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Characterization of Standard Reference Material 2942, Ce-ion-doped glass, spectral correction standard for UV fluorescence

Paul C. DeRose *, Melody V. Smith, Klaus D. Mielenz, Jeffrey R. Anderson, Gary W. Kramer

National Institute of Standards and Technology, 100 Bureau Dr., Gaithersburg, MD 20899-8312, United States

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ABSTRACT

Standard Reference Material (SRM) 2942 is a cuvette-shaped, Ce-ion-doped glass, recommended for use for relative spectral correction of emission from 320 to 430 nm and day-to-day performance verification of steady-state fluorescence spectrometers. Properties of this standard that influence its effective use or contribute to the uncertainty in its certified emission spectrum were explored here. These properties include its photostability, absorbance, dissolution rate in water, anisotropy and temperature coefficient of fluorescence intensity. The expanded uncertainties in the certified spectrum are about 9% around the peak maximum at 330 nm, using an excitation wavelength of 310 nm. The SRM also exhibits a strong resistance to photodegradation, with no measurable decrease in fluorescence intensity even after 25 h of irradiation with UV light > 280 nm from a Xe lamp.

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1. Introduction

The increasing use of fluorescence detection in quantitative assays has resulted in a greater need for fluorescence standards [1–4], particularly those suitable for day-to-day instrument performance verification and spectral correction of emission. NIST has certified Standard Reference Materials (SRMs) 2940 [5] and 2941 [6] for relative spectral correction of visible emission and recommended them for use as day-to-day intensity standards for instrument performance verification, due to their resistance to photodegradation. They are also commercially available from NIST, see URLs [5,6]. A high accuracy fluorescence spectrometer [7] was used to certify these standards.

Fluorescence standards are also needed in the UV region, where much of the intrinsic fluorescence in biological systems is found. The spectral shape and relative intensities of UV fluorescence are particularly distorted by most fluorometers due to the decreasing sensitivity of their detection systems and the decreasing intensity of their excitation sources in this region. The detection and quantitative analysis of tryptophan and other naturally fluorescing molecules have also become more common in many areas to determine amounts of these analytes in realtime in the field. Monitoring contaminants in drinking water and pathogens in food are two important examples. Spectral correction in the UV can be crucial to the accurate determination of such analytes.

Many of the fluorescence properties of SRM 2942 have been characterized here to understand better the uncertainties and limitations related to its use as a standard. Similar characterizations of SRMs 2940 and 2941 have been reported previously [8,9]. SRM 2942 is a ready-to-use, cuvette-shaped, Ce-ion-doped, solid glass standard, whose certified values can be used to correct fluorescence emission spectra for distortions in the measured spectral shape, i.e., relative intensity correction, due to the changing responsivity with wavelength of the detection system of a steady-state fluorescence spectrometer. SRM 2942 can be used in combination with SRMs 2940, 2941 and 2943 [10] to calibrate fluorescence instruments through the near UV and visible regions from 320 to 780 nm.

The certified values of SRM 2942 are to be used to obtain correction factors as a function of emission wavelength. The SRM is excited at a fixed wavelength of 310 nm while emission is collected from 320 to 430 nm, preferably using the instrument parameters given in the certificate [5]. The measured spectrum is then normalized to a peak intensity equal to one at 330 nm, i.e., divide all measured intensity values by the corresponding value at 330 nm. Each certified value is then divided by its corresponding normalized, measured value to obtain correction factors. The measured emission spectrum of an unknown sample that falls in the effective emission range of the SRM can then be corrected by multiplying its measured intensities by the correction factors at the corresponding emission wavelengths. Even though the correction factors must be determined using the SRM at a 310 nm

* Corresponding author. Tel.: +1 301 975 4572; fax: +1 301 977 0587.
E-mail address: paul.derose@nist.gov (P.C. DeRose).

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excitation wavelength, they may be applied to the spectral correction of emission independently of the excitation wavelength of a sample.

Desirable characteristics of artifact standards for fluorescence have been specified [11–13,1] and metal-ion-doped glasses have been suggested previously as fluorescence standards [14,11,15]. Many of the radiometric characteristics of glasses can influence their effectiveness as standards, including absorbance, fluorescence anisotropy, temperature dependence of fluorescence intensity, fluorescence lifetime and photostability [9]. All of these characteristics could cause observed fluorescence intensities and spectra to change with time or between instruments, which is not desirable for a standard. These characteristics among others will be explored here for the recently released SRM 2942.

This SRM is expected to be particularly useful to the luminescence community due to its excellent photostability under lamp irradiation at wavelengths longer than 280 nm, and because it covers much of the emission range of intrinsically fluorescent biomolecules.

2. Experimental

A more detailed experimental description of many of these procedures has already been reported [9]. All uncertainties given here are expanded using $k=2$, i.e., 2σ uncertainties, unless specified otherwise.

2.1. Samples

The glass was melted at 1300 °C in a high purity alumina crucible, using a base glass composition with mass fractions of $P_2O_5=71\%$ ($Ca(H_2PO_4)_2H_2O$ and NH_4 H_2PO_4 used), $CaO=26\%$ ($Ca(H_2PO_4)_2H_2O$ used) $Al_2O_3=3.0\%$ (Al_2O_3 used), and a dopant mass fraction of $CeO_2=0.00044\%$. A 95% N_2, 5% H_2 gas mixture was flowed into the crucible during melting. The glass was cut into cuvette-shaped pieces ($12.5\times12.5\times45.0$ mm^3) with three long sides polished and can be used with a 90° transmitting or a front-face detection geometry [16]. The final composition of the glass had mass fractions of $P_2O_5=76\%\pm15\%$, $CaO=18\%\pm4\%$, $Al_2O_3=6\%\pm1\%$ and other trace oxides$=0.3\%\pm0.1\%$, determined using X-ray fluorescence. Note that CeO_2 is at too low a concentration to be detected using this method.

2.2. Fluorescence measurements

All steady-state fluorescence spectra were taken on a SPEX Fluorolog 3 [17] (Jobin Yvon, Edison, NJ) spectrofluorometer using a continuous 450 W Xe lamp excitation source, except where noted. The relative radiometric accuracy as a function of wavelength of the reference (excitation) and signal (emission) detection systems was corrected using a calibrated detector and a calibrated light source, respectively, traceable to the NIST realization of the International System of Units (SI) [18–22]. The intensity of the calibrated light source was too weak below 344 nm to use for determining spectral correction of emission. Therefore, for wavelengths from 320 to 343 nm, the relative responsivity of the detection system was determined using a two-step method. In the first step, a calibrated detector was used to measure the spectral flux of the excitation beam at the sample, then a calibrated diffuse reflector (CR) was used to reflect the excitation beam into the detection system where the signal was measured [7,23]. It is useful to note that the correction factors determined using the two methods agreed within 5% from 340 to 350 nm, when normalized to be equal at 345 nm.

All fluorescence measurements were taken at 25 °C using a 90° transmitting geometry with the excitation beam incident on and normal to one of the polished glass surfaces. The excitation wavelength was 310 nm, which is not the excitation intensity maximum. The typical scanning range for emission spectra was from 320 to 430 nm, using excitation and emission bandwidths of 3 nm. A more detailed description of the qualification of the fluorescence spectrometer, related uncertainties and experimental conditions for certification and the determination of spectral correction factors is given elsewhere [7].

A fluorescence spectrometer with pulsed excitation (Varian Eclipse) was used with 5 and 2.5 nm bandwidths for excitation and emission, respectively, pulse duration$=2$ μs, PMT voltage$=$ 600–800 V, PMT gate$=40$ μs with no delay time between the excitation pulse and the gate. Correction factors for relative spectral correction were determined for this instrument using Federal Institute for Materials Research and Testing—Germany (BAM) certified reference materials (CRMs) [24], so corrected spectra could be compared between instruments using pulsed and continuous excitation. CRMs were used here to save the time needed to set up physical transfer standards, such as a calibrated light source. This emphasizes the ease-of-use of NIST SRMs and other CRMs, which can be measured in the same way as typical samples.

2.3. Polarizers

Glan Thompson polarizers were used just after the excitation monochromator and just before the emission monochromator to measure the fluorescence intensities I_{VV}, I_{VH}, I_{HV} and I_{HH}, which were then used to determine fluorescence anisotropy (r), where the first and second subscripts indicate the polarization setting of the excitation and emission polarizers, respectively, using V to indicate vertical or 0° polarization and H to indicate horizontal or 90° polarization. These measurements were taken at a fixed emission wavelength of 330 nm, corresponding to the peak maximum for SRM 2942. F and G values [25] were determined as described previously [7,9].

2.4. Photostability testing methods

The fluorescence intensity of SRM 2942 was measured periodically after several hours of continuous irradiation. These measurements were taken on the Fluorolog 3, after the sample was removed from the irradiation chamber and its temperature was allowed to equilibrate in the sample compartment of the fluorometer. The irradiation chamber used a 150 W Xe arc lamp with a Hoya U-340 bandpass filter between the lamp and chamber to pass wavelengths between 280 and 380 nm.

A fiber optic with a 400 μm diameter aperture attached to an Ocean Optics S2000 spectrometer with an 8 nm bandwidth was used to measure the irradiance of light incident on the samples as a function of wavelength. The relative spectral responsivity of the spectrometer was calibrated using a calibrated tungsten halogen lamp. The excitation irradiation incident on the samples when they were excited in our fluorometer at an excitation bandwidth and wavelength of 3 and 310 nm, respectively, was measured using both a calibrated Si detector and the fiber optic spectrometer. Comparison of the two measurements was used to calibrate the absolute responsivity of the fiber optic spectrometer.

3. Results and discussion

3.1. Corrected fluorescence spectra and uncertainties

The corrected emission spectrum has a single broad peak with a maximum at 330 nm and a half-width at half the maximum intensity of 31 nm on the red side of the peak. This spectral shape is consistent with those of Ce-ion-doped glasses reported previously, although the positions and broadness of peaks have been found to change with the base glass composition [26,27]. Homogeneity of the glass was measured on a centimeter scale by collecting the spectrum for each SRM in both a normal and a raised (0.5 cm) position and comparing them. Both spectra were found to be statistically identical for all SRMs, implying that they are spatially homogeneous.

The total uncertainty in the relative fluorescence intensity was calculated for each certified intensity value in the fluorescence spectrum by adding in quadrature the 1σ uncertainties due to 1) spatial uncertainty of the excitation beam′s position on the sample (causing secondary inner filter effect uncertainties), 2) variation of F and G polarization ratios between instruments, 3) temperature uncertainty, 4) excitation and emission wavelength and bandwidth uncertainty, 5) uncertainty in the spectral shape correction (due to uncertainty in the radiance and reflectance values of the calibrated light source and reflector) and 6) standard deviation of the certification data. The total 1σ uncertainties were then multiplied by an expansion factor $k=2$ to obtain the total expanded uncertainties (U_{95}). The spectrum of SRM 2942 and the associated uncertainties in the certified values are shown in Fig. 1 and reported in the certificate [28]. A sample of these values is given in Table 1. The values for U_{95} are about 9% near the peak maximum and 8% on the red side of the peak. The side or wing of the peak refers to the regions of the spectrum on either side of the peak maximum where the intensities are 10–20% of the peak maximum.

For this SRM, the blue wing of the spectrum was not measured since fluorescence intensities at emission wavelengths within 10 nm of the excitation wavelength include interference due to scattering, which reaches the detector from the excitation beam. In addition, absorbance of the excitation beam by the glass matrix becomes significant at wavelengths less than 300 nm, causing large inner filter effects. For these reasons, we chose an excitation wavelength of 310 nm and did not report the relative intensities at emission wavelengths less than 320 nm.

The certified values will yield effective spectral correction factors only when the SRM is excited at 310 nm, because the shape of the emission spectrum is excitation wavelength

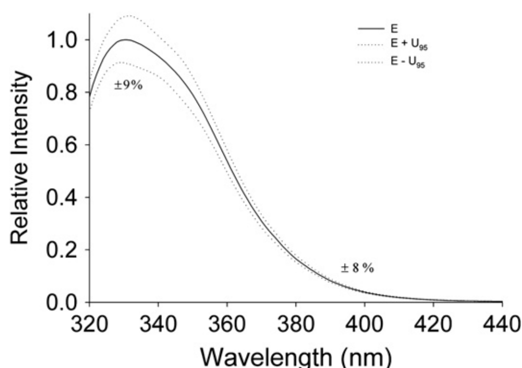

Fig. 1. Certified fluorescence spectrum of SRM 2942 with intensity in relative power units (E) and the corresponding uncertainty envelope obtained by adding and subtracting the total expanded uncertainty (U_{95}) to the certified values. The relative percent uncertainty is labeled in the peak and wing regions of the spectrum. The certified spectrum is normalized to one at the peak maximum at 330 nm.

Table 1
NIST certified relative intensity values (E) and uncertainties (U_{95}) at several emission wavelengths.

λ	E	U_{95}
320	0.783	0.057
330	1.000	0.088
360	0.538	0.043
390	0.083	0.007
430	0.0052	0.0006

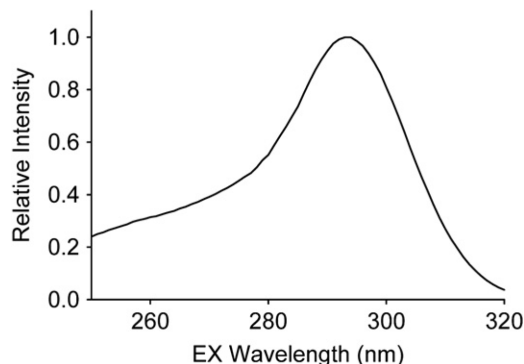

Fig. 2. Fluorescence excitation spectrum of SRM 2942 with emission collected at 330 nm. The spectrum is normalized to one at the peak maximum at 293 nm.

dependent. A 1.0 nm shift of the excitation wavelength in either direction causes the resulting emission spectrum to deviate from the certified values by 5% or less in the peak region (320–360 nm) and by as much as 9% at the wing out to 430 nm. Deviations due to a 1.0 nm change in the excitation bandwidth are much less than this, being 1% or less in the peak region and as much as 2% at the wing. Deviations due to a 1.0 nm change in the emission bandwidth are insignificant, being less than 0.3% across the entire emission spectrum.

The uncertainties near the peak maximum are larger than those at the red wing of the certified spectrum. Typically, the larger fluorescence intensities near a peak cause the uncertainties in this region to be smaller than at the wings. This is not the case here, because the sensitivity of the instrument decreases and the uncertainty in the calibration of the instrument increases as the fluorometer scans to shorter wavelengths in the UV. The decrease in sensitivity is due to a corresponding decrease in the responsivity of the detection system [7]. In addition, an increased calibration uncertainty of as much as 5% is caused by corresponding systematic errors associated with the use of a two-step calibrated detector/calibrated reflector based method for spectral correction [7]. For comparison, the uncertainties in the peak region for similar SRMs that fluoresce in the visible are 3–5%.

The excitation spectrum of SRM 2942, spectrally corrected for excitation intensity, has a peak maximum at 293 nm and a full-width at half the maximum intensity (FWHM) of 27 nm (see Fig. 2).

When excited at wavelengths shorter than 280 nm, the SRM does photodegrade. For instance, we observed a photodegradation rate of 0.9% per hour when excited at 260 nm with an irradiance of 0.5 mW cm^{-2}. Therefore, the SRM should not be exposed to less than 280 nm wavelength light and is not recommended for use as a day-to-day performance standard at these short excitation wavelengths.

3.2. Corrosion study

The weight of a Ce-ion-doped glass immersed in deionized water was measured over a period of 30 days. The rate of

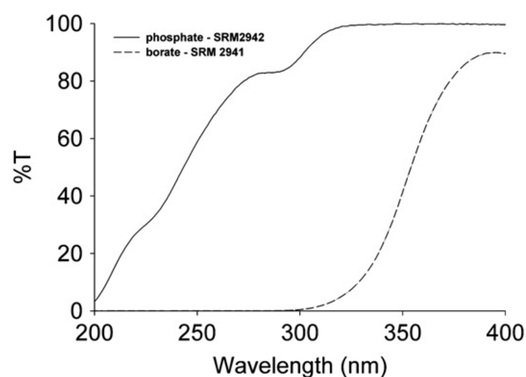

Fig. 3. Comparison of the transmittance spectrum of SRM 2942 with phosphate glass matrix to that of SRM 2941 with borate glass matrix.

dissolution was 0.011 $\mu g\,cm^{-2}\,min^{-1}$, which is equal to a log dissolution rate of $-8.0\,g\,cm^{-2}\,min^{-1}$. SRMs 2940 and 2941 both displayed a log dissolution rate of $-6.6\,g\,cm^{-2}\,min^{-1}$ [8,9]. These two SRMs are composed of a borate glass matrix, in contrast to SRM 2942, which uses a phosphate glass matrix. Our data suggest that the dissolution rate is dependent on the composition of the glass matrix and not on the dopant. Window glass is reported to have a log dissolution rate in the range of -8.0–$-8.5\,g\,cm^{-2}\,min^{-1}$ [29].

3.3. Absorbance and inner filter effects

The borate glass matrices of previous SRMs caused them to strongly absorb wavelengths less than 375 nm, with transmittance (T) dropping to 40% by 350 nm and to 1% or less below 300 nm (see Fig. 3). To make an SRM that would transmit excitation and emission below 350 nm, we needed to use a phosphate matrix for SRM 2942, which has a value of $T=96\%$ at 310 nm (see Fig. 3). The transmittance of the glass continues to decrease with decreasing wavelength, e.g., $T=83\%$ at 280 nm and $T=70\%$ at 260 nm. Absorbance was measured with a Lambda 900 (PerkinElmer) spectrophotometer using a 1 nm increment and a 3 nm bandwidth.

Inner filter effects (IFEs) are due to absorption by the sample of either the excitation beam before it reaches the detection region at the center of the sample, known as the primary IFE, or emission before it leaves the sample, known as the secondary IFE. Both cause the measured fluorescence intensity (F) to decrease, the extent of which can be easily calculated using the measured absorbances $A(\lambda_{EX})$ and $A(\lambda_{EM})$ of the sample at the excitation and emission wavelengths, respectively [30,31]. Samples with $A(\lambda_{EX})$ and $A(\lambda_{EM})$ values less than 0.04 ($T=91\%$), corresponding to intensity changes of less than 5%, are generally considered to be small enough to ignore, as is the case here.

SRM 2942 has a primary IFE at its excitation wavelength of 3%, but all IFEs will be observed with the same magnitude whenever SRMs are measured under the same conditions, so they should not matter when the conditions specified on the SRM certificate are followed. On the other hand, the positions of the excitation beam and detection path on the sample can change over time or between instruments, resulting in a corresponding change in IFE values. Ideally, the detection region should always be at the center of the cuvette, where the excitation beam and emission detection path overlap. In reality, this position can change due to misalignment of the excitation source, optics and sample over time or due to differences in optical alignment between samples. A 1 mm change in the position of the excitation beam or detection path would cause a 0.4% change in the measured fluorescence intensity at the peak maximum. These absolute intensity differences due to IFEs can affect the SRM when being used for day-to-day intensity verification of instrument performance, but the magnitude of this effect is relatively insignificant.

When these SRMs are being used with their certified values for relative spectral correction, only changes in relative intensity versus λ_{EM} are significant. This means that the primary IFE, which is independent of λ_{EM}, will not affect SRM performance. Only changes in the secondary IFE with λ_{EM} can affect the spectral correction when the position of the detection region changes. The percent error in the measured relative emission spectrum due to IFEs was calculated with the same 1 mm change in position. As might be expected, the relative IFE errors are even smaller than the corresponding absolute errors, given above, with those for SRM 2942 being less than 0.1%.

3.4. Photostability

Possibly, the most important characteristic of a solid, robust fluorescent reference material that is meant to be used repetitively in the field is photostability. The SRM was irradiated with UV light (>280 nm) from a 150 W Xe lamp to test the glasses under conditions close to those expected under normal use. Irradiation of SRM 2942 for 25 h showed no changes in spectral shape or fluorescence intensity, within the standard deviation (2σ) of our measurements ($\pm 0.5\%$ for fluorescence intensity at the peak). The irradiance incident on the SRM was measured to be about 1.5 $mW\,cm^{-2}$ at 310 nm, using a spectral bandpass of 8 nm, with a comparable irradiance throughout the region between 280 and 380 nm. The exposure time in the irradiation chamber (25 h) corresponds to about 50 h (2.1 days) of continuous excitation in our fluorometer. This correspondence was calculated by considering the intensity in the irradiation chamber at other excitation wavelengths where sample absorption produces fluorescence. The intensity values of the excitation fluorescence spectrum at each wavelength were used to weight the corresponding excitation intensity values in the irradiation chamber. Ultraviolet (UV) light shorter than 280 nm from the Xe lamp was blocked, using a bandpass filter, to prevent UV solarization of the glass, which is known to change the absorption of metal-ion-containing glasses [32,33]. SRM 2942 should not be employed as a performance verification standard at excitation wavelengths below 280 nm due to this effect.

3.5. Anisotropy and polarization effects

Samples with non-zero values for fluorescence anisotropy (r) will show different fluorescence intensities and spectral shapes on different instruments, since each fluorometer has its own polarization ratios or factors, where $I_{V,EX}/I_{H,EX}$, referred to as the F factor, is the ratio of the vertically to horizontally polarized components of the excitation intensity and R_V/R_H , referred to as the G factor, is the ratio of the responsivities of the detection system to vertically and horizontally polarized light. The values of these polarization factors are dependent on the unique components of individual instruments, such as gratings, other optics, lamps and detectors.

The r value for SRM 2942 was measured to be 0.000 ± 0.002 at its fluorescence peak maximum. The anisotropy of SRM 2942 did not change significantly with emission wavelength (λ_{EM}). Y error bars representing 1σ standard deviations for the average r values and a trendline are also given in Fig. 4. With excitation and emission polarizers in place, the intensity of detected fluorescence from the Ce glass became too weak at emission wavelengths greater than 400 nm to measure r values accurately,

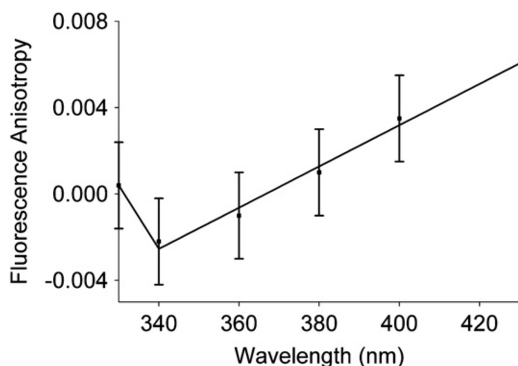

Fig. 4. Dependence of the fluorescence anisotropy (r) of SRM 2942 on emission wavelength The uncertainty in wavelength is smaller than the point size used.

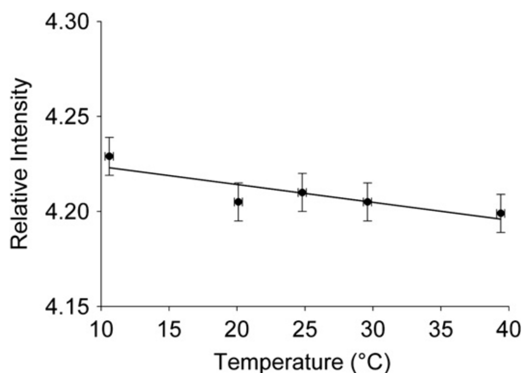

Fig. 5. Temperature dependence of the fluorescence intensity of SRM 2942 at the peak maximum.

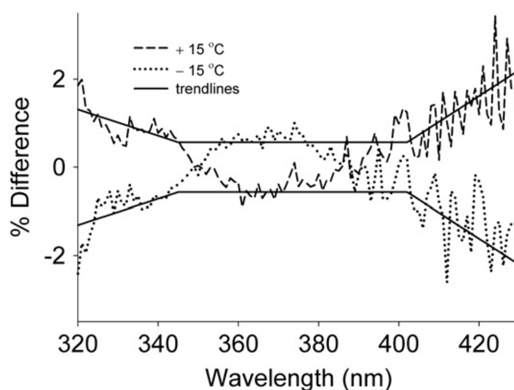

Fig. 6. Percent difference in the fluorescence spectrum of SRM 2942 caused by a ± 15 °C change in temperature from 25 °C.

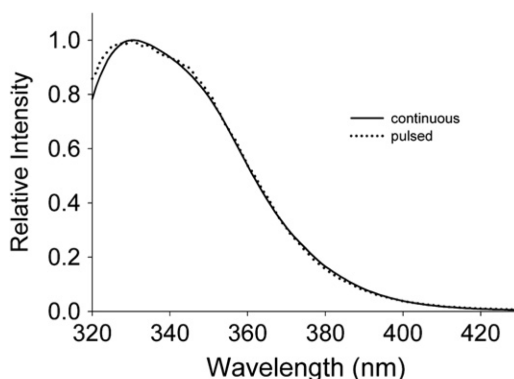

Fig. 7. Spectrally corrected fluorescence spectra of SRM 2942 taken on instruments with pulsed and continuous excitations.

so r was assumed to follow the linear trendline shown in Fig. 4 from 400 to 430 nm.

The F factor at 310 nm is 1.2. The range of G factors for our instrument is from 3.5 to 1.3 over the emission wavelength range of the SRM. These F and G values are typical for monochromator-based systems [34]. We estimated $\pm 25\%$ to be a typically expected instrument-to-instrument difference between the F and G values of our instrument and those of other users for conventional fluorometers designed to cover the emission region from about 350 to 750 nm with greatest sensitivity. With this assumption, differences in the absolute intensity at the peak maximum and in the relative intensities across the emission spectrum that can be expected due to variations in F and G values between instruments were calculated [35]. The absolute intensity difference at the peak maximum was calculated to be less than 0.01% for SRM 2942. The differences in the relative intensity across the emission spectrum normalized to one at the peak maximum were calculated to be 0.12% or less. These instrument-to-instrument-polarization related uncertainties are insignificant compared to the total uncertainties related with fluorescence intensity measurements.

3.6. Temperature dependence

The fluorescence peak intensity as a function of temperature was measured between 10 and 40 °C (see Fig. 5). The slope of the linear least-square fitted straight line to the plotted points was taken to be the temperature coefficient. This value corresponds to $-0.02\%/°C \pm 0.02\%/°C$ for SRM 2942 at 25 °C.

By graphing the percent difference between the spectra, normalized to one at the peak maximum, at other temperatures with that at 25 °C, the temperature dependence of the spectral shape as a function of emission wavelength was determined. To average out noise, the percent difference plots at 40 and 10 °C were both fitted to polynomials (a set of three trendlines) using least-square fits, see Fig. 6. SRM 2942 was certified at 25 °C with an uncertainty of ± 0.5 °C. The temperature dependence of the percent difference was found to be linear with changes in temperature. In addition, the fit at 40 °C shows a slightly larger percent difference than that at 15 °C, with both temperatures deviating from the certification temperature by 15 °C. Therefore, the percent difference fit at 40 °C, as the larger of the two, was used to calculate the uncertainty in the certified values corresponding to the uncertainty in temperature, by taking the percent difference values as a function of wavelength and dividing each by 30 (15/0.5 °C=30). Spectral differences due to a ± 0.5 °C change in temperature were found to be insignificant, less than 0.1%, for SRM 2942 across its emission wavelength range.

3.7. Fluorescence lifetimes and pulsed excitation

The corrected emission spectrum for SRM 2942 was also determined on an instrument with pulsed excitation and compared to the certified spectrum taken on the Fluorolog 3. The fluorescence spectra look very similar using either pulsed or continuous excitation (see Fig. 7) with the relative intensity values from the pulsed instrument differing by less than 10% throughout the spectrum from the certified values. They differ by 2% or less in the peak region except for the blue edge of the spectrum from 325 to 320 nm, where the difference is as much as 9%. This is due to a combination of increased uncertainties in this wavelength region, as discussed in an earlier section, and

scattered light from the excitation beam reaching the detector of the pulsed instrument, causing the measured intensity to be greater than the true intensity as the emission wavelength nears the excitation wavelength. These differences are within the combined uncertainties of the certified values and the uncertainties related with the pulsed instrument measurements. These results imply that the fluorescence emitted within 40 μs of the excitation pulse has the same spectral profile as that of the longer, time-averaged fluorescence. The 40 μs PMT gate duration was chosen as a typical value for conventional pulsed fluorometers, suggesting that SRM 2942 can also be used as a spectral correction standard for instruments with pulsed excitation. This conclusion is consistent with the fluorescence lifetimes of Ce-doped glasses reported in the literature with values from 20 to 50 ns [26,36]. Since these fluorescence decay times are much shorter than that of the PMT gate, no difference between microsecond pulsed and continuous excitation instruments is expected.

4. Conclusion

SRM 2942, a Ce-ion-doped glass in the shape of a standard cuvette, has been certified as a relative spectral correction standard for fluorescence emission from 320 to 430 nm. The expanded uncertainties in the certified values are about 9% near the peak maximum at 330 nm. Errors in the measured emission spectrum due to changing inner filter effects and polarization ratios (*F* and *G* factors), experienced over time or between instruments, were found to be insignificant. This assumes a 1 mm displacement of the detection region from the center of the cuvette for inner filter effects and a 25% difference between the *F* and *G* values of our instrument and those of other conventional fluorometers. The fluorescence anisotropy and temperature coefficient of fluorescence intensity for the SRM were measured to be $0.000\% \pm 0.002\%$ and $-0.02\%/^{\circ}C \pm 0.02\%/^{\circ}C$, respectively, at the peak maximum at 25 °C. SRM 2942 possesses good photostability with no photodegradation observed under common lamp-based excitation conditions at wavelengths greater than 280 nm.

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