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Comparison of Methods and Achievable Uncertainties for the Relative and Absolute Measurement of Photoluminescence Quantum Yields

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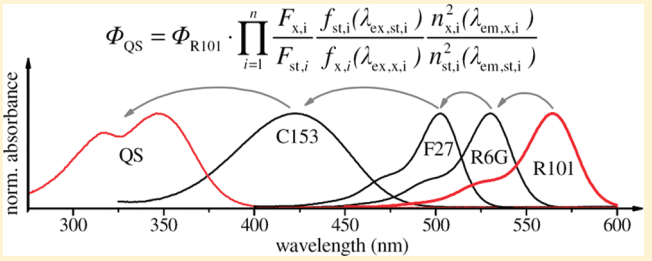
Supporting Information

ABSTRACT: The photoluminescence quantum yield (Φ_f) that presents a direct measure for the efficiency of the conversion of absorbed photons into emitted photons is one of the spectroscopic key parameters of functional fluorophores. It determines the suitability of such materials for applications in, for example, (bio)analysis, biosensing, and fluorescence imaging as well as as active components in optical devices. The reborn interest in accurate Φ_f measurements in conjunction with the controversial reliability of reported Φ_f values of many common organic dyes encouraged us to compare two relative and one absolute fluorometric method for the determination of the fluorescence quantum yields of quinine sulfate dihydrate, coumarin 153, fluorescein, rhodamine 6G, and rhodamine 101. The relative methods include the use of a chain of Φ_f transfer standards consisting of several "standard dye" versus "reference dye" pairs linked to a golden Φ_f standard that covers the ultraviolet and visible spectral region, and the use of different excitation wavelengths for standard and sample, respectively. Based upon these measurements and the calibration of the instruments employed, complete uncertainty budgets for the resulting Φ_f values are derived for each method, thereby providing evaluated standard operation procedures for Φ_f measurements and, simultaneously, a set of assessed Φ_f standards.



The direct measure for the efficiency of the conversion of absorbed photons into emitted photons by a chromophore is the photoluminescence quantum yield (termed here fluorescence quantum yield Φ_f).[1−4] Together with the molar (decadic) absorption coefficient $\varepsilon(\lambda_{ex})$ of the fluorophore at the excitation wavelength and the fluorophore concentration, Φ_f determines the achievable sensitivity in luminescence analysis as characteristics of the chromophore. Thus, the brightness, i.e., the product of $\varepsilon(\lambda_{ex})$ and Φ_f, is frequently used for dye comparison.[5] This underlines the importance of Φ_f as fluorometric key parameter that controls the suitability of a molecule or material, for example, for application as a label, probe, or sensor or as a converter material in light-emitting devices.

$$\Phi_f = \frac{N_{em}}{N_{abs}} \quad (1)$$

Φ_f can be determined fluorometrically either relative to a fluorescent standard with a known fluorescence quantum yield using the same excitation wavelength (method 1a) or different excitation wavelengths (method 1b) for sample and standard,[5−10] respectively, or absolutely (method 2) measuring the number of emitted photons N_{em} per number of absorbed photons N_{abs};[11−15] see eq 1. Alternatively, Φ_f can be obtained indirectly exploiting the dissipated heat using photoacoustic spectroscopy or thermal lensing.[15]

In the majority of cases, method 1a is employed because of its comparative simplicity, low costs, and high sensitivity, thereby minimizing the amount of sample and also enabling measurements of small fluorescence quantum yields. This procedure is often regarded as being established, at least in expert spectroscopy laboratories, for the most simplest case of transparent dilute solutions of small organic dyes. However, for the broad community of fluorescence spectroscopists, this seems not to be achieved yet. This is revealed, for example, by the still controversial Φ_f values reported for long known dye classes such as coumarins[15] and recent efforts by the International Union of Pure and Applied Chemistry (IUPAC; project no. 2004-021-1-300) to develop technical notes for the performance of reliable Φ_f measurements.[6,7,9,15−18] The most error-prone steps are the correction of the measured emission spectra for the instrument-specific spectral responsivity and the reliability of the Φ_f value of the standard that is typically taken from the literature.[1,10,19,20] Even the Φ_f values of many recommended quantum yield standards are still debated, partly because these compounds are often not well characterized with respect to all Φ_f-affecting parameters such as dye purity, excitation wavelength, and temperature.[15,20] Certified quantum yield standards that could overcome this problem are presently not available. Moreover, method 1a commonly relies on samples and standards that absorb within the same wavelength regions (because of the need

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for identical excitation wavelengths).[10] Similar wavelength regions are also recommended for the emission spectra in order to reduce systematic deviations related to the spectral correction of the emission spectra of sample and standard.[5] To cover an extended wavelength region with method 1a, a chain of Φ_f transfer standards can be built up from several dyes, the fluorescence quantum yields of which being measured pairwise starting from a golden quantum yield standard of reliably known Φ_f. This "advanced" method 1a that requires dyes with excitation wavelength-independent quantum yields was recently introduced by us for the determination of the Φ_f values of a set of CdTe quantum dots of various size and thus emission wavelengths.[10]

A principally more efficient strategy is the use of different excitation wavelengths for standard and sample (method 1b). This method, however, makes a challenging excitation correction mandatory to account for the wavelength dependence of the photon flux reaching the sample.[19,21−25]

The most straightforward method for the determination of Φ_f values presents the absolute measurement of N_{abs} and N_{em} in eq 1 with an integrating sphere setup (method 2).[11−14,26−31] This circumvents uncertainties related to the use of fluorescence standards inherent to all relative methods; also, this is the only fluorometric method suitable for measurements of Φ_f values of scattering systems. Key factors determining the accuracy of absolute Φ_f measurements are the reliability of the radiometric characterization of the integration sphere setup and proper consideration of reabsorption effects.[11,31,32] Non- or inaccurate consideration of these factors can result in considerable systematic errors, especially for nonexpert laboratories that completely rely on the instrument calibration and standard operation procedures for the measurement of Φ_f provided by the instrument manufacturers.

Despite the reborn interest in reliable Φ_f values of common and new chromophores, only few data addressing sources of uncertainty and associated values have yet been reported, even for the common method 1a.[10,15,33] For the evaluation and comparison of the different fluorometric methods of Φ_f determination, however, complete uncertainty budgets are needed that address contributions from all possible sources of variation such as instrument calibration, fluorophore purity, and the actual fluorescence measurement. To the best of our knowledge, no such attempts have been described yet for the transfer standard dye approach or for the use of different excitation wavelengths for standard and sample. Also for absolute measurements of Φ_f, data on typical uncertainties are very scarce. Moreover, the majority of these measurements have been performed with custom-built instruments and not with commercialized setups.[12,31] This encouraged us to systematically evaluate and compare fluorometric methods 1a, 1b, and 2 based upon Φ_f measurements of dilute solutions of five representative fluorophores of different Stokes shift and varying dye concentration. To render these data especially valuable for the broad community of fluorescence spectroscopists, we used a novel commercialized instrument for the measurement of the absolute fluorescence quantum yields and the commercialized, not further purified dyes quinine sulfate dihydrate (QS), coumarin 153 (C153), fluorescein 27 (F27), rhodamine 6G (R6G), and rhodamine 101 (R101). These dyes are among the best characterized fluorophores.[1,9,15,17,34−37] Based upon these measurements, complete uncertainty budgets are derived and achievable uncertainties are discussed. The overall goal here is to provide the broad community of fluorescence spectroscopists with evaluated standard operation procedures including achievable uncertainties for Φ_f.

MATERIALS AND INSTRUMENTATION

Materials. Rhodamine 101 (R101), rhodamine 6G (R6G), fluorescein 27 (F27), coumarin 153 (C153), and coumarin 120 (C120) were purchased from Lambda Physik GmbH and were of the highest purity available. Quinine sulfate dihydrate (QS; equaling SRM936a) was obtained from the National Institute of Standards and Technology (NIST).[34] The solvents used, i.e., ethanol in the case of R101, R6G, C153, and C120, 0.105 M perchloric acid for QS, and 0.1 M NaOH for F27, were of spectroscopic grade and purchased from Sigma Aldrich Inc. and Merck KGaA, respectively. Prior to use, all solvents were checked for fluorescent impurities.

Instrumentation. *Relative Measurement of Φ_f.* Absorption spectra were recorded on a Cary 5000 spectrometer (spectral bandwidth 1 nm, stepsize 0.5 nm). The accuracy of the intensity and wavelength scale of this instrument is regularly controlled with certified absorption standards from Hellma GmbH. Fluorescence emission and excitation spectra were measured with a Spectronics Instruments 8100 spectrofluorometer recently described, equipped with Glan Thompson polarizers in the excitation and emission channel and a separately addressable and adjustable reference channel;[10,22] see also Supporting Information. For the determination of the relative fluorescence quantum yields and the excitation spectra, the excitation polarizer was set to 0° and the emission polarizer to 54.7° (magic angle conditions) to render detected emission intensities independent of a possible emission anisotropy of the sample.[38] The calibration of this instrument including the determination of the wavelength and polarization dependence of the spectral responsivity of the detection system (emission correction) and the wavelength dependence of the photon flux at the sample position (excitation correction) is detailed in the Supporting Information. These measurements were always performed with identical settings of the reference channel as used for subsequent measurements of fluorescence quantum yields (method 1b) or excitation spectra of the dyes to minimize measurement uncertainties.

All fluorescence spectra presented are corrected for emission from the solvent and dark counts from the detector (blank correction) and for instrument-specific contributions (emission and excitation correction, see Supporting Information).[19,22] Relative Φ_f values were calculated employing the formula of Demas and Crosby,[6] see eq 2 in Results and Discussion. For 0.105 M perchloric acid and 0.1 M NaOH, we used the wavelength-dependent refractive index of water.[37,39] Also for ethanol, a wavelength-dependent refractive index was employed.[40]

Absolute Measurement of Φ_f. Absolute Φ_f values were obtained with an integrating sphere setup C9920-02 from Hamamatsu Photonics K.K. previously described;[31] see Supporting Information. The wavelength accuracy of the setup, the linearity of the detection system, and the spectral responsivity of the integrating sphere-detector assembly (emission correction) were determined by the instrument manufacturer in Japan and controlled by us. These emission correction curves that were incorporated in the data evaluation software, were automatically applied to the measured data. The fluorescence quantum yields were calculated from the spectra of the blank (solvent-filled cuvette) and the sample according to eq 1 using a procedure implemented by the instrument manufacturer.[31]

Table 1. Dyes, Solvents, and Excitation Wavelengths Used in This Study[a]

		R101 in ethanol	R6G in ethanol	F27 in 0.1 M NaOH	C153 in ethanol	QS in 0.105 M $HClO_4$
method 1a	λ_{ex} (nm)	–	490, 505, 530	465, 480, 502	455, 465, 475	370, 375, 380
method 1b	$\lambda_{ex,x}$, $\lambda_{ex,st}$ (nm)	–	500, 525	475, 525	423, 525	348, 525
method 2	λ_{ex} (nm)	525, 565	465, 480, 490, 502, 505, 530	455, 465, 475, 480, 502	422, 455, 465, 475	315[a]

[a] Excitation wavelength recommended by us for integrating sphere measurements.[31]

Measurement Conditions. All absorption and fluorescence measurements were performed with air saturated dye solutions at $T = (25 \pm 1)$ °C using 10 mm × 10 mm quartz cuvettes from Hellma GmbH for methods 1a and 1b and 10 mm × 10 mm long-necked quartz cuvettes from Hamamatsu Photonics K.K. for method 2, respectively, always filled with 3.5 mL of solvent or dye solution.

The absorbances of the dye solutions used for the relative Φ_f determinations were within the range of 0.02 to 0.1 (at the longest wavelength absorption maximum). For absolute Φ_f measurements, solutions of R6G, F27, and C153 with absorbances of 0.03 to 0.1 were employed; for R101 and QS solutions, the absorbances were varied from 0.036 to 1.6 and from 0.013 to 0.31, respectively. The excitation spectra of C120, C153, F27, R6G, and R101 exploited for control of the excitation correction of the spectrofluorometer were recorded with very dilute dye solutions displaying absorbances below 0.01 within the excitation wavelength region covered. Under these conditions, the attenuation of the excitation light at the center of a 10 mm cuvette reaches a maximum of 1%. These measurements were repeated multiple times to obtain very smooth spectra.

All measurements were carried out with fresh dye solutions to avoid additional uncertainties, for example, due to acid−base equilibria in the case of the xanthenes dyes.[36] If not stated otherwise, the deviations of the resulting Φ_f values were typically determined from at least six independent measurements for absolute and three for relative determinations of Φ_f, respectively.

Dye Purity. HPLC analysis of R101, R6G, F27, and C153 detailed in the Supporting Information was performed with a HPLC system from Knauer equipped with a diode array detector. For selected dyes, also a mass spectrometer was used for peak detection and analysis. This yielded the following purities: R101, 95.5% (525 nm) and 97.4% (565 nm); R6G: >98.5% (480 nm, 530 nm); F27: >84% (455 nm, 465 nm); C153: >99% (422 nm) and >99.5% (455 nm), respectively. The purity of QS of at least ≥98% follows from the NIST certificate and report.[34] All dyes were used without further purification.

Safety Considerations. Proper safety procedures for handling, storage, and disposal of the organic dyes should be observed.

RESULTS AND DISCUSSION

To address achievable measurement uncertainties of fluorometric methods 1a, 1b, and 2 for the determination of Φ_f and to introduce a chain of Φ_f transfer standards for the ultraviolet (UV)/visible (vis) region referenced to R101, we determined the fluorescence quantum yields of QS, C153, F27, R6G, and R101 with all three methods using different concentrations/absorbances of these dyes (Table 1).

The normalized absorption spectra (C120 instead of QS; see below) and the normalized corrected emission spectra of these chromophores (most dilute dye solutions used) which cover the spectral region of 320 to 600 nm (excitation) and 380 to 700 nm (emission), respectively, are summarized in Figure 1 (lower panels). The excitation wavelengths selected that are indicated with arrows are given in Table 1.

Relative Determination of Φ_f (methods 1a and 1b). Relative determination of Φ_f includes the following steps: (i) measurement of the absorption and emission spectrum of the sample, (ii) choice of a suitable fluorescence quantum yield standard, the quantum yield of which should be reliably known for the measurement conditions to be used (e.g., solvent/matrix, excitation wavelength, temperature, chromophore concentration) as well as preferably also its purity,[18,20] (iii) choice of the measurement conditions for standard and sample (e.g., excitation wavelength(s), fluorometer settings) and measurement of the corresponding absorption and emission spectra of sample and standard, (iv) data evaluation (e.g., blank correction: subtraction of the emission spectrum of the solvents from that of the sample and standard to remove possible background signals, i.e., scattering and fluorescence from the solvent and dark counts at the detector; spectral correction),[10] and (v) calculation of the relative fluorescence quantum yield according to the formula of Demas and Crosby (eq 2).[5]

$$\Phi_{f,x} = \Phi_{f,st}\frac{F_x}{F_{st}}\frac{f_{st}(\lambda_{ex,st})}{f_x(\lambda_{ex,x})}\frac{n_x^2}{n_{st}^2}\frac{q_{p,st}(\lambda_{ex,st})}{q_{p,x}(\lambda_{ex,x})} \quad (2)$$

In eq 2, the subscripts x and st denote sample and standard, ex and em excitation and emission wavelength(s), $\Phi_{f,st}$ equals the fluorescence quantum yield of the standard. F presents the integrated spectral fluorescence photon flux $q^f_{p,\lambda}(\lambda_{em})$ at the detector that is obtained from the blank and dark-count corrected signal of the emission detector $I_u(\lambda_{em})$ multiplied with the photon energy hc_0/λ_{em} and divided by the spectral responsivity $s(\lambda_{em})$ of the emission channel (emission correction). This quotient was integrated over the complete emission wavelength range of the respective dye (see right panel of Figure 1).[19,25]

$$F = \int_{\lambda_{em_1}}^{\lambda_{em_2}} q^f_{p,\lambda}(\lambda_{em})\mathrm{d}\lambda_{em} = (hc_0)^{-1}\int_{\lambda_{em_1}}^{\lambda_{em_2}} \frac{I_u(\lambda_{em})}{s(\lambda_{em})}\lambda_{em}\mathrm{d}\lambda_{em} \quad (3)$$

The absorption factor $f(\lambda_{ex})$ in eq 2 provides the fraction of the excitation light absorbed by the chromophore that is given in good approximation by the absorbance $A(\lambda_{ex})$ at the excitation wavelength by eq 4. For a more exact calculation of $f(\lambda_{ex})$ considering the spectral bandwidth of the excitation light, see eq 4S in the Supporting Information.

$$f(\lambda_{ex}) = 1 - 10^{-A(\lambda_{ex})} \quad (4)$$

$q_{p,st}(\lambda_{ex,st})$ and $q_{p,x}(\lambda_{ex,x})$ in eq 2 are the photon fluxes (the photon flux $q_p(\lambda)$ is the integral of the photon irradiance $E_p(\lambda)$ over the illuminated area) at sample position for standard and sample at the chosen excitation wavelengths. These fluxes are identical for method 1a and have to be considered only for method 1b that uses different λ_{ex} for sample and standard and thus relies on an excitation correction (see Supporting Information). The

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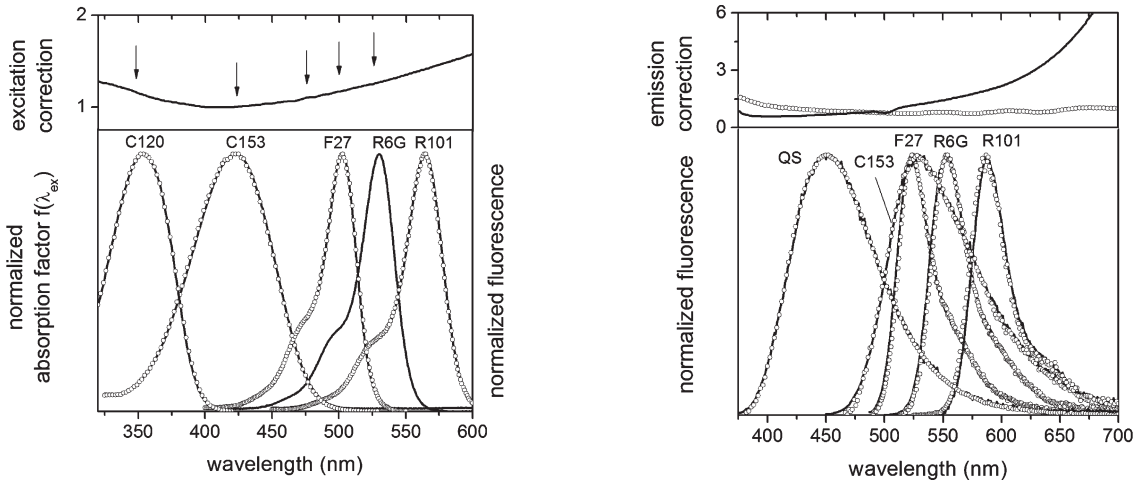

Figure 1. Left, bottom: Normalized absorption factors (solid lines) and normalized corrected excitation spectra (open symbols) of C120, C153, F27, R6G, and R101 measured with spectrofluorometer 8100. Left, top: Excitation correction curve (solid line) of the fluorometer and the excitation wavelengths used (arrows) for method 1b, respectively. Right, bottom: Normalized corrected emission spectra of QS, C153, F27, R6G, and R101 measured with the fluorometer (solid lines) and with the integrating sphere setup from Hamamatsu (open symbols). Right, top: Emission correction curves of fluorometer 8100 (solid line) and the integrating sphere setup (open symbols). For the measurement of the corrected emission spectra of the dyes with the integrating sphere setup shown here, only very dilute dye solutions were used to minimize reabsorption effects.

refractive index correction term (n_i^2) in eq 2 has to be applied if different solvents are used for sample and standard.[41] To reduce uncertainties due to possible emission anisotropies of the sample, all measurements have to be performed under the magic angle conditions (see Materials and Instrumentation).

Method 1a. Requirements for step ii: a fluorescence quantum yield standard absorbing within a similar wavelength region as that for the sample; for step iii: use of identical instrument settings including identical λ_{ex} for sample and standard and typically matching absorbances of standard and sample at λ_{ex}; for step iv, a spectral emission correction according to eq 3.[10,22] The quantities $q_p(\lambda_{ex,st})$ and $q_p(\lambda_{ex,x})$ in eq 2 are identical. For our Φ_f transfer standard chain procedure consisting of n dye pairs, eq 2 can be written as

$$\Phi_{f,x} = \Phi_{f,st} \prod_{i=1}^{n} \frac{F_{x,i}}{F_{st,i}} \frac{f_{st,i}(\lambda_{ex,st})}{f_{x,i}(\lambda_{ex,x})} \frac{n_{x,i}^2(\lambda_{em,x})}{n_{st,i}^2(\lambda_{em,st})} = \Phi_{f,st} \prod_{i=1}^{n} C_i \quad (5)$$

Consequently, C_i has to be determined n times, i.e., steps i−v need to be performed for each pair of standard and reference dye.

For the determination of the fluorescence quantum yield of QS versus R101 with method 1a, we used the following Φ_f transfer chain or "standard dye" versus "reference dye" pairs: R6G versus R101, F27 versus R6G, C153 versus F27, and QS versus C153. These standards were chosen to display excitation wavelength-independent quantum yields. For $\Phi_{f,st}$ in addition to the absolutely determined (method 2) quantum yield, the fluorescence quantum yield of our "golden standard" R101 was taken from the literature ($\Phi_f = 0.96$)[10,35,42,43] to underline the influence of the fluorescence quantum yield value used for the standard on the resulting relative Φ_f values.

Method 1b. Requirements for step ii: this enables free choice of the fluorescence quantum yield standard regarding its absorption spectrum; for step iii: use of identical instrument settings for sample and standard except for λ_{ex} is mandatory; for step iv: an emission correction (see eq 3) and an excitation correction (see eq 2 and eq 2S in the Supporting Information) must be performed, thereby accounting for the different photon fluxes $q_p(\lambda_{ex})$ at the excitation wavelengths $\lambda_{ex,st}$ and $\lambda_{ex,x}$; see also Supporting Information.[10,21−23,25] The following measurements were performed: R6G versus R101, F27 versus R101, C153 versus R101, and QS versus R101. For $\Phi_{f,st}$, always the absolutely determined (method 2) quantum yield of R101 was used.

Absolute Determination of Φ_f (method 2). The measurement of absolute fluorescence quantum yields of dilute dye solutions according to eq 1 with the commercialized integrating sphere setup consists of the following steps: (i) determination of the of the excitation light peak ($I_x(\lambda_{ex})$) and the emitted light ($I_x(\lambda_{em})$) of the sample and a blank (solvent-filled cuvette; ($I_b(\lambda_{ex})$; $I_b(\lambda_{em})$) under identical measurement conditions (e.g., excitation wavelength, temperature), each within a single scan, (ii) data evaluation including choice of the excitation and emission wavelength region used for signal integration and spectral emission correction; (iii) calculation of the fluorescence quantum yield based upon eq 6, using the software of the instrument manufacturer Hamamatsu.[12]

$$\Phi_f = \frac{\int_{\lambda_{em_1}}^{\lambda_{em_2}} \frac{(I_x(\lambda_{em}) - I_b(\lambda_{em}))}{s(\lambda_{em})} \lambda_{em} d\lambda_{em}}{\int_{\lambda_{ex}-\Delta\lambda}^{\lambda_{ex}+\Delta\lambda} \frac{(I_b(\lambda_{ex}) - I_x(\lambda_{ex}))}{s(\lambda_{ex})} \lambda_{ex} d\lambda_{ex}} = \frac{N_{em}}{N_{abs}} \quad (6)$$

N_{em} is obtained upon integration of the blank-corrected (subtraction of $I_b(\lambda_{em})$) and spectrally corrected emission spectrum of the sample ($I_x(\lambda_{em})$). N_{abs} follows from the integrated difference between the excitation light resulting from measurements with the blank ($I_b(\lambda_{ex})$) and the sample ($I_x(\lambda_{ex})$).

TYPICAL AND METHOD-INHERENT SOURCES OF UNCERTAINTIES

For the determination of uncertainty budgets for methods 1a, 1b, and 2, calibration-, measurement-, and sample-related uncertainties are discussed in the following sections for each method and summarized in Table 2. A detailed description of the individual

Table 2. Summary of the Determined Calibration- and Measurement-Related Uncertainties[a]

method	source	uncertainty	symbol
1a	emission correction	≤2% (see Table 2S)	u_{em}
	max. relative experimental standard deviation of $\Phi_{f\text{-}1a}$	≤2.5%	$\Delta C_{std,max}/C$
1b	emission correction	≤2% (see Table 2S)	u_{em}
	excitation correction	≤2% (see Table 2S)	u_{ex}
	max. relative experimental standard deviation of $\Phi_{f\text{-}1b}$	≤3.5%	$\Delta D_{std,max}/D$
2	emission correction	5.5%[b]	u_{em}
	max. relative experimental standard deviation of $\Phi_{f\text{-}2}$	≤2.4%	$\Delta\Phi_{std,max}/\Phi$

[a] For a complete summary, see section 5 in the Supporting Information. [b] Information from the instrument manufacturer.

contributions is given in the Supporting Information (Tables 1S, 2S, and 3S).

Calibration-Related Systematic Variations. Calibration-related systematic variations in all cases are composed of uncertainties from (i) the accuracy of the wavelength scales of the spectrometers used (excitation and emission channel of the fluorometer and the integration sphere setup; absorption spectrometer), (ii) the range of linearity of the detection system(s) of the instruments used (for methods 1a and 2, the emission detection system; for method 1b, in addition also the reference detector; see Supporting Information) that can have a much more pronounced influence on measurement results for method 2 compared to methods 1a and 1b, due to the strongly different intensities of the transmitted and scattered excitation light and the dye emission, and (iii) the determination of $s(\lambda_{em})$ (emission correction). For method 1b, additionally (iv) systematic uncertainties arising from the determination of $q_p(\lambda_{ex})$ (excitation correction) have to be considered.[21,22] As the size of these calibration-related uncertainties are directly affected by the calibration uncertainties of the transfer standards used, we employed only high quality physical transfer standards for the determination of the emission and excitation correction, thereby minimizing these uncertainty contributions.[18,19] Although the calibration-related uncertainties are wavelength-dependent, for simplicity reasons, we provide here only mean, i.e., wavelength-averaged, values for each uncertainty contribution for the excitation and emission wavelength ranges used (e.g., Table 2 and Tables 2S and 3S and Figure 3S in the Supporting Information).

Emission Correction. The reliability of our emission correction was recently controlled in an international multilaboratory comparison of all National Metrology Institutes active in the area of spectrofluorometry, determining the corrected emission spectra of eight dyes in the wavelength region of 300 to 770 nm. The spectral deviations between the corrected emission spectra of QS, C153, F27, R6G, and R101 recorded with our calibrated fluorometer (solid lines) and the corrected emission spectra obtained with the integrating sphere setup (symbols) are shown in Figure 1 (right panel, bottom). Generally, the comparability is good. The deviations at the left wing of the emission spectra (see, for example, C153) are ascribed to difficulties to accurately separate excitation and emission with the integrating sphere setup and to reabsorption effects.

Excitation Correction. The excitation correction curve was controlled by comparing the corrected excitation spectra of C120, C153, F27, R6G, and R101 with the corresponding absorption factors $f(\lambda)$ shown in Figure 1 (Figure 1, left panel, bottom).[44] C120 was employed for the wavelength region of 320 to 380 nm and not QS, as the latter can display an ambiguous wavelength dependence of its fluorescence spectrum.[34] The resulting spectral deviations between these spectra are summarized in Figure 2. The deviations of maximum 2.5% underscore the good quality of our excitation correction.

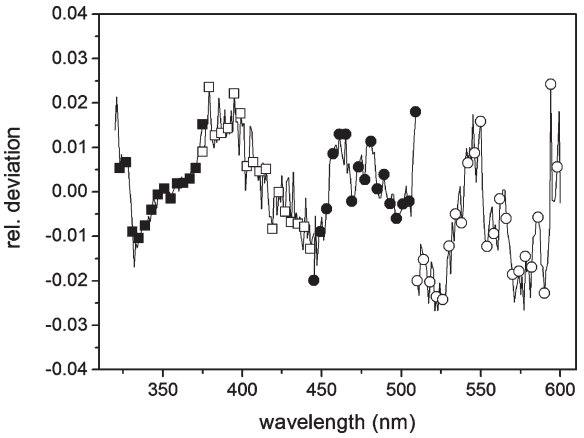

Figure 2. Relative spectral deviations of the excitation spectra and the absorption factors for C120 (full squares), C153 (open squares), F27 (full circles), and R101 (open circles).

Measurement- and Sample-Inherent Uncertainties. For the relative methods 1a and 1b, measurement- and sample-inherent uncertainties are composed of uncertainties related to the measurement of the absorption factor at the excitation wavelength, the emitted photon flux, and the reliability of the Φ_f value(s) employed for the standard. The influence of the assumed Φ_f value of the standard is detailed in the following sections. For method 2, the most important measurement-inherent sources of uncertainty are sample-specific reabsorption effects, the reproducibility of sample positioning (for a sample-blank pair), and the choice of the integration limits (e.g., fluorescence signal >3 × noise, spectral separation of emission and scattered excitation light). For the measured transparent dilute dye solutions, the missing separation of direct and indirect excitation of fluorescence[14] that is critical, for example, for scattering samples, is not expected to introduce significant uncertainties.

Sample-related uncertainties include dye purity for all methods and dye-specific, concentration-dependent reabsorption effects. Temperature effects, the possible influence of oxygen, and dye self-quenching[1,35,45] that similarly affect all measurements were beyond the scope of this comparison.

Reabsorption Effects in an Integrating Sphere. The fluorescence spectra of C153 and QS that display a strongly Stokes-shifted emission are independent of dye concentration

Table 3. Φ_f Values Determined with Methods 1a, 1b, and 2 Including Overall Absolute Uncertainties[a]

	method 1a	method 1b	method 2	literature
R101	–	–	0.900 ± 0.050	0.96,[35,42] 1.00 ± 0.05[13]
R6G	0.893 ± 0.053	0.894 ± 0.058	0.897 ± 0.050	0.95,[35,42] 0.94,[11,51] 0.88[27]
F27	0.824 ± 0.049	0.804 ± 0.054	0.785 ± 0.045	0.81,[37] 0.86,[11] 0.91 ± 0.05[13]
C153	0.514 ± 0.031	0.538 ± 0.037	0.521 ± 0.029	0.40,[52] 0.26,[53] 0.58[47]
QS	0.587 ± 0.037	0.599 ± 0.040	0.633 ± 0.035	0.60[9]

[a] For methods 1a and 1b, we used the Φ_f value of 0.90 (Φ_{f_2}) for R101 determined by us absolutely. Solely in the case of method 1a, we also report the corresponding Φ_f values obtained with a typical literature Φ_f value of 0.96 used for R101 in the text.

within the concentration range studied for both setups. For the xanthenes F27, R6G, and R101, showing a strong spectral overlap between absorption and emission like most bioanalytically relevant dyes such as BODIPYs and cyanines as well as quantum dots,[46] however, only the corrected emission spectra of very dilute dye solutions obtained with the integrating sphere closely match the spectra determined with the spectrofluorometer (Figure 1, right panel, bottom). The considerable influence of reabsorption effects on fluorescence measurements with an integrating sphere setup is detailed in the Supporting Information (Figure 2S). The extent of such reabsorption-related systematic variations depends on the size of the spectral overlap of the fluorophore absorption and emission spectrum, on the dye concentration, and on the distance traveled by the emitted photons within the sample prior to detection.[31] The latter is also affected by the wavelength- and material-dependent reflectivity of the sphere walls and the sample volume.

For dye solutions showing a reabsorption-induced red-shift of the emission maxima compared to the spectra obtained with the spectrofluorometer, we employed a reabsorption-correction of the determined absolute $\Phi_{f,obs}$ values (see eq 7).[32]

$$\Phi_{f,corr} = \frac{\Phi_{f,obs}}{1 - a + a\Phi_{f,obs}} \quad (7)$$

Here, a is the reabsorption probability that can be determined by comparison of an undisturbed (spectrally corrected) emission spectrum measured, for example, with a spectrofluorometer and the disturbed (spectrally corrected) emission spectrum determined with the integrating sphere spectrometer. The determination of a solely with an integrating sphere setup requires the measurements of concentration series.

Uncertainties of Φ_f Values for Method 1a. To calculate complete uncertainties of method 1a (see Supporting Information, eq 9S) contributions of the quantum yield standard $\Delta\Phi_{f,st}$, calibration-related systematic variations (u_{em}) of the emission correction (arising from $s(\lambda_{em})$ in eq 3), and uncertainties related to the measurement of Φ_f itself have to be considered. The latter are due to, for example, cuvette positioning. Consequently, the combined standard deviations of the Φ_f values increase with the number n of dye pairs (eq 5 and eq 9S in the Supporting Information). For our Φ_f transfer standard chain, consisting of five dyes and thus four standard−reference dye pairs for the determination of Φ_f of QS versus R101, the standard deviation of Φ_{f_1a} varies between 1% and 2.5% (Table 2). This suggests that the standard deviation of C_i (eq 5) cannot be expressed solely by contributions from relative standard deviations of the emitted integrated spectral photon flux F (eq 3; 1.3%, Table 3S in the Supporting Information) and the relative standard deviations of the absorption factor f (0.3%, measured at the same wavelength for standard and sample; Table 3S in the Supporting Information). Also uncertainties arising from the different wavelength accuracies of the absorption spectrometer and the spectrofluorometer used and the size of the spectrofluorometer excitation bandpass in conjunction with the different slopes of the absorption spectra at the excitation wavelength of the standard−reference dye pairs contribute to the uncertainty of C_i.

As the emission bands of each pair of reference and standard dyes are typically located within a comparatively narrow spectral window, deviating by at most ca. 50 nm (Figure 1, right panel, bottom), systematic wavelength-dependent deviations due to the uncertainty of the emission correction are often also small, at least in the visible region up to ca. 650 nm, where the wavelength dependence of the spectral responsivity of many fluorometers is not very pronounced (e.g., Figure 1, right panel, top). This underlines the importance of the knowledge of the emission correction curve for the proper choice of quantum yield standards.[10] Taking into account all uncertainty contributions summarized in Table 2S in the Supporting Information, the relative uncertainty of the emission correction curve u_{em} is determined to <2% for the wavelength region of 400 to 700 nm.

Uncertainties of Φ_f Values for Method 1b. To ease this discussion for method 1b, eq 2 is written as

$$\Phi_{f,1b} = \Phi_{f,st}\frac{F_x}{F_{st}}\frac{f_{st}(\lambda_{ex,st})}{f_x(\lambda_{ex,x})}\frac{n_x^2}{n_{st}^2}\frac{q_{p,st}(\lambda_{ex,st})}{q_{p,x}(\lambda_{ex,x})} = \Phi_{f,st}D \quad (8)$$

For the calculation of the complete uncertainties of the Φ_f values determined with method 1b (see also eq 10S in the Supporting Information) in addition to the systematic uncertainty of the emission correction (u_{em}) of <2%, also systematic variations arising from the excitation correction (u_{ex}) and the experimental standard deviation of D must be considered (see Table 2 and Table 2S in the Supporting Information). The relative uncertainty of the excitation correction curve (Figure 1, left panel, top) was determined to <2% in the relevant spectral region of 350 to 600 nm. The determination of $f_{st}(\lambda_{ex,st})$ and $f_x(\lambda_{ex,x})$ involves two independent measurements at different wavelengths. Consequently, the absolute uncertainty of the absorbance measurements must be considered additionally to the standard deviation of $f_{st}(\lambda_{ex,st})$. The absolute measurement accuracy of our instrument is on the order of 0.8% (Table 3S, Supporting Information).

Uncertainties of Φ_f Values for Method 2. The combined relative uncertainties of absolute Φ_f measurements can be expressed by the relative experimental deviation of the mean of Φ_f and systematic uncertainties arising from the instrument calibration, (see eq 11S, Supporting Information). The uncertainty of the calibration of the integrating sphere setup was 5.5% for the relevant wavelength region of ca. 370 to 700 nm (see Table 3S, Supporting Information).[31] The uncertainty

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Table 4. Relative Uncertainties of the Mean of the Φ_f Values Determined with Methods 1a, 1b, and 2 Considering and Disregarding the Uncertainty of the Standards' Φ_f Value

	method 1a		method 1b		method 2
	rel uncertainty without standard	rel uncertainty with standard	rel uncertainty without standard	rel uncertainty with standard	rel uncertainty
R101	–	–	–	–	5.52
R6G	2.05	5.88	3.8	6.44	5.57
F27	2.23	5.95	4.17	6.67	5.67
C153	2.32	5.99	4.1	6.66	5.59
QS	2.53	6.07	4.25	6.72	5.51

arising from the reabsorption correction was not considered, as we did not observe a systematic, concentration-dependent change of Φ_f.[12,31] We assume that this uncertainty contribution is covered by the standard deviation of 2.4% (see Table 2 and Table 3S in the Supporting Information) obtained from three independent measurements of at least two different dye concentrations.

Evaluation of the Determined Φ_f Values. The Φ_f values of QS, C153, F27, R6G, and R101 determined with methods 1a, 1b, and 2 are summarized in Table 3 together with the deviations of the mean values derived from the calibration- and measurement-related uncertainties. The fluorescence quantum yields of our five fluorophores are in excellent agreement for all three methods when using a Φ_f value of 0.90 for R101 as determined by us with method 2. The minimum deviations between the results of methods 1a, 1b, and 2 are covered by the derived uncertainties. Moreover, all Φ_f values are in good agreement with the literature data provided in Table 3 except for F27. Whether the minimally smaller Φ_f values found by us for this dye could be ascribed to dye purity remains to be shown. This, however, does not affect our systematic comparison of methods 1a, 1b, and 2.

When the uncertainty of the standards' Φ_f values is neglected, the relative uncertainty of the mean Φ_f value of our Φ_f transfer chain method 1a rises from 2.1% (R6G) to 2.5% (QS), the relative uncertainty of method 1b lies between 3.8% (R6G) and 4.3% (C153), and for method 2, the relative uncertainty reaches 5.7% (Table 4, uncertainty of the standards' Φ_f values not considered). The accuracy of method 1a exceeds that of method 1b, because of the additionally required excitation correction in the latter case (see eq 2) despite the additive character of the standard deviation of C_i. The enhanced uncertainties found for method 2 compared to methods 1a and 1b, when the uncertainty of the standards' Φ_f values is neglected, are ascribed to the enlarged calibration uncertainty of the integrating sphere setup, as the size of the standard deviation of Φ_f is rather similar for all three methods (see Table 2). This uncertainty contribution can be possibly further reduced by elaborate calibration procedures as demonstrated by us.[22] When similar calibration uncertainties are assumed, relative uncertainties of 3% to 5% for absolute quantum yield measurements seem to be accomplishable.

Consideration of the uncertainty of the Φ_f value of the quantum yield standard used for methods 1a and 1b results in an increase of the systematic variations of the relative Φ_f values (Table 4). Use of the absolute Φ_f value of R101 determined with method 2 yields combined relative uncertainties of 5.9% to 6.1% for R6G, F27, C153, and QS for method 1a. For method 1b, the relative uncertainties amount to 6.4% to 6.7%. Obviously, for relative measurements of Φ_f, the uncertainty of the Φ_f value of the quantum yield standard presents the key uncertainty contribution, at least in cases where uncertainties from instrument calibrations are minimized.

To demonstrate the considerable influence of the Φ_f value of the fluorescence standard on the resulting Φ_f values for method 1a, we additionally calculated the fluorescence quantum yields of R6G, F27, C153, and QS using a Φ_f value of 0.96 for our golden standard R101 as reported in the literature.[9,13,35,42,47] This value differs from our absolutely determined Φ_f value of R101 of 0.90 by about 7%. In this case, Φ_f values of 0.95, 0.87, 0.54, and 0.57, respectively, are obtained for the studied fluorophores. Use of Φ_f values from the literature as reference values despite the fact that the Φ_f values of the individual dye batches employed are not exactly known can yield considerable systematic variations for relative methods. This highlights the need for commercially available fluorescence quantum yield standards with certified Φ_f values for the UV/vis/NIR spectral region to improve the reliability of Φ_f data.

CONCLUSIONS AND OUTLOOK

We systematically examined two relative methods (method 1a: same excitation wavelength for standard and sample; e.g., Φ_f transfer standard chain; method 1b: different excitation wavelengths for standard and sample and excitation correction) and one absolute method (method 2) for the fluorometric determination of the fluorescence quantum yields of rhodamine 101, rhodamine 6G, fluorescein 27, coumarin 153, and quinine sulfate and derived complete uncertainty budgets. With all three methods, uncertainties below 7% could be achieved if typical calibration- and measurement-inherent sources of uncertainty were minimized. The minimum deviations between the Φ_f values of rhodamine 101, rhodamine 6G, fluorescein 27, coumarin 153, and QS obtained with methods 1a, 1b, and 2 are covered by the derived measurement uncertainties. Simultaneously, with this comparison, we provide a set of evaluated fluorescence quantum yield standards with assigned uncertainties for the ultraviolet and visible region.

For most commonly used relative methods, we identified the reliability of the Φ_f value of the quantum yield standard as the key source of systematic variation. This underscores the need for commercially available fluorescence quantum yield standards with certified Φ_f values for the UV/vis/NIR spectral region.

For relative measurements of Φ_f, method 1a (the Φ_f transfer chain approach) is recommended by us as method of choice. The uncertainties given by us for method 1b, which is principally more flexible than method 1a, can only be accomplished if excitation calibration-related uncertainties are minimized. Although a dye-based emission correction can be simply obtained with the aid of certified emission standards such as the spectral

fluorescence standards F001 to F005 supplied by BAM,[19,48,49] an excitation correction can present a considerable challenge and can thus yield enhanced measurement uncertainties. Use of emission and excitation correction curves implemented by instrument manufacturers is only recommended if the method of determination, instrument settings employed, and the respective radiometric reference quantities are known. In any case, an evaluation of these curves is necessary prior to use.

Eventually, in the near future, absolute measurements of dilute dye solutions can become a very attractive alternative to relative measurements provided that properly evaluated integrating sphere setups are available with openly accessible procedures for instrument calibration and data assessment. This also requires implementation of a reabsorption correction, as the majority of (bio)analytically relevant fluorophores display a considerable spectral overlap between absorption and emission. With this respect, also an international interlaboratory comparison of expert and field laboratories using such setups may be very helpful to systematically assess achievable uncertainties for commonly used fluorophores.[50] This could also provide the basis for standard operation procedures for the reliable determination of absolute Φ_f values with such setups.

■ ASSOCIATED CONTENT

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

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