State-of-the Art Comparability of Corrected Emission Spectra – Part II: Field Laboratory Assessment of Calibration Performance Using Spectral Fluorescence Standards

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Abstract

In the second part of this two part series on the state-of-the-art comparability of corrected emission spectra, we have extended this assessment to the broader community of fluorescence spectroscopists, by involving twelve field laboratories that were randomly selected based upon their fluorescence measuring equipment. These laboratories performed a reference material (RM)-based fluorometer calibration with commercially available spectral fluorescence standards following a standard operating procedure that involved routine measurement conditions, and the data evaluation software *LINKCORR* developed and provided by the Federal Institute for Materials Research and Testing (BAM). This instrument-specific emission correction curve was subsequently used for the determination of the corrected emission spectra of three test dyes X, QS, and Y, revealing an average accuracy of 6.8% for the corrected emission spectra. This compares well with the relative standard uncertainties of 4.2 % for physical standard (PTS)-based spectral corrections demonstrated in the first part of this study involving an international group of four expert laboratories. The excellent comparability of the measurements of the field laboratories also demonstrates the effectiveness of RM-based correction procedures.

Keywords

Fluorometry, emission spectra, measurement capability, laboratory intercomparison, reference materials, emission standards, spectral correction, uncertainty.

Introduction

Fluorescence techniques have transformed over the last 20 years from mainly qualitative, research tools with limited use, to widely-employed, qualitative and quantitative analytical tools. This change has been particularly evident in the life and material sciences. [1-4] These techniques are only minimally invasive, comparatively inexpensive, and easy to use with high sensitivity. They also have the ability to provide spectral, temporal and spatial information, suitable for multiplexing and remote sensing. This has led to a substantial increase in fluorescence applications with the interdisciplinary expansion in the use of fluorescence techniques thereby making standardization increasingly important. [3, 4]

For each fluorescence technique, reproducible, accurate results require compensation and control of instrument-specific and fluorophore-specific contributions to the analyte signals. [5, 6 7, 830] At the same time, general difficulties exist in measuring absolute fluorescence intensities accurately. [9] This limits the comparability of fluorescence data across instruments and for the same instrument over time. As a result, quantification from measurements of fluorescence must be performed using corrected relative intensities, requiring calibration standards.

In response to this need, there has been a renewed interest in research activities dedicated to the development of fluorescence standards and quality practices, [10-24] leading toward accreditation and traceability. [25, 26] This is evidenced by the increasing number of publications and workshops dedicated to these topics. Recent activities include the development of technical notes by the International Union of Pure and Applied Chemistry (IUPAC; e.g. project #2004-021-1-300) on a broad variety of fluorescence topics including quality criteria for fluorescence standards, [26, 27] a standard guide to fluorescence (E2719) from ASTM International for

instrument qualification, [28] and the evaluation of optical methods and equipment for the determination of the fluorescence quantum yield. [15, 16, 29]

At the same time, there is a growing need for suitable and simple instrument calibration and performance verification procedures (preferably with low and readily accomplishable uncertainties) for different types of instruments. [30] Also, parameters and instrument quantities that can affect the intensity, spectral shape, and position of measured fluorescence signals must be better understood by users. [6, 26, 31, 32] This includes the (relative) spectral responsivity of the emission channel, the (relative) spectral radiant power of the excitation beam reaching the sample, the accuracy of the excitation and emission wavelength scales and spectral bandwidths, and the linearity of the detection system. [1, 2, 6, 25, 28] These needs triggered the recent development of new spectral fluorescence standards by both the National Institute of Standards and Technology (NIST; glass-based reference materials (RM)) [19-21] and the Federal Institute for Materials Research and Testing (BAM-Germany; liquid RM). [6, 18, 25, 26] These materials are chromophore-based spectral radiance transfer standards, which enable the straightforward determination of the spectral responsivity of the detection channel of fluorescence instruments at routinely used instrument settings in the wavelength region from 300 nm to 800 nm.

The reliable determination of spectral responsivity provides the basis for the comparability of emission spectra and the determination of accurate fluorescence quantum yields, [11, 30] In part 1 of this series, the importance of linking fluorescence measurements to physical scales encouraged four National Metrology Institutes (NMIs) to evaluate the state-of-the-art comparability of corrected fluorescence emission spectra among expert laboratories. They used physical transfer standards (PTS), such as standard lamps, and chromophore-based spectral radiance transfer standards (chemical transfer standards referred to as RMs). The results of Part I

provided fluorescence users with representative data demonstrating readily achievable uncertainties and simple, validated methods and tools to improve the overall comparability of fluorescence measurements in the spectral region of *ca*. 300 nm to 720 nm. In part II of this study, we evaluate the corrected emission spectra of our three test dyes X, QS, and Y among field laboratories using an RM-based calibration only. To render these data especially valuable for the broad fluorescence community, only common commercial spectrofluorometers, routine measurement conditions as well as commercially available RMs [33] and data evaluation software were employed.

Instrumentation and Materials

Expert laboratories. The instrumentation and calibration methods including physical transfer standards used by the NMIs: NIST, NRC, PTB, and BAM, as well as the measurement protocols for the determination of the corrected emission spectra of BAM RMs: F001 to F005,and test dyes X, QS, and Y, are described in detail in part I of this series

Field laboratories. The field laboratories in this study were randomly selected by BAM from academia and industry, based upon their fluorescence measuring equipment, in order to include a diversity of common commercial spectrofluorometers, and are not identified in this report. The following instruments were used: spectrofluorometers CARY Eclipse (Varian Inc.),[30] LS50B (Perkin Elmer Ltd.), AB2 (SLM Aminco Inc.), all equipped with a pulsed excitation light source, single monochromators in the excitation and emission channel, a red sensitive PMT as detector, and a reference channel to account for fluctuations of the excitation light source; a single photon counting (PC) spectrometer CD900 (Edinburgh Instruments Ltd); a Fluorolog 2 spectrometer

(Horiba Jobin Yvon; continuous excitation light source, double monochromators in the excitation and emission channel, red sensitive PMT as detector, reference channel, PC mode); and a Fluoromax 3 spectrometer (Horiba Jobin Yvon; pulsed excitation light source, single monochromators in the excitation and emission channel, red sensitive PMT as detector, reference channel, PC mode). One field laboratory that had a new spectrofluorometer with the instrument manufacturer's internal emission correction function included as a spectral correction option in the software , was also asked to measure the emission spectra of the three test dyes using two different modes of instrument correction. The first mode implemented the same RM-based correction procedure used by the other participants, and the second mode applied the manufacturer's internal emission correction function to the data. This internal correction function had been obtained by the manufacturer with a calibrated light source and a calibrated white standard for randomly chosen instrument settings. These additional measurements provided a preliminary estimate of the quality of such internal emission correction curves.

Materials. For the RM-based emission correction, F001 to F005 (solvent ethanol), covering the wavelength region between 300 nm and 770 nm, [18] were provided by BAM as solutions. 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), [34] and BAM dye Y (solvent acetonitrile), which were chosen to cover the spectral region used for calibration. These dyes were also provided as solutions. For the interlaboratory comparison of different field spectrofluorometers, dye solutions with an absorbance of 0.04 at the longest wavelength absorption maximum were employed.

In the interlaboratory comparison of the NMIs, these eight dyes were measured and the resulting data were pre-processed by each NMI according to detailed standard operating

procedures (SOPs) evaluated and provided by BAM and NIST. For this initial study, RMs and test dyes with an absorbance of 0.04 and 0.08 at the longest wavelength absorption maximum were used, with the former measured on research-grade commercial spectrofluorometers for analytical applications (NIST, PTB, BAM) and with the latter measured on the less sensitive custom-built NRC reference spectrofluorometer for colorimetric applications using a 45°/0° measurement geometry, as described in part I of this series. [35, 36]

For the interlaboratory comparison of the field laboratories detailed here, the participants obtained solutions of F001 to F005 [18] commercially available from BAM and Sigma-Aldrich (i.e., Calibration Kit Spectral Fluorescence Standards, product number 97003-1KT-F) and the standard operating procedure (SOP) supplied with these RMs as well as solutions of the three test dyes X, QS, and Y and a SOP for their use. Each laboratory was instructed to perform fluorescence measurements of F001 to F005 and of the test dyes at identical, yet routinely used instrument settings that were not further specified, see also Supplementary Information (SI). Each participant was asked to verify the accuracy of the fluorometer´s emission wavelength scale prior to instrument calibration and to ensure that the emission measurements were performed within the linear range of the fluorometer´s emission detection system.

Data Analysis

Principles of data assessment. The field laboratories provided the following data i.) measured uncorrected emission spectra of F001 to F005, ii.) measured uncorrected emission spectra of dyes X, QS, and Y, and iii.) the corresponding solvent or blank spectra (see also part I). Based upon the data sets i.) and iii.), BAM determined the relative spectral responsivity of each

laboratory's fluorometer by employing the BAM software *LINKCORR* distributed with the BAM RMs and used this emission correction curve for the subsequent determination of the solvent and spectrally corrected emission spectra of dyes X, QS, and Y. [6, 18] In addition, selected participants were asked to independently calculate the corrected emission spectra of the test dyes and these results were compared with the BAM corrected data. This comparison did not yield any differences, thereby demonstrating the robustness of the *LINKCORR*-based data evaluation procedure. The BAM-corrected emission spectra of the dyes X, QS, and Y for each participant were then assessed against the corresponding intercomparison reference functions from the NMI study (ICRF-NMI), see part I. From this comparison, an average systematic deviation was calculated for each participant.

Data pre-treatment. The blank and spectrally corrected, non-normalized emission spectra of the study participants [6] varied in format due to different start and end points and different spacing/step widths. To realize common start and end points, the emission spectra were truncated by simply cutting possible edges/tails below a 5% relative signal level or at the smallest and largest wavelength position at which data points were available for all participants. Subsequently, the truncated emission data were projected onto a common sampling grid with a spacing corresponding to the data available for the ICRF-NMI (1 nm for dye X, 2 nm for the other dyes). For the preliminary analysis, the data were normalized by dividing by the maximum value observed in the measured spectra.

Determination of the intercomparison reference functions (ICRF). The ICRF represents the best estimate of the true value for each fluorophore based upon the available comparison data. This was determined by considering all the spectral data measured by the participants regardless of their position in the 2D space spanned by the functional relationship. The determination of the

ICRF for the intercomparison of the NMIs (ICRF-NMI) is described in part I of this series and the ICRF of this field laboratory intercomparison (ICRF-FL) was calculated following a similar procedure. Let y_{jk} be the values measured by lab k at setting j of the independent variable x (here, the nominal wavelength setting λ_{jk}). Both the measurements of the y_{jk} and the nominal settings of the x_{jk} are somehow distorted from their "true" values because of the uncertainty of measurement, along with the random scatter, Here, for each fluorometer used, different correction factors f_k were assumed that reflect their different spectral responsivities and a different correction function $\varphi(x_k)$ to take account of the uncertainties in their wavelength scale. The $\varphi(x_k)$ can be any reasonable transformation of the independent variable, i.e., additive, multiplicative, combined, or even non-linear. Both the f_k and $\varphi(x_k)$ are characteristic for each participant. For each laboratory, f_k and $\varphi(x_k)$ and for all laboratories, an ICRF was determined such that the sum of the squared deviations (SSD) was minimized, see equation 1.

$$SSD = \sum_{j,k}^{J,K} [\, f_k \cdot y_{jk}(\varphi(x_{jk})) - ICRF(\varphi(x_{jk}))]^2 = \min \qquad \text{(eq. 1)}$$

In this minimization process, the same restrictions apply as discussed in part I of this series.

Models and adjustments. Finding appropriate f_k values is essential in the process of defining the ICRF. If one allows for adjustments in the independent variable, ambiguity arises. A unique solution can only be obtained when additional constraints are used, e.g. by allocating the minimum-variance solution at a certain point on the x scale. This corresponds to the mean value of the original maximum positions of the spectra. Besides a possible shift of the spectra, one might also assume a distortion of the individual wavelength scale rendering the minimization problem even more ambiguous. Therefore, adjustments were limited to i) a scaling factor in $y(f_k)$, and ii) an additive shift in $x(\delta_k)$. The ICRF-NMI was then fitted against adjusted data of the form

$$\varphi(x_{jk}) = x_{jk} + \delta_k \quad : \quad x_{jk} \rightarrow x_{jk} + \delta_k$$
$$y_{jk} \rightarrow f_k \cdot y_{jk} \qquad \text{(eq. 2)}$$

The adjustment vector thus consisted of n scaling factors f_k and n individual shift values δ_k with n being the number of participating laboratories.

2D averaging: A 2D averaging procedure was used to determine the ICRF and the values of f_k and δ_k. This 2D procedure consisted of the following steps: i.) Each spectrum included in the optimization procedure was made "continuous" by a straight-line interpolation between the experimentally determined data points.. ii.) The emission spectra were subjected to scaling (y-axis) and shifting (x-axis). iii.) At any of the iteration steps, each intensity value of the measured emission spectra was attributed to the closest actual sampling position (normally the sampling points of the original, non-shifted grid).. The wavelength position and value of the ICRFs were calculated as the corresponding mean in both the x and the y directions according to equation 3.

$$\overline{f_k \cdot y_{jk}} = ICRF_j(\overline{x_{jk} + \delta_k}) \qquad \text{(eq. 3)}$$

Here, the average was taken over k, i.e., the data points in the close vicinity of the actual sampling point of all the measured spectra. Note, that the average of the shifted positions is normally different from the sampling points on the original grid. According to equation 3, the ICRF is then represented by a frequency polygon. iv.) Individual deviations of each laboratory from the ICRFs and the total SSDs were estimated. v.) The total SSDs were minimized by adjusting the parameters f_k and δ_k in an iterative procedure. vi.) After reaching convergence, i.e., finding best-fit estimates for f_k and δ_k, the joint confidence region (JCR) for each of the points, which make up the ICRF, was determined. Upper and lower confidence intervals of the point on the ICRF were then estimated as the points where the bisecting line of the ICRF frequency polygon passed through the JCR.

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.

Results and Discussion

The blank-corrected, [6] yet spectrally uncorrected emission spectra of the three test dyes X, QS, and Y and the corrected emission measured by the twelve field laboratories are highlighted in Figures 1 and 2 together with the ICRFs-NMI (lines) and the corresponding expanded (k = 2, confidence interval of 95%) uncertainties or confidence intervals (CIs; dashed lines, see Figure 2). The test dyes were chosen according to the following criteria: i.) coverage of the spectral region of the set of BAM RMs, i.e., *ca*. 300 nm to 700 nm, ii.) large Stokes shift (dyes QS and Y) to minimize reabsorption of emitted light (inner filter effects), and iii.) common use: QS was the only certified emission standard until 2006 [23, 34] and is one of the best studied fluorophores and dye Y is a common laser dye. In addition, dye X was selected for its slightly structured emission spectrum, thereby, enabling a more stringent check of wavelength accuracy and the influence of spectral resolution on the reliability of the spectral emission correction..

< Insert Figure 1 >

As highlighted in Figure 1 for one representative laboratory, all uncorrected emission spectra of the field laboratories deviate from the ICRFs of the corrected emission spectra determined by the NMIs. The spectral deviations are most pronounced in the ultraviolet at wavelengths < 400 nm and at wavelengths > *ca*. 500 nm. Here, many detectors and other optical components of spectrofluorometers display strong wavelength dependences of their spectral responsivity or transmission profiles, whereas, between *ca*. 400 nm and 500 nm, these profiles are relatively flat.

Figure 2 summarizes the RM-based normalized corrected emission spectra of the three test dyes X, QS, and Y of selected participating laboratories (symbols) together with the ICRF-NMI (lines) and the corresponding expanded (k = 2, confidence interval of 95%) uncertainties or confidence intervals (CIs; dashed lines). The seven laboratories whose data are shown were selected as a representative sampling of the data from all twelve field labs. As seen in Figure 2, the comparability of the corrected emission spectra is excellent, especially in the case of dyes QS and Y. The most pronounced deviations, on the order of 10 % (e.g. laboratory 5), are observed for dye X, which displays a structured emission spectrum in the wavelength region of *ca*. 310 nm to 410 nm. All RM-based corrected emission spectra clearly resolved the vibrational fine structure. In the case of dye QS, the corrected emission spectra of all field laboratories appears to be blue-shifted with respect to the ICRF-NMI, touching the left wings of the CI; this deviation was also observed in the NMI intercomparison (part I), for this dye. [34]

< Insert Figure 2 >

The accuracy of the RM-based spectral correction and resulting emission spectra of the field laboratories for dyes X, QS, and Y, is determined, too a large extent, by the accuracy of the BAM-corrected emission spectra of F001 to F005. Figure 3 displays the relative spectral deviations of the PTS-based corrected emission spectra of the NMIs from the BAM-certified corrected emission spectra of these dyes (top panel), and the relative spectral deviations of the BAM-corrected emission spectra of F001 to F005 from the ICRF-NMI for these dyes (PTS-based correction; center panel). The overall excellent comparability of these data underlines the reliability of the BAM-certified values. The agreement of these data can be further improved by introducing a wavelength shift of 0.2 nm to 0.8 nm with increasing wavelength of the BAM-certified emission spectra (Figure 3, bottom;). This is related to the fact that the BAM data have not been corrected for the wavelength calibration of the BAM spectrofluorometer. The inaccuracy of the wavelength scale of this instrument was as much as 0.4 nm when determined with a low pressure atomic discharge Hg-Ar lamp at the sample position (see Table S2, Supplementary Information of part I of this series).

< Insert Figure 3 >

The deviations of the corrected emission spectra of the selected field laboratories from the internal ICRFs (ICRF-FL), i.e., the reference functions calculated on the basis of the data from all twelve field laboratories for each test dye, are highlighted in Figure 4. In principle, these deviations should be symmetrical since the reference is an average of the individual data.

< Insert Figure 4 >

Figure 4 clearly demonstrates that the field laboratories are able to achieve a reproducibility almost comparable to those of the NMIs in part I of this study. Interestingly, the quality of the performance of certain field laboratories reveals a slight wavelength dependence. For instance, laboratories 4, 5, and 6 display an excellent relative deviation in the spectral region from 310 nm to 410 nm for the most challenging test dye X with its structured emission spectrum. In this region, however, there are also exceptions such as laboratory 1 performing worse than average. Nevertheless, for the spectral region from *ca*. 400 nm to 710 nm, laboratory 1 displays an excellent agreement with the ICRF-NMI, similar to that achieved by the NMIs, whereas, the performance of laboratory 5 is below average for the emission regions corresponding to the wings of the spectra of dyes QS and X.

<Insert Table 1>

In Table 1, for each test dye, the comparability (or between-laboratory reproducibility) of the corrected emission spectra attained in the initial NMI study (Table 1, "NMI comparability", average over the four laboratories) is compared with that demonstrated here by the field laboratories in this peer-to-peer evaluation (Table 1, "ILC comparability", by laboratory and averaged over all participants). The average bias with respect to the ICRF-NMI is also given in Table 1. Both averaged parameters (reproducibility and bias, i.e. precision and trueness) were then combined into an average accuracy for all participating field laboratories and for the spectral region governed by each dye´s emission.

The performance characteristics of the field laboratories and NMIs, summarized in Table 1, are specified in accordance with the GUM (ISO Guide 98-3:2008), but consider the specificity of spectral responses, namely to be multi-variate and two-dimensional, They are calculated as follows:

Performance characteristic	**per lab**	**total**
NMI comparability	-	$\sqrt{\frac{\sum_{m}(\vec{x}_k(\lambda_i)-\vec{R}(\lambda_i))^T\cdot(\vec{x}_k(\lambda_i)-\vec{R}(\lambda_i))}{M\cdot I}}$
ILC comparability s_R	$\sqrt{\frac{(\vec{y}_k(\lambda_i)-\vec{I}(\lambda_i))^T\cdot(\vec{y}_k(\lambda_i)-\vec{I}(\lambda_i))}{I}}$	$\sqrt{\frac{\sum_{k}(\vec{y}_k(\lambda_i)-\vec{I}(\lambda_i))^T\cdot(\vec{y}_k(\lambda_i)-\vec{I}(\lambda_i))}{K\cdot I}}$
average bias (assessed against the ICRF of the pilot study) s_b	$\sqrt{\frac{(\vec{y}_k(\lambda_i)-\vec{R}(\lambda_i))^T\cdot(\vec{y}_k(\lambda_i)-\vec{R}(\lambda_i))}{I}}$	$\sqrt{\frac{\sum_{k}(\vec{y}_k(\lambda_i)-\vec{R}(\lambda_i))^T\cdot(\vec{y}_k(\lambda_i)-\vec{R}(\lambda_i))}{K\cdot I}}$
average accuracy s_A	-	$\sqrt{s_b^2+s_R^2}$

where $\vec{x}_k(\lambda_i)$ is the vector of the values of the blank-corrected and normalized spectral emission at wavelengths λ_i determined by laboratory *m* in the NMI study, $\vec{R}(\lambda_i)$ the vector of values of the ICRF at wavelengths λ_i as determined in the NMI study, $\vec{y}_k(\lambda_i)$ the vector of the values of the blank-corrected and normalised spectral emission at wavelengths λ_i determined by laboratory *k* in the ILC, and $\vec{I}(\lambda_i)$ the vector of values of the ICRF-FL at wavelengths λ_i.

These performance characteristics provide a measure of i) the average absolute bias of the field laboratories relative to the ICRF-NMI, and ii) the average between-laboratory reproducibility with respect to the ICRF-FL. The average accuracies of 6.8 %, 6.2 %, and 5.2% for dyes QS, X, and Y, respectively, given in Table 1 underline the trends highlighted in Figures 2 and 4. The observed deviations from the NIST-certified corrected emission spectrum of QS exceed the average accuracies

and were similar to those found in the NMI intercomparison. This is ascribed to the use of the BAM-certified corrected emission spectra of F001 to F005 as the PTS-based corrected emission spectra of QS measured by BAM also displayed this trend (see e.g. part I, Figure 5).

The spectral emission data of the field laboratories display less overall accuracy than that of the NMIs, but the differences are surprisingly small. The RM- and PTS-based spectrally corrected emission spectra of dyes X, QS, and Y measured by the four NMIs show relative standard uncertainties of 4.2 % and 2.4 %, respectively. By comparison, an average accuracy ranging from 5.2 % to 6.8 % is achieved by the field laboratories for the three test dyes. This is an impressive result for certified reference materials (CRMs) which are easier to use than physical transfer standards and do not require specialized expertise. Moreover, it has to be kept in mind that the NMIs used instruments for the RM-based calibration that were previously calibrated with PTS (see part I) and dedicated much more time to instrument characterization, The field laboratories, on the other hand, performed the RM-based instrument calibration and the measurements of the test dyes within one or two days. In conclusion, the performance of the field laboratories is fully satisfactory for most if not all intended fluorescence applications.

< Insert Figure 5 >

To gain some insight into the reliability of internal emission correction curves that have been implemented into many different spectrofluorometers, one field laboratory equipped with a relatively new spectrofluorometer was asked to use the manufacturer´s emission correction curve to determine the corrected emission spectra of the three test dyes. Such emission correction curves are typically obtained by averaging the PTS-based correction curves for several instruments at a fixed set of instrument settings. Information on the calibration standard(s), measurement conditions, and reference quantities that were used are not typically provided. The results obtained using the manufacturer-supplied correction were subsequently compared with the corresponding RM-based corrected emission spectra of

dyes X, QS, and Y, see Figure 5. While the RM-corrected emission spectra display relative spectral deviations that are random and most likely due to noise, whereas the corrected emission spectra obtained with the implemented emission correction curve display more pronounced systematic deviations from the ICRF-NMI.. These data, however, are only an example and are not intended to be representative of the general quality of internal emission correction curves provided by instrument manufacturers.

Conclusions and Outlook

A first interlaboratory comparison of fluorescence measurement capabilities of twelve field laboratories randomly selected from academia and industry revealed an average accuracy of the corrected emission spectra of 6.8 % or better for three test dyes in the wavelength region from 310 nm to 720 nm. Common commercial spectrofluorometers equipped with single and double monochromators, different detectors (different types of PMTs) and with and without polarizers were used under routine measurement conditions. Emission correction curves were determined with certified reference materials (CRMs) for spectral correction of emission (BAM F001 to F005) and with a commercial data analysis software (*LINKCORR* from BAM). These results only slightly exceed the relative standard uncertainties of 4.2 % for physical transfer standard (PTS)-based spectral corrections and 2.4 % for standardized RM-based spectral corrections demonstrated in part I of this study by an international interlaboratory comparison of four expert laboratories. Even though an improved comparability is expected when using the same standards and procedures, resulting in smaller interlaboratory bias, the degree of comparability achieved in this field laboratory assessment is noteworthy. In addition, the trueness (accuracy) of measurement results was increased significantly by using CRMs fully traceable to the international system of units, here to the radiometric scale of the spectral radiance.

Prerequisites for this excellent comparability of emission data over a wide wavelength range in the UV and visible regions are 1) well characterized CRMs meeting the criteria of spectral fluorescence standards; [26, 27, 37] 2) suitable data analysis software; 3) the use of similar instrument conditions for the measurement of the CRMs and the samples (these include optical components, settings and measurement geometries); and 4) the operation of the spectrofluorometers within the linear range of their detection systems. These findings are in agreement with part I of this series on the comparability of spectral correction. Here, an accurate wavelength scale, instrument operation within the linear range of detection, and consistency (invariance) of these parameters during system calibration and measurement collection, as well as the use of identical instrument settings for instrument calibration and measurement of samples, were identified as the critical instrument operating and design parameters for a reliable determination of the spectral responsivity of fluorescence measurement systems.

When the wavelength accuracy was carefully controlled and the detection system was operated in its linear range, the data from the field labs did not display larger uncertainties related with the instrument design by any laboratory. Such correlations were also beyond the scope of this study. In principle, similar relative standard uncertainties may be achieved with an instrument equipped with a charge coupled device (CCD) as long as all measurements are performed using the same wavelength grid / spectral window of the CCD. However, it needs to be considered that CCDs operate over a relatively narrow dynamic range compared with PMTs and are more susceptible to stray light errors compared with spectrofluorometers equipped with monochromators, such as those used in both Parts I and II of this study.

This study provides the broad community of fluorescence users with simple and validated methods and tools to improve the comparability of fluorescence measurements with commercial RMs. For the longer term goal of standardizing fluorescence measurements, future steps may include a similar study of the comparability and accuracy of the determination of corrected emission spectra in the near infrared spectral region of *ca*. 650 nm to 900 nm, which is becoming increasingly important, and the

determination of fluorescence quantum yields of transparent dye solutions of common fluorophores. Moreover, our preliminary results on the quality of internal emission correction curves, indicate that it would be worthwhile to further investigate the accuracy of these correction curves supplied by manufacturers for different instruments.[38]

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(38) 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 are necessarily the best available for the purpose.

Table 1. Performance characteristics of the inter-laboratory comparisons of corrected emission spectra between NMIs and between field laboratories (ILC)

dye X								
	lab 1	lab 4	lab 5	lab 6	lab 7	lab 10		**total**
NMI comparability	-	-	-	-	-	-		**1.94%**
ILC comparability	6.62%	2.21%	1.42%	2.24%	3.94%	2.42%		**3.59%**
average bias (ILC vs. NMI)	7.25%	4.57%	4.49%	2.49%	5.41%	4.55%		**4.99%**
average accuracy								**6.15%**
dye QS								
	lab 1	lab 4	lab 5	lab 6	lab 7	lab 10	lab 12	**total**
NMI comparability	-	-	-	-	-	-	-	**2.41%**
ILC comparability	1.42%	2.61%	4.11%	2.60%	3.20%	3.56%	1.59%	**2.88%**
average bias (ILC vs. NMI)	3.13%	4.90%	5.50%	7.71%	7.51%	6.73%	6.68%	**6.21%**
average accuracy								**6.84%**
dye Y								
	lab 1	lab 4	lab 5	lab 6	lab 7	lab 10	lab 12	**total**
NMI comparability	-	-	-	-	-	-	-	**1.46%**
ILC comparability	1.15%	2.12%	4.73%	3.54%	3.20%	2.48%	1.19%	**2.89%**
average bias (ILC vs. NMI)	4.29%	3.68%	7.00%	4.70%	2.22%	3.57%	3.17%	**4.32%**
average accuracy								**5.20%**

Figure Captions

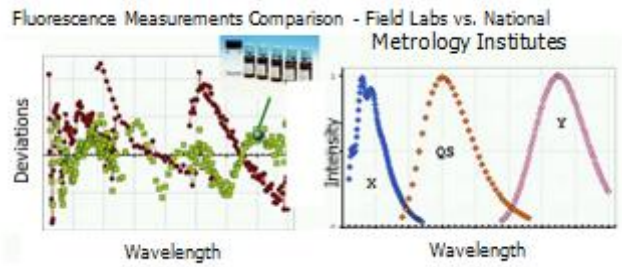
Figure 1. Normalized, blank corrected, yet spectrally uncorrected emission spectra of dyes X, QS, and Y obtained by one of the field laboratories (hollow squares) and the intercomparison reference function of the NMI intercomparison (grey diamonds).

Figure 2. Normalized and corrected emission spectra of dyes X, QS, and Y obtained by a representative sub-set of the field laboratories (different symbols represent different participants, comp. legend on the right-hand side), and the intercomparison reference function (ICRF) of the NMI intercomparison (bold line) and the ICRF-NMI confidence interval (bold dotted line) for dye X (upper panel, left), dye QS (upper panel, right), and dye Y (lower panel, left). Note that, for better visibility, the confidence bounds in the graphical representation are expanded by a factor of two.

Figure 3. Top: Relative deviations of the corrected emission spectra obtained by the NMIs from the BAM-certified values of dyes F001 to F005; Centre: Relative deviations of the corrected emission spectra of dyes F001 to F005 measured by BAM from the ICRF-NMI in Part 1; Bottom: Relative deviations of the BAM certified values of dyes F001 to F005 from the ICRF-NMI with a variable wavelength shift being applied to the certified values, ranging from 0.2 to 0.8 nm.

Figure 4. Relative deviations of the spectra obtained by the field laboratories from the corresponding ICRF-NMI for dye X (upper panel, left), dye QS (upper panel, right), and dye Y (lower panel, left). Different symbols represent different laboratories, comp. legend in the left bottom field.

Figure 5 Relative spectral deviations of the corrected emission spectra of dyes X, QS, and Y obtained a.) using an emission correction curve supplied by a spectrofluorometer manufacturer (open circles connected with lines) and b.) an RM-based spectral correction (grey full squares). The corrected spectra are then subtracted from the ICRF-NMI yielding the shown relative spectral deviations.


Fluorescence Measurements Comparison - Field Labs vs. National Metrology Institutes
Deviations
Wavelength
Intensity
X
QS
Y
Wavelength

For TOC only

Figure 1

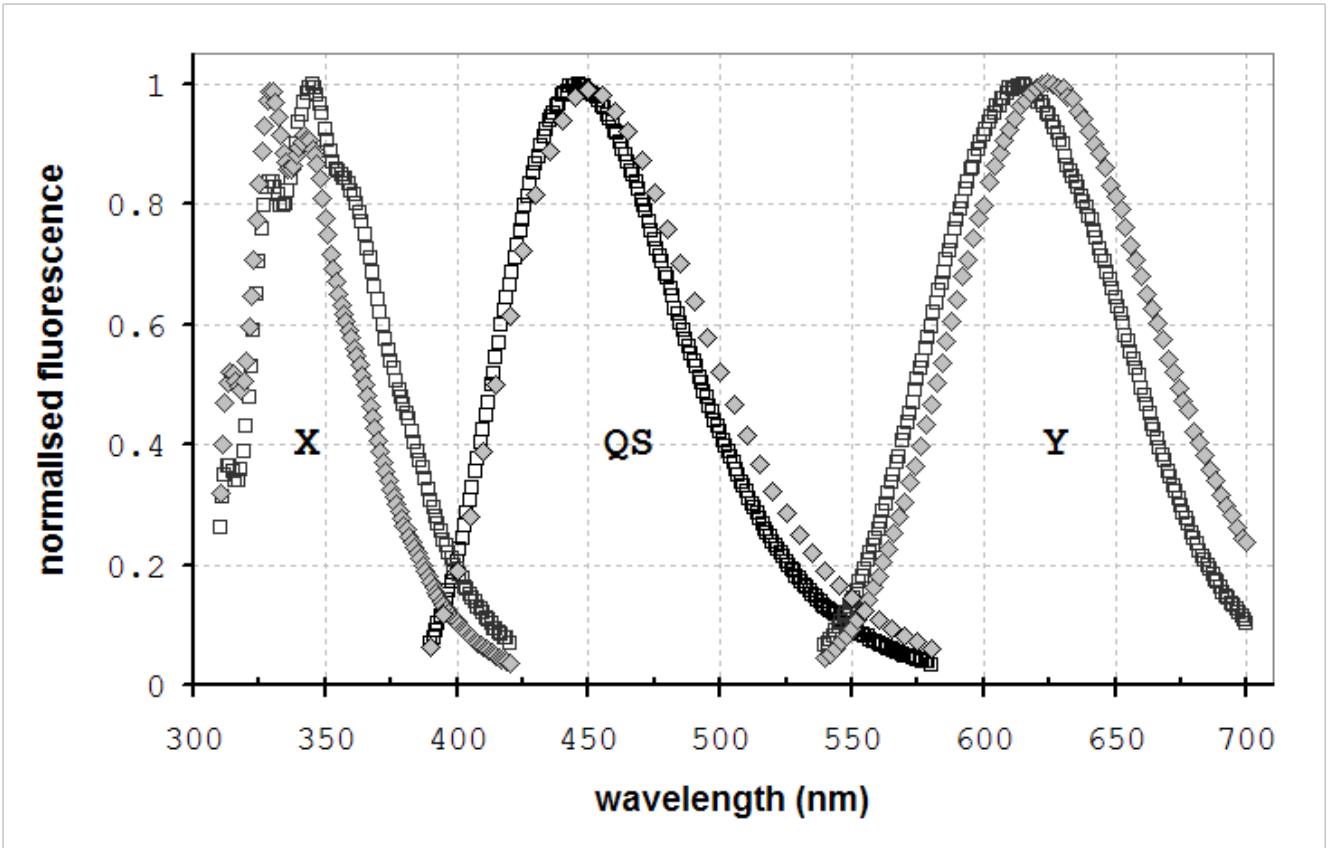

normallsed fluorescence
1
0.8
0.6
0.4
0.2
0
X
QS
Y
300
350
400
450
500
550
600
650
700
wavelength (nm)

Figure 2

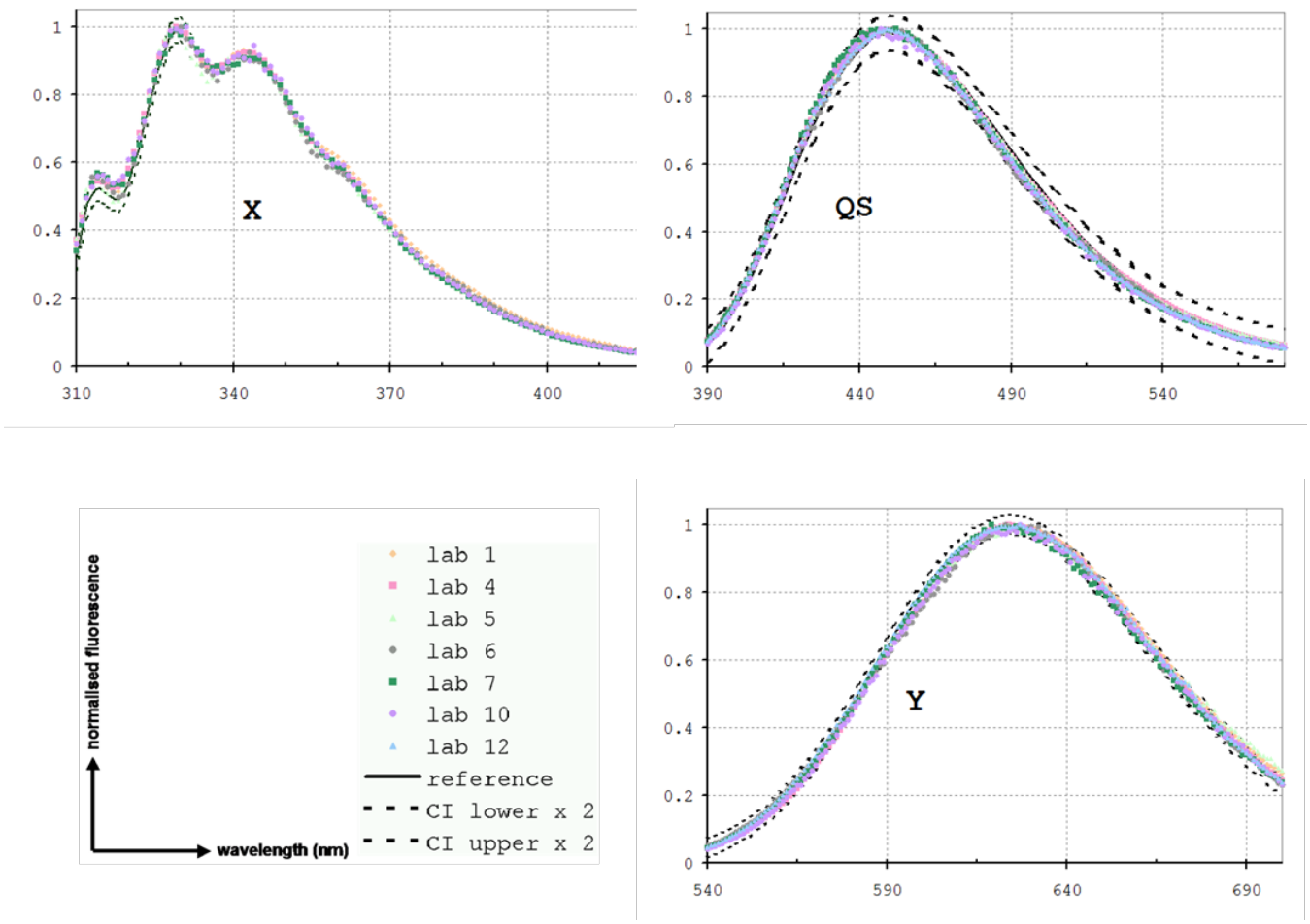

X
QS
Y
normalised fluorescence
wavelength (nm)
lab 1
lab 4
lab 5
lab 6
lab 7
lab 10
lab 12
reference
CI lower x 2
CI upper x 2

Figure 3

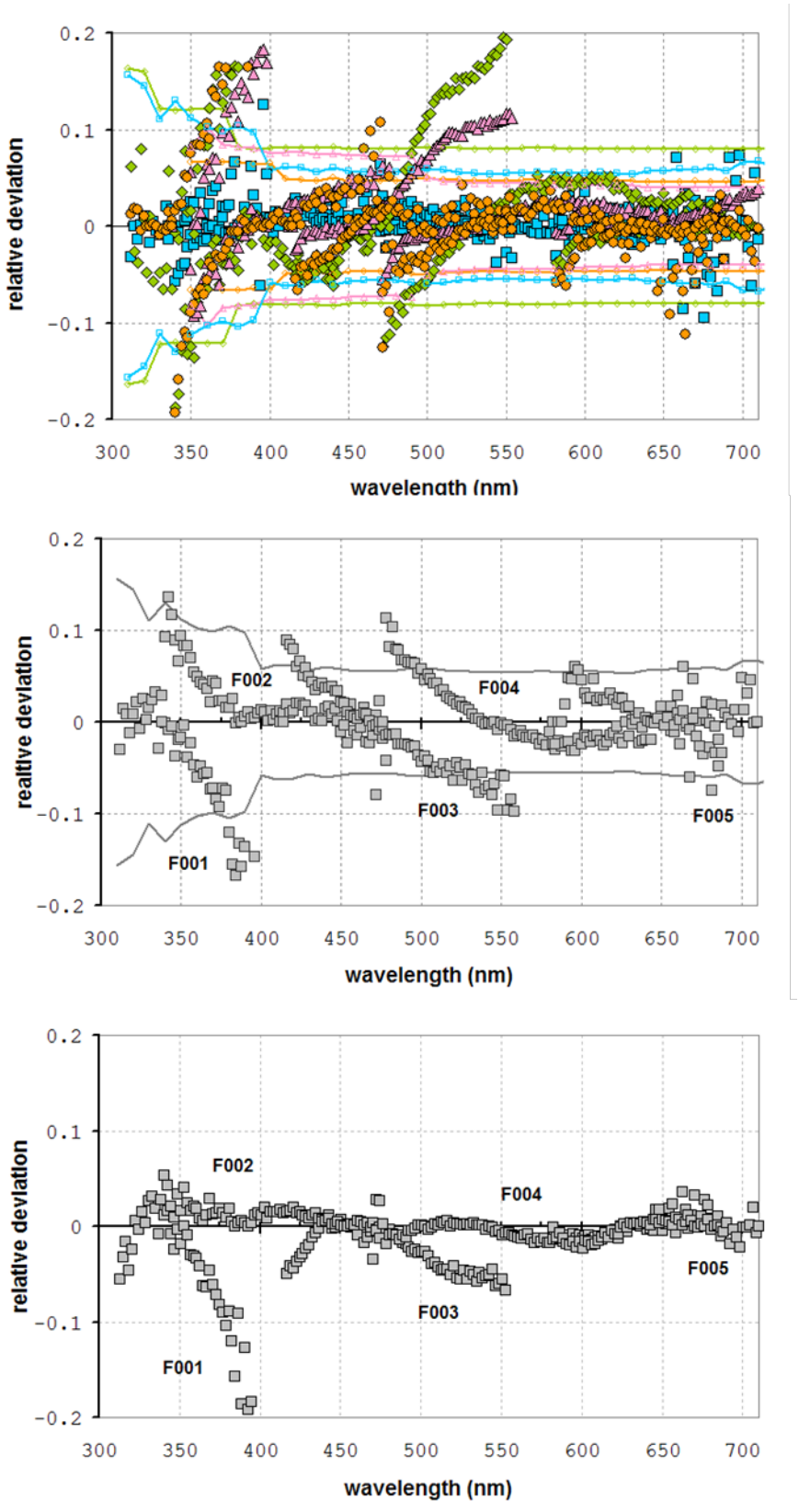


Figure 4

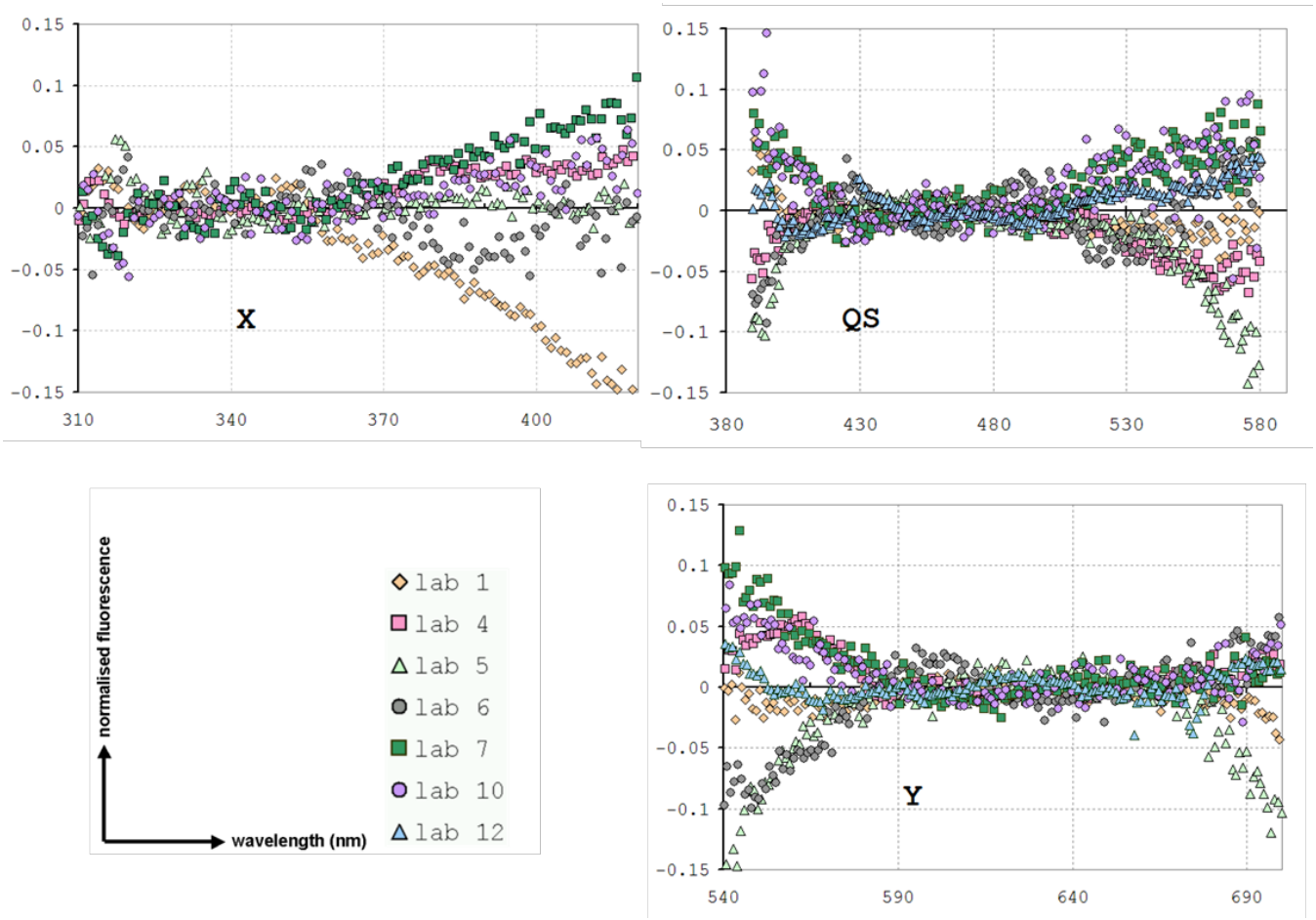
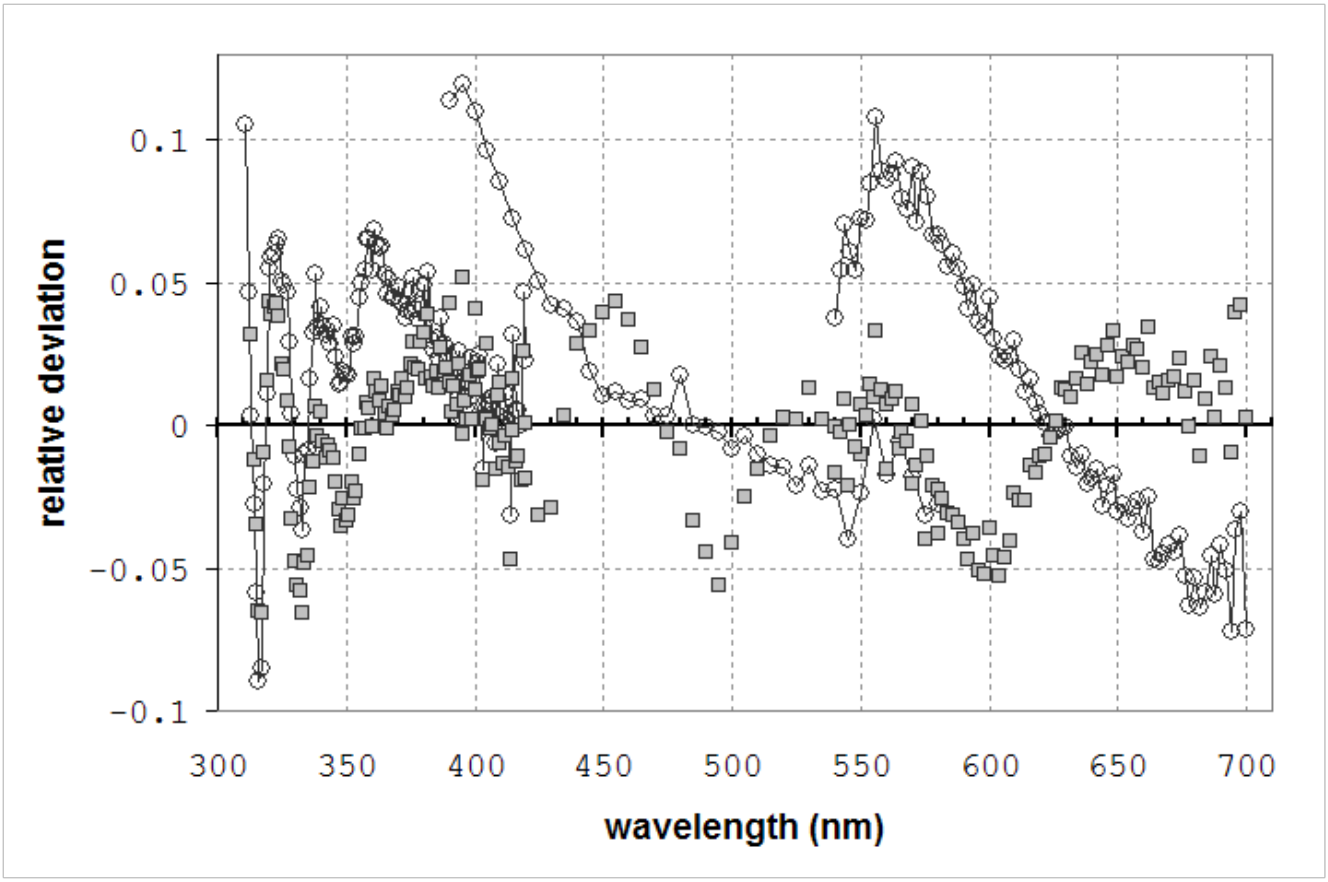

0.15
0.1
0.05
0
-0.05
-0.1
-0.15
310
340
370
400
X
380
430
480
530
580
QS
normalised fluorescence
wavelength (nm)
lab 1
lab 4
lab 5
lab 6
lab 7
lab 10
lab 12
540
590
640
690
Y

Figure 5


relative deviation
0.1
0.05
0
-0.05
-0.1
300
350
400
450
500
550
600
650
700
wavelength (nm)