are analyzed together, the gain in variance from the previously described 2D averaging procedure is significant, but moderate (Table 1). Nearly 80% of the

Table 1. Initial and Best Fit Results for Dyes F001 to F005 for the PTS-Based Calibrations

	NMI A (■)	NMI B (◆)	NMI C (▲)	NMI D (●)
Initial Results ($\text{SSD}^a = 0.151$)				
factor f_k	1	1	1	1
wavelength shift δ_k	0	0	0	0
average SD ^b	0.0114	0.0125	0.0061	0.0085
Best Fit Results ($\text{SSD} = 0.111$)				
factor f_k	0.9968	1.0011	0.9934	0.9982
wavelength shift δ_k	0.529	−0.336	−0.051	−0.142
average SD	0.0087	0.0109	0.0056	0.0080

^aSum of the squared deviations. ^bStandard deviation.

reduction in the sum of the squared deviations is caused by the adjustment or shift in x . Only 20% of the SSD reduction results from further adjustment or scaling in y . This is largely due to the fact that all emission spectra were already pretreated and scaled by a normalization procedure.

The average standard deviations of the emission spectra of F001 to F005 about the ICRFs are included in Table 1 for each participant. The shifts δ_k are all well below 0.6 nm and thus physically reasonable with respect to typical uncertainties of the wavelength accuracy of emission monochromators of high-precision spectrofluorometers. The absolute deviations between the nominal and observed spectral positions of the atomic emission lines used for the calibration of the emission wavelength scale of the participating spectrofluorometers are below 0.5 nm; see Figure 1 and the Supporting Information (Table 2S). These deviations did not provide a consistent trend and, therefore, did not justify the assumption of a linear (or even higher order) distortion of the wavelength scales of the participating spectrofluorometers.

In Figure 2, the ICRFs and the adjusted measurement results of the participants are illustrated for dye X, the only fluorophore

Figure 1. Absolute deviation between the nominal and the observed positions of atomic emission lines used for wavelength calibration. Each laboratory is represented by a different symbol (see Table 1). One laboratory (triangles) submitted only a single point check of the wavelength scale.

Figure 2. Measured data, ICRF (bold black), and confidence interval of the ICRF (dashed lines) for dye X, all data being determined with a PTS-based calibration. Lower plots: relative deviations from the ICRF of the data obtained by the four participating laboratories. See Table 1 for the meanings of the symbols.

in this study that reveals a slightly structured emission spectrum. The importance of this data evaluation procedure is twofold. First, 2D averaging of the PTS-corrected data enabled accommodation of incomplete spectra such as the spectrum of dye X measured by one participant only for wavelengths ≥ 350 nm. Second, the adjustment procedure led to a reduction of the initial SSDs by a factor of more than 7. The wavelength shifts δ_k (nm) of 0.64, −0.24, −0.48, and 0.07 for the dye X spectrum for each of the four laboratories are comparable in size with the wavelength shifts obtained when determining the ICRF for the spectra of the dyes F001 to F005 over the full spectral range covered and the experimentally determined deviations in wavelength accuracy shown in Figure 1.

Interlaboratory Comparability of PTS-Corrected Emission Spectra. The calculated ICRFs of the PTS-based corrected emission spectra of dyes F001 to F005, X, QS, and Y are displayed in the left panel of Figure 3. The right panel of Figure 3 illustrates the deviations of the corrected emission spectra of F001 to F005 relative to the ICRFs of the corresponding emission spectra (original, nonadjusted data) measured and corrected by the participants (Figure 3, right panel, symbols) and the expanded ($k = 2$; confidence interval of 95%)

Figure 3. Left panel: ICRFs for dyes F001 to F005 (◆) and the test dyes X, QS, and Y (◇) for the PTS-based calibration. Right panel: Deviations (symbols) of the individual PTS-based corrected emission spectra of dyes F001 to F005 measured by the participating laboratories, the corresponding ICRFs of these dyes, and the expanded ($k = 2$) combined uncertainties (lines) of the measured spectra. See Table 1 for the meanings of the symbols in the right panel.

Figure 4. Participants' relative deviations from the ICRF for dyes X (left panels), QS (middle panels), and Y (right panels) using a PTS-based correction (top) and an RM- or a dye-based correction (bottom). See Table 1 for the meanings of the symbols.

combined uncertainties (Figure 3, right panel, lines). This figure reveals a certain deviation "pattern" for each participant, yet these trends clearly go in different directions. This explains why the gain in the SSD from the optimization procedure was only moderate. For the desired estimation of accomplishable comparability of spectrally corrected emission spectra, it is of particular interest whether all participants were able to match the ICRFs within their stated uncertainties, i.e., whether the observed differences are covered by the respective uncertainties of instrument calibration and performance of emission measurements. Possible sample-related uncertainties were minimized here by the provision of liquid samples and solvents always originating from the same batch and detailed protocols for dye measurement (see the Supporting Information, section S4). As follows from the right panel of Figure 3, for more than 95% of the data points provided, the deviations

between the measurement results of the laboratories and the deviations with respect to the best estimate of the ICRFs are within the stated uncertainties or are covered by them. This underlines the general suitability of the instrument characterization procedures developed by the participants, which provides the basis for the very satisfying comparability of the PTS-based corrected emission spectra of F001 to F005. Moreover, this confirms the reliability of the BAM-corrected emission spectra of the emission standards F001 to F005 used for the common RM-based spectral correction presented in the following section and for the field laboratory intercomparison detailed in part 2 of this series (subsequent paper in this issue).

Comparison of PTS- and RM-Based Spectral Correction. To further examine the influence of instrument calibration and data evaluation procedures on the uncertainties

of corrected emission spectra, the previously assessed spectral fluorescence standards F001 to F005 were used for the determination of an RM-based spectral correction of the emission spectra of the test dyes X, QS, and Y. These results (Figure 4d–f, lower panels), including the average relative deviations of the participants' results from the ICRFs, were compared to the corresponding PTS-based corrected emission spectra of these fluorophores (Figure 4a–c, top panels); see also Table 2. For the PTS calibration, the results of the

Table 2. Comparison of PTS- and RM-Based Spectral Corrections: Average Relative Deviations (%) of the Corrected, Unshifted Original Emission Spectra of the Participating Laboratories from the ICRFs for the PTS- and RM-Based Calibrations

	NMI A (■)	NMI B (◆)	NMI C (▲)	NMI D (●)
Dye X				
PTS-based calibration	5.9	2.9		4.9
RM-based calibration				
comparability	2.3	1.8	1.8	2.0
accuracy	5.5	5.3	5.3	5.4
Dye QS				
PTS-based calibration	4.2	6.6	1.2	3.1
RM-based calibration				
comparability	3.4	1.6	1.2	2.8
accuracy	5.4	4.5	4.4	5.1
Dye Y				
PTS-based calibration	1.6	1.5	1.9	1.8
RM-based calibration				
comparability	1.8	1.0	1.5	1.5
accuracy	3.5	3.2	3.3	3.4

participating laboratories were comparable within a relative standard uncertainty of 4.2% for all dyes measured except for the values in the (extreme) wings of the emission spectra (emission intensity $\leq 10\%$ of the intensity at the emission maximum). This high degree of comparability is impressive taking into account that each laboratory applied different calibration procedures and one laboratory used a different measurement geometry. Hence, the estimates given in Table 2 not only characterize the degree of comparability between the different laboratories, but are also a measure of accuracy. Therefore, the ICRF derived from the data obtained by the four independently calibrated instruments can be considered the best estimate for the true values. Such a result is only achievable by expert laboratories that dedicate considerable resources to the development and evaluation of instrument characterization procedures with the goal of minimizing calibration uncertainties.

The RM-based correction procedure with all participants using the BAM dyes as a common reference significantly improved the data comparability, yielding a relative standard uncertainty of 2.4% over the three test dyes studied, except for the (extreme) wings of the emission spectra. The observed deviations confirm the general trend of a substantially reduced average between-laboratory scatter for the RM-based spectral correction. This is most pronounced for dye X with its structured emission

spectrum, the vibrational fine structure of which was resolved considerably better by all participants (see Figure 4a,d) with the RM-based correction compared to the PTS calibration. The improved comparability of data accomplished with the RM-based correction is largely due to three factors: (i) Dye measurements were performed using high-precision spectrofluorometers already calibrated to perform the PTS-based comparison of corrected emission spectra. (ii) A common reference procedure was applied for spectral correction of the test dyes by all four laboratories, thereby reducing systematic differences in the procedures implemented in the laboratories. (iii) The spectral fluorescence standards used for the RM-based correction were designed to simulate the spectral radiances and radiating volumes of typical fluorescent samples to reduce some of the major sources of uncertainty inherent in PTS-based spectral correction, such as effects related to optical geometry and detector nonlinearity.^{6,9,33,38,54}

In the case of the RM-based calibration, the average deviation from the ICRFs (Table 2) is a measure of comparability only, since all deviations refer to the same standard. For assessing the accuracy, the uncertainty of the reference standard itself must be considered. This has been realized in Table 2 in the second line for the RM-based calibrations using uncertainties given in the BAM certificates of F001 to F005.⁶⁶ The accuracies given in Table 2 for the RM-based calibrations slightly exceed those obtained by the PTS-based calibrations, which is in excellent agreement with the measurement capabilities demonstrated in this intercomparison.

Comparison of Corrected and Certified Spectra for QS. Until recently,^{6,9,33,45–55,67} dye QS was the only commercial fluorescence standard supplied with a normalized certified corrected emission spectrum including wavelength-dependent uncertainties. For this reason, comparison of the study result to this universally used emission standard was a necessity. Figure 5 summarizes the relative deviations of the participants' PTS-based (top) and RM-based (bottom) corrected emission spectra of dye QS from the NIST-certified⁶⁸ reference spectrum (dotted gray line) and the expanded ($k = 2$) uncertainties of the reference spectrum (data interval of 5 nm).¹⁷ For most parts of the spectral region covered by the emission spectrum of QS, these deviations are within the NIST-stated uncertainties. Interestingly, the relative deviations of the corrected emission spectra of all participants display a similar trend in the wavelength region from 390 to 480 nm, whereas between 480 and 570 nm the deviations show different trends, with one laboratory deviating from the rest, particularly in the red wing. At wavelengths below 420 nm and at wavelengths above 500 nm, the relative deviations of two laboratories exceed the expanded combined uncertainties of the corrected emission spectrum of SRM 936a. The observed trend of the relative deviations of the participating laboratories suggests a common wavelength shift between the reference spectrum and the spectra obtained in this intercomparison. This assumption is supported by the observed reduction in residual SSD by introducing a common wavelength shift. The residual SSDs reach a minimum for wavelength shifts of 1.237 and 1.777 nm when compared to the ICRFs for PTS-based and RM-based calibrations, respectively. The size of this wavelength shift exceeds the combined uncertainties in the wavelength accuracy provided by the participants (<0.6 nm) and determined (0.2 nm) during the certification of SRM 936a using atomic discharge lamps (see the Supporting Information, Table S2). This suggests that a wavelength shift may be a contributing factor,

Figure 5. Top panel, left: Relative deviation of the participants' PTS-based corrected emission spectra of dye QS from the reference spectrum (large-dashed gray line) as certified by NIST in 1979 and expanded ($k = 2$) uncertainty of the reference spectrum (small-dashed gray line). Top panel, right: Corresponding "wavelength-shifted" corrected spectra applying a wavelength shift of 1.237 nm that corresponds to the minimum SSD. Bottom panel, left: Relative deviation of the participants' RM-based corrected emission spectra of dye QS from the reference spectrum (dotted gray line) as certified by NIST in 1979 and expanded ($k = 2$) uncertainty of the reference spectrum. Bottom panel, right: Corresponding "wavelength-shifted" corrected spectra applying a wavelength shift of 1.777 nm that corresponds to the minimum residual SSD. See Table 1 for the meanings of the symbols.

but other unknown factors may also be involved. In the case of the RM-based spectral corrections (Figure 5, lower panels), the wavelength uncertainties of the corrected emission spectra of F001 to F005 of BAM play a role. NIST discontinued SRM 936a in 2009 and currently recommends SRM 2943 for spectral correction in the emission wavelength range covered by SRM 936a.

Typical sources of Calibration Uncertainty and Influence on Corrected Emission Spectra. During the course of this study and additional studies with nonexpert or field laboratories, we identified two major sources of uncertainty for spectral correction: (i) instrument characterization and measurement of the emission spectra under nonidentical experimental conditions and (ii) nonlinearities of the emission detection system that could result in distorted emission spectra. Uncertainties of the wavelength scale or wavelength accuracy are typically not problematic for monochromator/PMT-based detection systems. However, CCD-based instruments, which did not participate in the study but are becoming increasingly popular, require careful detector alignment and focusing to ensure acceptable uncertainties in wavelength and bandwidth accuracy, making their inclusion in a future comparative study of particular interest.

Different Instrument settings for Instrument Calibration and Fluorescence Measurements. Mandatory for the spectral correction of emission spectra with minimum uncertainties are the determination of the instrument's spectral responsivity and the measurement of samples under identical measurement conditions, independent of the type of transfer standards used, i.e., PTSs or RMs.^{6,9,38,54} These conditions include identical optical components in the respective instrument channel and identical instrument settings. In the case of spectrofluorimeters equipped with charge-coupled device (CCD) detectors, there is the further requirement that identical wavelength ranges and CCD pixel grids be used for calibration and subsequent fluorescence measurements. The fulfillment of this requirement is not difficult for an RM-based spectral correction, yet imposes very stringent conditions for suitable instrument characterization procedures using PTSs, especially for source-based calibrations as the spectral radiances of common calibrated light sources exceed that of a typical dilute organic dye solution by as much as 2 (single integrating sphere-mounted lamp) to 4 (tungsten ribbon lamp) orders of magnitude.²⁷ Accordingly, the avoidance of detector nonlinearities presents a considerable challenge when measuring the emission spectrum of a calibrated source under typical fluorescence measurement conditions without the introduction of additional spectral components, e.g., attenuators.

Thus, in many cases, different measurement conditions are used for instrument characterization and subsequent fluorescence measurements, such as different spectral bandpasses of the emission monochromator, different filter settings, or even different monochromator gratings. The importance of this requirement is explained further in the Supporting Information, where different monochromator gratings were used to measure the emission spectra of dye QS and the RMs F001 to F005 (Figure 2S). The resulting corrected emission spectrum of QS deviates by as much as 25% from the corresponding data of the other participants and the ICRF.

Detector Nonlinearities. Larger spectral radiances incident on a photodetector can result in significant deviations from a linear responsivity with intensity.^{6,38} The onset of the upper limit of the detector's linear range depends on the instrument design, especially on the type of detector used, the detection mode (photon counting; analog), and in the case of photomultiplier tubes the applied voltage. In addition, the considerable changes of the spectral radiances of PTSs and RMs with wavelength must be considered. The high spectral radiances of most commercially available, calibrated light sources are again of particular concern.²⁷ Even for dilute dye solutions, the influences of detector nonlinearities on measured fluorescence signals can easily result in relative variations (especially at the emission maxima) of 10–20% as shown for F003 in the Supporting Information (section S7 and Figure 3S). In the case of an RM-based spectral correction, detector nonlinearities typically do not play a significant role. Elegant procedures for the determination of the linear range of detection systems that are detailed in the Supporting Information (section S7, Figure 3S and Figure 4S) include (i) the measurement of dye emission spectra as a function of concentration and the comparison of the resulting normalized emission spectra and (ii) the measurement of ratios of signals or emission intensities for a controlled variation of the spectral radiance.⁶ Candidates for the former method are fluorophores, such as dyes F001 to F005, QS, and Y, revealing concentration-independent emission spectra (no dimer formation or aggregation; minimum overlap between absorption and emission bands to circumvent inner filter effects and reabsorption) and the use of only comparatively small absorbances.^{6,9,38}

CONCLUSIONS AND OUTLOOK

A first international interlaboratory comparison of measurement capabilities in fluorescence spectroscopy among four National Metrology Institutes used four different high-precision spectrofluorometers with two measurement geometries (0°/90° and 45°/0° typical for conventional spectrofluorometry and bispectral fluorescence) and different PTSs and procedures for instrument calibration, demonstrating a comparability of corrected emission spectra in the wavelength region from 300 to 770 nm of better than 5%. This excellent comparability within the participants' stated uncertainties is largely due to the careful instrument characterization procedures that have been developed by the participating laboratories over the years. These results also confirmed the corrected emission spectra of the five liquid spectral fluorescence standards F001 to F005 certified by BAM, which are commercially available. The comparison of PTS-based and RM-based spectral correction of the emission spectra of three test dyes clearly demonstrates the efficacy of certified reference materials (CRMs), in general, and the BAM spectral fluorescence standards, in particular, for the determination of the wavelength dependence of the spectral responsivity of emission detection systems of different designs.

Such an RM-based characterization of fluorescence instruments is more convenient and less error-prone and can provide a better comparability between instruments and laboratories than PTS-based methods. The use of certified spectral fluorescence standards, such as BAM's RMs or NIST's SRMs, present a reliable, simple, and robust means to improve the overall comparability of emission spectra among the broad community of fluorescence spectroscopists. This is also demonstrated in part 2 of this study (subsequent paper in this issue) by the results from a comparison of 12 field laboratories randomly selected from academia and industry using common spectrofluorometers and the BAM dyes F001 to F005.

The most critical requirements for a high level of data comparability as identified during the course of this study are (i) the use of reliable and well-characterized standards such as the PTSs and RMs evaluated in this study, (ii) the performance of an instrument characterization under conditions identical to those employed for subsequent fluorescence measurements (these include instrument components such as monochromator gratings and filters, instrument settings, and measurement geometries), (iii) the calibration of wavelength accuracy to better than 1 nm (preferably to 0.5 nm), and (iv) the operation of the emission detection system within its linear range. These critical requirements should also serve as recommendations to users for obtaining emission spectra with higher accuracy.

In addition, it was shown that an external method validation via an intercomparison of expert laboratories is preferable to any internal validation procedure in uncovering unknown biases. 2D averaging enabled the determination of intercomparison reference functions that account for possible sources of uncertainty. Fit parameters can be chosen to identify and correct for a number of systematic errors, thereby considerably reducing deviations between laboratories, as demonstrated here, but the number of fit parameters should be strictly limited to be physically reasonable.

This comparison presents a first important step toward standardized fluorescence measurements. In the future, similar comparisons may be performed to establish the state-of-the-art of the comparability of emission measurements for other types of fluorescence instruments, such as those covering the near-infrared spectral region, which is becoming increasingly popular, in conjunction with suitable standards.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ute.resch@bam.de (U.R.-G.); paul.derose@nist.gov (P.C.D.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank all participating laboratories for the efforts spent on participation in this laboratory intercomparison. BAM gratefully acknowledges financial support from the German Ministry of Economics and Labor (BMWi; Grants BMWi VI A2-18 and VI A2-17/03). BAM and PTB express their gratitude to Dr. A. Höpe from PTB for the calibration of their diffuse reflectance

standards. NIST thanks Dr. D. Duewer for advice on data analysis.

REFERENCES

- (1) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer Science+Business Media: New York, 2006.
- (2) Valeur, B. *Molecular Fluorescence: Principles and Application*; Wiley-VCH: Weinheim, Germany, 2002.
- (3) Resch-Genger, U. *Standardization and Quality Assurance in Fluorescence Measurements I: Techniques*, 1st ed.; Springer-Verlag: Berlin, Heidelberg, 2008.
- (4) Resch-Genger, U. *Standardization and Quality Assurance in Fluorescence Measurements II: Bioanalytical and Biomedical Applications*, 1st ed.; Springer-Verlag: Berlin, Heidelberg, 2008.
- (5) Resch-Genger, U.; Grabolle, M.; Cavaliere-Jaricot, S.; Nitschke, R.; Nann, T. *Nat. Methods* 2008, 5, 763–775.
- (6) Resch-Genger, U.; Pfeifer, D.; Monte, C.; Pilz, W.; Hoffmann, A.; Spieles, M.; Rurack, K.; Hollandt, J.; Taubert, D.; Schonenberger, B.; Nording, P. J. *Fluoresc.* 2005, 15, 315–336.
- (7) DeRose, P. C.; Early, E. A.; Kramer, G. W. *Rev. Sci. Instrum.* 2007, 78, 033107.
- (8) Resch-Genger, U.; Hoffmann, K.; Niefeld, W.; Engel, A.; Neukammer, J.; Nitschke, R.; Ebert, B.; Macdonald, R. J. *Fluoresc.* 2005, 15, 337–362.
- (9) Resch-Genger, U.; Pfeifer, D.; Hoffmann, K.; Flachenecker, G.; Hoffmann, A.; Monte, C. In *Standardization and Quality Assurance in Fluorescence Measurements I: Techniques*; Resch-Genger, U., Ed.; Springer Verlag: Berlin, Heidelberg, 2008; Vol. 5, pp 65–100.
- (10) Waggoner, A. *Curr. Opin. Chem. Biol.* 2006, 10, 62–66.
- (11) Nagl, S.; Schaeferling, M.; Wolfbeis, O. S. *Microchim. Acta* 2005, 151, 1–21.
- (12) Sapsford, K. E.; Berti, L.; Medintz, I. L. *Angew. Chem., Int. Ed.* 2006, 45, 4562–4588.
- (13) Resch-Genger, U. *J. Fluoresc.* 2005, 15.
- (14) Parker, C. A.; Rees, W. T. *Analyst* 1960, 85, 587–600.
- (15) Melhuish, W. H. *J. Phys. Chem.* 1960, 64, 762–764.
- (16) Chapman, J. H.; Förster, T.; Kortüm, G.; Parker, C. A.; Lippert, E.; Melhuish, W. H.; Nebbia, G. *Appl. Spectrosc.* 1963, 17, 171–171.
- (17) Velapoldi, R. A.; Mielenz, K. D. *NBS Spec. Publ. (U.S.)* 1980, 260–64, 1–115.
- (18) Velapoldi, R. A. *Liquid Standards in Fluorescence Spectrometry*; Elsevier Science Publishers B.V.: Amsterdam, 1987.
- (19) Miller, J. N., Ed. *Standards in Fluorescence Spectrometry*; Chapman and Hall: New York, 1981.
- (20) Argauer, R. J.; White, C. E. *Anal. Chem.* 1964, 36, 368–371.
- (21) Hofstra, J. W.; Latuhihin, M. J. *Appl. Spectrosc.* 1994, 48, 436–447.
- (22) Gardecki, J. A.; Maroncelli, M. *Appl. Spectrosc.* 1998, 52, 1179–1189.
- (23) ISO 17025, I. I., 2nd ed.; International Organization of Standardization: Geneva, 2005.
- (24) Zwinkels, J. C. In *Standardization and Quality Assurance in Fluorescence Measurements I: Techniques*; Resch-Genger, U., Ed.; Springer-Verlag: Berlin, Heidelberg, 2008; pp 163–192.
- (25) Rich, D. C.; Martin, D. *Anal. Chim. Acta* 1999, 380, 263–276.
- (26) NCCLS I/LA24-A, 2004, vol. 24, No. 26.
- (27) Monte, C.; Resch-Genger, U.; Pfeifer, D.; Taubert, D. R.; Hollandt, J. *Metrologia* 2006, 43, S89–S93.
- (28) Pfeifer, L.; Stein, K.; Fink, U.; Welker, A.; Wetzl, B.; Bastian, P.; Wolfbeis, O. S. *J. Fluoresc.* 2005, 15, 423–432.
- (29) Boens, N.; Qin, W. W.; Basaric, N.; Hofkens, J.; Ameloot, M.; Pouget, J.; Lefevre, J. P.; Valeur, B.; Gratton, E.; Vandeven, M.; Silva, N. D.; Engelborghs, Y.; Willaert, K.; Sillen, A.; Rumbles, G.; Phillips, D.; Visser, A.; van Hoek, A.; Lakowicz, J. R.; Malak, H.; Gryczynski, I.; Szabo, A. G.; Krajcarski, D. T.; Tamai, N.; Miura, A. *Anal. Chem.* 2007, 79, 2137–2149.
- (30) Luchowski, R.; Szabelski, M.; Sarkar, P.; Apicella, E.; Midde, K.; Raut, S.; Borejdo, J.; Gryczynski, Z.; Gryczynski, I. *Appl. Spectrosc.* 2010, 64, 918–922.
- (31) Giebler, R.; McGown, E.; French, T.; Owicki, J. C. *J. Fluoresc.* 2005, 15, 363–375.
- (32) Velapoldi, R. A.; Tonnesen, H. H. *J. Fluoresc.* 2004, 14, 465–472.
- (33) Resch-Genger, U.; Hoffmann, K.; Pfeifer, D. In *Reviews in Fluorescence*; Geddes, C. D., Ed.; Springer Science+Business Media: New York, 2009; Vol. 4, pp 1–32.
- (34) Grabolle, M.; Spieles, M.; Lesnyak, V.; Gaponik, N.; Eychmüller, A.; Resch-Genger, U. *Anal. Chem.* 2009, 81, 6285–6294.
- (35) Gaigalas, A. K.; Wang, L. L.; He, H. J.; DeRose, P. J. *Res. Natl. Inst. Stand. Technol.* 2009, 114, 215–228.
- (36) Suzuki, K.; Kobayashi, A.; Kaneko, S.; Takehira, K.; Yoshihara, T.; Ishida, H.; Shiina, Y.; Oishi, S.; Tobita, S. *Phys. Chem. Chem. Phys.* 2009, 11, 9850–9860.
- (37) Würth, C.; Lochmann, C.; Spieles, M.; Pauli, J.; Hoffmann, K.; Schutttrigkeit, T.; Franzl, T.; Resch-Genger, U. *Appl. Spectrosc.* 2010, 64, 733–741.
- (38) DeRose, P. C.; Resch-Genger, U. *Anal. Chem.* 2010, 82, 2129–2133.
- (39) Noble, J. E.; Wang, L.; Cerasoli, E.; Knight, A. E.; Porter, R. A.; Gray, E.; Howe, C.; Hannes, E.; Corbisier, P.; Wang, J.; Wu, L.; Altieri, I.; Patriarca, M.; Hoffman, A.; Resch-Genger, U.; Ebert, B.; Voigt, J.; Shigeri, Y.; Vonsky, M. S.; Konopelko, L. A.; Gaigalas, A. K.; Bailey, M. J. *A. Clin. Chem. Lab. Med.* 2008, 46, 1033–1045.
- (40) Würth, C.; Grabolle, M.; Pauli, J.; Spieles, M.; Resch-Genger, U. *Anal. Chem.* 2011, 83, 3431–3439.
- (41) Resch-Genger, U.; DeRose, P. C. *Pure Appl. Chem.* 2010, 82, 2315–2335.
- (42) Eaton, D. F. *Pure Appl. Chem.* 1988, 60, 1107–1114.
- (43) Eaton, D. F. *Pure Appl. Chem.* 1990, 62, 1631–1648.
- (44) Hollandt, J.; Taubert, R. D.; Seidel, J.; Resch-Genger, U.; Gugg-Helminger, A.; Pfeifer, D.; Monte, C.; Pilz, W. *J. Fluoresc.* 2005, 15, 301–313.
- (45) Certificate of Analysis, Standard Reference Material 2940, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Orange Emission; National Institute of Standards and Technology: Gaithersburg, MD, 2007; https://www-s.nist.gov/srmors/view_detail.cfm?srn=2940. Accessed Feb. 10, 2012.
- (46) Certificate of Analysis, Standard Reference Material 2941, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Green Emission; National Institute of Standards and Technology: Gaithersburg, MD, 2007; https://www-s.nist.gov/srmors/view_detail.cfm?srn=2941. Accessed Feb. 10, 2012.
- (47) Certificate of Analysis, Standard Reference Material 2942, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Ultraviolet Emission; National Institute of Standards and Technology: Gaithersburg, MD, 2009; https://www-s.nist.gov/srmors/view_detail.cfm?srn=2942. Accessed Feb. 10, 2012.
- (48) Certificate of Analysis, Standard Reference Material 2943, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Blue Emission; National Institute of Standards and Technology: Gaithersburg, MD, 2009; https://www-s.nist.gov/srmors/view_detail.cfm?srn=2943. Accessed Feb. 10, 2012.
- (49) Certificate of Analysis, Standard Reference Material 2944, Relative Intensity Correction Standard for Fluorescence Spectroscopy: Red Emission; National Institute of Standards and Technology: Gaithersburg, MD, 2011; https://www-s.nist.gov/srmors/view_detail.cfm?srn=2944. Accessed Feb. 10, 2012.
- (50) DeRose, P. C.; Smith, M. V.; Mielenz, K. D.; Anderson, J. R.; Kramer, G. W. *J. Luminesc.* 2011, 131, 2509–2514.
- (51) DeRose, P. C.; Smith, M. V.; Mielenz, K. D.; Anderson, J. R.; Kramer, G. W. *J. Luminesc.* 2011, 131, 1294–1299.
- (52) DeRose, P. C.; Smith, M. V.; Mielenz, K. D.; Blackburn, D. H.; Kramer, G. W. *J. Luminesc.* 2009, 129, 349–355.
- (53) DeRose, P. C.; Smith, M. V.; Mielenz, K. D.; Blackburn, D. H.; Kramer, G. W. *J. Luminesc.* 2008, 128, 257–266.
- (54) Pfeifer, D.; Hoffmann, K.; Hoffmann, A.; Monte, C.; Resch-Genger, U. *J. Fluoresc.* 2006, 16, 581–587.

(55) BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005 ready-made from Sigma-Aldrich GmbH (former Fluka GmbH) and certified by BAM in June 2006 are available from BAM or from Sigma-Aldrich. The corresponding product numbers from Sigma-Aldrich are 97003-1KT-F for the Calibration Kit and 72594, 23923, 96158, 74245, and 94053 for BAM-F001, BAM-F002, BAM-F003, BAM-F004, and BAM-F005, respectively. Certificate of Analysis, Certified Reference Materials BAM-F001 to BAM-F005, Calibration Kit Spectral Fluorescence Standards for the determination of the relative spectral responsivity of fluorescence instruments, Federal Institute for Materials Research and Testing (BAM), 2006. All materials were recertified in 2009, and F002 was exchanged for F002a, the emission spectrum of which closely matches that of F002.

(56) The Physikalisch-Technische Bundesanstalt is the National Metrology Institute of Germany. The Federal Institute for Materials Research and Testing is the designated institution for the field of metrology in chemistry (CIPM MRA, 1999).

(57) 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, BAM, PTB, or NRC, Canada, nor does it imply that the materials or equipment identified is necessarily the best available for the purpose.

(58) DeRose P. C.; Early E. A.; G.W., K. *Proceedings of the 5th Oxford Conference on Spectrometry*; Crown: UK, 2006; pp 1–7.

(59) Wuerth, C.; Grabolle, M.; Pauli, J.; Spieles, M.; Resch-Genger, U. *Anal. Chem.* **2011**, *83*, 3431–3439.

(60) *Colorimetry*, 3rd ed.; CIE Publication 15:2004; Central Bureau of the CIE: Vienna, Austria, 2004.

(61) ASTM E-991-98: *Color Measurement of Fluorescent Specimens*, ASTM Standards on Color and Appearance Measurement, 6th ed.; ASTM International: Philadelphia, PA, 1998.

(62) Zwinkels, J. C.; Gauthier, F. *Anal. Chim. Acta* **1999**, *380*, 193–209.

(63) Zwinkels, J. C.; Gignac, D. S.; Nevins, M.; Powell, I.; Bewsher, A. *Appl. Opt.* **1997**, *36*, 892–902.

(64) Zwinkels, J. C.; Gauthier, F. *Proceedings of the 5th Oxford Conference on Spectrometry*; Crown: UK, 2006; pp 21–34.

(65) Zwinkels, J. C.; Gignac, D. S. *Appl. Opt.* **1991**, *30*, 1678–1687.

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

(67) DeRose, P. C.; Resch-Genger, U.; Wang, L.; Gaigalas, A. K.; Kramer, G. W.; Panne, U. In *Standardization and Quality Assurance in Fluorescence Measurements I: Techniques*; Resch-Genger, U., Ed.; Springer-Verlag: Berlin, Heidelberg, 2008; Vol. 5, pp 33–64.

(68) Certificate of Analysis, Standard Reference Material 936, quinine sulfate dihydrate, National Institute of Standards and Technology, Gaithersburg, MD, 1979. A new certificate was issued in 1994: Certificate of Analysis, Standard Reference Material 936a, quinine sulfate dihydrate, National Institute of Standards and Technology, Gaithersburg, MD, 1994.