A METHOD FOR ROUTINE CORRECTIONS OF INNER FILTER EFFECTS IN MEASUREMENTS OF EXCITATION AND FLUORESCENCE SPECTRA

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The described procedure serves for corrections of effects of light absorption and reflections inside the sample cell on the fluorescence intensity. The sample transmittance data necessary for the corrections are obtained from the ratio of signals of fluorescence spectrophotometer measured at two positions of the sample in which the excitation light and/or the emitted light pass through various width of solution (the method of cell shift), or from two fluorescence intensity measurements one of which is weakened by insertion of another cell with the measured solution in the path of the excitation or emitted light (the method of two cells). The results are evaluated off-line with a computer along with correction of spectral dependence of the excitation beam intensity and spectral dependence of sensitivity of detection part of the apparatus. Magnitude of the experimental error of the corrected results is discussed with the presumption that reemission effect can be neglected.

Correction of fluorescence spectra with respect to various types of distortion has fundamental importance in all studies necessitating molecular parameters of fluorescence[1] as *e.g.* those of kinetics of radiative and non-radiative processes, relative intensities of vibration bands in fluorescence spectrum, quantum yield of fluorescence *etc.* Furthermore the corrected results are necessary for comparison of data from different laboratories.

Correction of excitation spectra with respect to spectral dependence of the excitation beam intensity and correction of emission spectra with respect to spectral dependence of sensitivity of emission part of the apparatus (correction of the apparatus distortion) have become routine procedures now. A number of commercial spectrophotometers are equipped with automatic correction of these effects. The inner filter effects[2] *i.e.* weakening of the excitation beam on passing through the sample to the point scanned by the emission part of the apparatus and weakening of the fluorescence radiation by the sample reabsorption can be avoided in a number of cases by measurements in sufficiently diluted solutions[3]. However, sample producing weak fluorescence signals in the detector must be measured at concentration corresponding to maximum signal, which is accompanied by the sample absorbing considerable part of the light. The inner filter effects must also be corrected with the samples containing absorbing admixtures and often with respect to fluorescence of solvent, too.

Expressions of correction of the inner filter effects were derived for various experimental arrangements: *e.g.* those given in refs[4-9] for the fluorescence scanned perpendicularly with respect to the excitation beam. Eastman[9] used the extrapolation method for this correction. Hunt and Hill[10] dealt with correction for the effect of repeated reflections of the light beam incident at right angle on the cell wall and expressed the overall light intensity with a geometric

series. Slageren and coworkers[11] give an expression correcting both the inner filter effects and the effect of light reflections inside the cell.

Both the computer off-line and on-line arrangements were used by most authors for correction of the excitation and fluorescence spectra distortion caused by the apparatus[12-17]. Holland and coworkers[18,19] used computer also for correction of intensity decrease of the excitation beam passing through the sample. These authors developed a correction method for an apparatus of their own construction enabling simultaneous measurements of absorption and fluorescence.

The aim of this work was to find a method enabling routine corrections of the inner filter effects using a commercial fluorescence spectrophotometer.

THEORETICAL

A simplified model of experimental arrangement is sketched in Fig. 1. In this model the excitation radiation and a part of the fluorescence radiation (which is scanned by the detector) have the character of a parallel monochromatic beam. The beam width δl_x and δl_m is negligible with respect to the cell width L. The excitation beam and the scanned beam of emitted radiation are mutually perpendicular and incide at right angles on the cell walls. Fluorescence is emitted isotropically, and the re-emission effect is neglected. Furthermore constant sensitivity of the emission part of the apparatus is presumed for the whole scanned sample volume, and the spectral data are corrected with respect to the distortion caused by the apparatus.

With very dilute samples it is not necessary to consider effect of light absorption in the sample; in this case the effect of reflections on the cell walls is also compensated in the cells made of non-absorbing material. Under these conditions the fluorescence

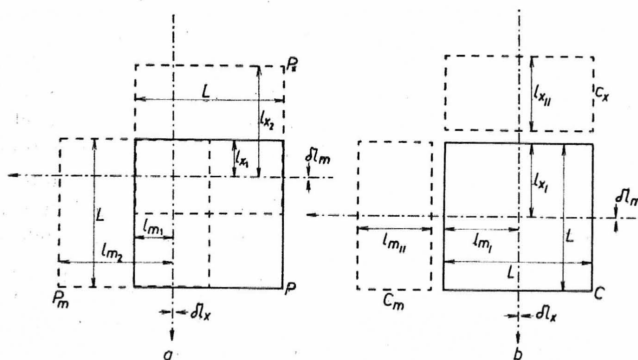

FIG. 1

Experimental Arrangement for (*a*) the Method of Cell Shift and (*b*) the Method of Two Cells

The excitation and emitted beams are represented by dot-and-dash lines.

radiation intensity measured by the detector is expressed by the following relation*

$$I_0 = 2{\cdot}3(k/R_m) \,.\, \varphi_{m_0} \,.\, \Phi_0 \,.\, i_x \,.\, \varepsilon_{x_0} \,.\, c \,.\, \delta l_m \,, \quad (1)$$

$$\varphi_{m_0} = I_{F_0}(\lambda_m) \,.\, \Delta\lambda_m \Big/ \int_{\lambda_{m_1}}^{\lambda_{m_2}} I_{F_0}(\lambda_m) \,.\, d\lambda_m \,.$$

At higher concentrations Eq. (*1*) is transformed into Eq. (*2*) giving explicitly the corrected fluorescence radiation flux I_{corr}.

$$I_{corr} = R_m \,.\, K_x \,.\, K_m \,.\, I = 2{\cdot}3k \,.\, \varphi_m \,.\, \Phi \,.\, i_x \,.\, \varepsilon_x \,.\, c \,.\, \delta l_m \,. \quad (2)$$

The factors K_x and K_m can be expressed by Eq. (*3*) (ref.[11]).

$$K_q = (1 - \varrho_{q_{sa}}^2 \,.\, T_q^2)/[\tau_q \,.\, T_q^{l_q/L}(1 + \varrho_{q_{sa}} \,.\, T_q^{2(L-l_q)/L})] \,. \quad (3)$$

The Eq. (*3*) and the subsequent equations containing the general index q are formally similar for the excitation light ($q = x$) and for the emitted light ($q = m$). The terms τ_q and $\varrho_{q_{ij}}$ (which are characteristical especially for the cell and depend little on refractive index of usual solvents) are given by Eq. (*4*) (ref.[10]).

$$\tau_q = \tau_{q_{ij}} = \tau_{q_{ji}} = \tau^*(1 - f_i)(1 - f_j)/(1 - \tau^{*2} \,.\, f_i \,.\, f_j) \,, \quad (4)$$

$$\varrho_{q_{ij}} = f_i + \tau^{*2}(1 - f_i)^2 f_j/(1 - \tau^{*2} \,.\, f_i \,.\, f_j) \,,$$

where

$$f_i(\lambda) = (n(\lambda) - n_i(\lambda))^2/(n(\lambda) + n_i(\lambda))^2 \,.$$

The transmittance data $T_x(\lambda)$ and $T_m(\lambda)$ necessary for correction of the excitation and emission spectra, respectively, are characteristical for every sample and must be measured by a suitable method prior to calculation of Eq. (*3*). It makes no difference here if the absorption of the sample is due to the fluorescent component or to an admixture or solvent.

The Method of Cell Shift

From Eqs (*2*) and (*3*) a general expression (*5*) can be obtained for the flux ratio of fluorescence radiation I_{x_2}, I_{x_1} and I_{m_2}, I_{m_1} measured for two positions of the sample cell (P_x, P and P_m, P, respectively) (Fig. 1*a*).

* See the List of symbols.

$$I_{q_2}/I_{q_1} = (R_{q_1}T_q^{l_{q2}/L}/R_{q_2}T_q^{l_{q1}/L})\,[(1 + \varrho_{q_{sa}}T_q^{2(L-l_{q2})/L})/(1 + \varrho_{q_{sa}}T_q^{2(L-l_{q1})/L})]\,, \quad (5)$$

which can be transformed to Eq. (*6*) for small $\varrho_{q_{sa}}$ values.

$$T_q \approx t_q[(1 + \varrho_{q_{sa}}t_q^{2(L-l_{q1})/L})/(1 + \varrho_{q_{sa}}t_q^{2(L-l_{q2})/L})]^{L/(l_{q2}-l_{q1})}\,,$$
$$t_q = (R_{q_2}I_{q_2}/R_{q_1}I_{q_1})^{L/(l_{q2}-l_{q1})}\,. \quad (6)$$

$R_{x_2} = R_{x_1}$, because the factor R is independent of the distance l_x.

The Method of Two Cells

In this case a second cell with the measured solution is inserted in the trajectory of the excitation or emitted light (position C_x and C_m, respectively, in Fig. 1*b*). Mathematical analysis considering all possible light reflections leads to complicated expressions which can be simplified in the case of equal transmissivity and reflectivity of walls of the sample cell and the added cell and by neglecting all terms containing ϱ_q^4 and ϱ_q^6.

$$T_q \approx t'_q[(1 - \varrho_{q_{as}}^2 - (\varrho_{q_{sa}}^2 + \varrho_{q_{as}}\cdot\varrho_{q_{sa}}\cdot\tau_q^2)\,(t_q'^2 + t_q'^{2l_{qII}/L}) -$$
$$- \varrho_{q_{sa}}^2\cdot\tau_q^4\cdot t_q'^{2(L+l_{qII})/L})/(1 - \varrho_{q_{sa}}^2\cdot t_q'^2)]^{L/l_{qII}}\,,$$
$$t'_q = (R_{qT}\cdot I_{qT}/\tau_q^2\cdot R_q\cdot I_{qI})^{L/l_{qII}}\,, \quad (7)$$

the terms R_{xT} and R_x being equal.

The both methods for calculation of corrected excitation and fluorescence spectra start from two spectra records. Using the transmissivity values $T_x(\lambda)$ and $T_m(\lambda)$ given in Eqs (*6*) and (*7*) the excitation and fluorescence spectra measured with the arrangement with a thinner absorbing layer (l_{q_1} and l_{q_I} in the method of cell shift and the method of two cells, respectively) are corrected according to Eqs (*2*) and (*3*). Weakening of the excitation beam by the sample absorption during measurement of fluorescence spectra and reabsorption of the fluorescence radiation during measurement of the excitation spectra must only be corrected in some types of measurements as *e.g.* measurement of the quantum yield of fluorescence and subtraction of the fluorescence background of solvent from the sample fluorescence. For the latter purpose, generally four spectral records are needed (the subtraction is carried out with corrected spectra of both sample and solvent). This number can be reduced, if the light absorption by solvent or even by sample is negligible (3 or 2 measurements, respectively).

EXPERIMENTAL

The fluorescence measurements were carried out with a fluorescence spectrophotometer Hitachi Perkin–Elmer MPF-2A extended with a punched tape data output (a digital voltameter Metra

MT 100, a tape punch Daro Cellatron 1215). This arrangement enables the data collection to be synchronized with motion of the excitation or emission monochromator of the apparatus with equidistant intervals of 1 nm. The resulting spectra were analyzed point by point using a computer Hewlett–Packard 2116 B.

The measurement by the method of cell shift was carried out in standard cells 1 × 1 cm in a cell holder of our own construction enabling sample cell to shift between two end stops in direction of both the excitation and the scanned part of the emitted beam. The values l_{x_1} and $l_{m_1}^{eff}$, which are not directly measurable with sufficient accuracy, were estimated and gradually refined so that the shape of excitation spectrum of quinine sulphate in 0·5M sulphuric acid and that of fluorescence spectrum of anthracene in ethanol were independent of concentration within experimental error. From the known distances between the end stops of the cell holder the values l_{x_2} and $l_{m_2}^{eff}$ (in cm) could be calculated ($l_{x_1} = 0{\cdot}25$, $l_{x_2} = 0{\cdot}75$, $l_{m_1}^{eff} = 0{\cdot}36$, $l_{m_2}^{eft} = 0{\cdot}78$).

This procedure proved to be the simplest one and, at the same time, sufficiently accurate. The way of determination of l_{x_1} and $l_{m_1}^{eff}$ represents an inversion of the above-described method of determination of the correction factors K_x and K_m, respectively, and it started from measurements carried out under the optimum conditions (the used compounds have high quantum yields of fluorescence, a series of samples with absorbances within 0·1 to 2·0). All that guarantees that systematical deviation of K_x (or K_m) due to inaccurate determination of l_{x_1} ($l_{m_1}^{eff}$) will never exceed a random deviation of K_x (K_m), in fact, it will be considerably lower. Applicability of this procedure also results from that it gives the effective value $l_{m_1}^{eff}$ for the scanned beam of the emitted radiation which is divergent in the real case. Approximative calculation for the used experimental arrangement shows that the l_m^{eff} values will be greater than the corresponding l_m values by 1 to 2%. The effective values l_m^{eff} involving this correction were used for calculation of K_m.

For the measurements by the method of two cells a standard cell 1 × 1 cm ($l_{xI} = l_{mI} = 0{\cdot}5$ cm) was used along with the cell holder supplied by the manufacturer. The second cell ($l_{xII} = l_{mII} = 0{\cdot}5$ cm) was inserted in the optical trajectory in close vicinity of the sample cell but not in complete contact with the latter to avoid interference phenomena.

The height dimension of the excitation beam was reduced by placing a mask in the inlet window of the excitation monochromator in such a way that the detector might scan full height of the fluorescence trail of the excitation beam passing through the sample. This arrangement suppresses the dependence of the measured part of fluorescence radiation on the change of position of the sample in direction of the excitation beam as far as the inner filter effect is negligible (the real excitation beam is not quite parallel, and its height dimension depends on refractive index of the solution and position of the cell). The described modification also eliminates effect of non-uniform distribution of the light intensity along the height of the excitation beam. The masking of the excitation beam reduced sensitivity of the apparatus roughly by the factor of 2.

Slit width of the monochromators was chosen to make the spectral-dependent quantities in Eqs (*1*) to (*9*) constant within the measured wavelength interval. The real width of beams Δl_x and Δl_m is less than or equal to 0·2 cm with usual slit adjustment ($\Delta\lambda \leqq 10$ nm), and it does not strictly fulfil the condition $\delta l_q \ll L$ which was presumed in Theoretical. For this case it would be necessary to multiply the right-hand side of Eq. (*3*) at least by the approximate relation

$$\alpha_q = 2{\cdot}3(l_q/L) \log T_q/(T_q^{\Delta l_q/2L} - T_q^{-\Delta l_q/2L}) \quad (8)$$

which respects the effect of Δl_q on the beam reduction due to light absorption and leaves unchanged the correction of reflexion effects. Equations (*2*), (*5*), (*6*) and (*7*) would have to be correspondingly changed. However, the value α_q differs from unity by less than 1% even in the least favourable case ($\Delta l_q = 0{\cdot}2$ cm, $\varepsilon_q c = 2$ cm^{-1}) so that it was not necessary to consider the factor α_q.

With respect to divergence of the emitted beam it is necessary to make correction for the effect of refractive index of the sample. If the detector scans full height of the fluorescence trail of the excitation beam, then R_m is given by Eq. (9) (ref.[20]) where $F = 3{\cdot}7$ cm for the apparatus used.

$$R_m = [n_s F - l_m(n_s - 1)]/F\,. \tag{9}$$

RESULTS AND DISCUSSION

The concentration dependence of fluorescence spectrum of Rhodamine B in ethanol (Fig. 2) illustrates the correction of reabsorption. The fluorescence intensity decrease in the short-wave part of the uncorrected spectrum due to increasing concentration of the sample (10^{-6}M, $2 \,.\, 10^{-6}$M, $4 \,.\, 10^{-6}$M, $6 \,.\, 10^{-6}$M, $8 \,.\, 10^{-6}$M, 10^{-5}M) can be seen in Fig 2*a*, whereas Fig. 2*b* gives the average curve obtained by correction of the spectra for the reabsorption effect. The corrected spectra agree with the spectrum which can be obtained in limit case of very dilute Rhodamine B solution. Length of the vertical ordinates characterizes standard deviation of the resulting spectral data.

Subtraction of the solvent fluorescence superposed on fluorescence spectrum of a sample is shown in Fig. 3. In this model case the solvent consisted of $2 \,.\, 10^{-5}$M anthracene solution in 9 : 1 mixture of ethanol and 0·5M sulphuric acid. In this solvent quinine sulphate ($2 \,.\, 10^{-5}$M) was dissolved as the sample. From the figure it can be

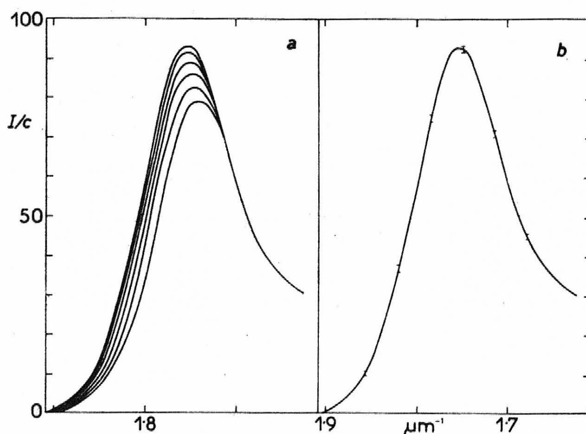

FIG. 2

Fluorescence Intensity Lowering of Rhodamine B in Short-Wave Part of Spectrum due to Reabsorption Depending on Concentration (*a*), Average Spectral Curve after Correction for Reabsorption (*b*)

Length of the vertical lines characterizes standard deviation of the resulting spectral data.

seen that the non-corrected fluorescence spectrum of solvent (curve *1*) has, in the region of the first vibronic band of anthracene, a higher intensity than the non-corrected fluorescence spectrum of the sample (curve *2*). Simple subtraction of fluorescence intensity of solvent from that of the sample would give negative values in this case. The fluorescence spectrum of the sample obtained as a difference of the corrected spectra (curve *3*) lacks these deviations towards negative values and forms a smooth curve without oscillations characteristic for fluorescence spectrum of anthracene, which otherwise would indicate that the subtraction was carried out incorrectly. This spectrum also agrees with the fluorescence spectrum of quinine sulphate measured in the solvent mixture ethanol +0·5M sulphuric acid (9 : 1).

Magnitude of random error of the corrections is affected decisively by magnitude of standard deviation of determination of the correction factors K_x. K_m. Estimates of this deviation can advantageously start from Eq. (*3*) which can be simplified to Eq. (*10*) by neglecting reflexion effects and putting $\tau_q = 1$.

$$K_q \approx 1/T_q^{l_{q1}/L} . \qquad (10)$$

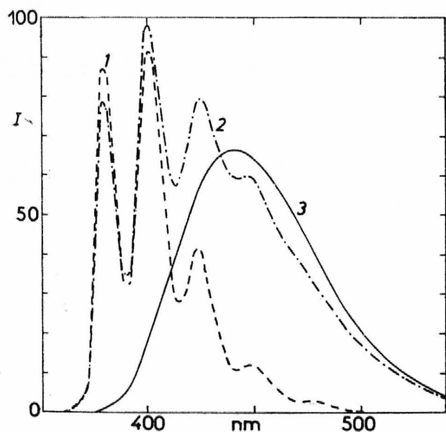

FIG. 3

Subtraction of Solvent Fluorescence from Sample Fluorescence (Model System)

1 the solvent fluorescence spectrum (solution of anthracene in mixture ethanol —0·5M sulphuric acid 9 : 1), *2* the fluorescence of quinine sulphate in the given solvent, *3* the resulting fluorescence spectrum of quinine sulphate obtained as difference of the spectra *1* and *2* after correction for inner filter effects.

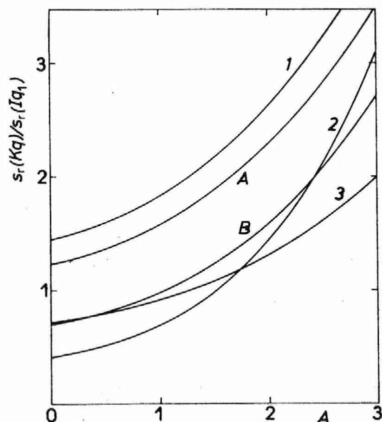

FIG. 4

Dependence of Ratio $s_r(K_q)/s_r(I_{q1})$ on Sample Absorbance in 1 cm Thickness ($s_r(K_q)$ Relative Standard Deviation of Correction Factor, $s_r(I_{q1})$ Relative Standard Deviation of Fluorescence Intensity)

1 $l_{q1} = 0·4$, $l_{q2} = 0·8$; *2* $l_{q1} = 0·2$, $l_{q2} = 0·9$; *3* $l_{q1} = 0·2$, $l_{q2} = 0·6$; *A* $l_{q1} = 0·36$; $l_{q2} = 0·78$; *B* $l_{q1} = 0·25$, $l_{q2} = 0·75$ (l given in cm).

With respect to the relation $s(I_{q2}) = s(I_{q1})(I_{q2}/I_{q1})^{1/2}$ and to Eqs (6) and (7) the standard deviation of the correction factor is given by Eq. (11). A relation of the same form is valid for the both methods (assuming $l_{q1} = l_{qI}$, $l_{q2} = l_{qI} + l_{qII}$, $I_{q1} = I_{qI}$, $I_{q2} = I_{qT}$).

$$s(K_q) \approx s_r(I_{q1}) [l_{q1}/(l_{q2} - l_{q1})] T_q^{-l_{q1}/L} (1 + T_q^{(l_{q2}-l_{q1})/L})^{1/2} \qquad (11)$$

For various l_{q1} and l_{q2} values Fig. 4 gives the sample absorbance dependence of the ratio of relative standard deviation of the correction factor $s_r(K_q)$ *vs* that of the fluorescence intensity measurement $s_r(I_{q1})$. The graph shows rapid increase of $s_r(K_q)$: : $s_r(I_{q1})$ with increasing l_{q1}. Increase of the difference $(l_{q2} - l_{q1})$ causes a decrease of $s_r(K_q)/s_r(I_{q1})$ in the region of low absorbance values. In the region of higher absorbances, on the contrary, it causes the ratio $s_r(K_q)/s_r(I_{q1})$ to increase due to rapid increase of the relative deviation $s_r(I_{q2})$. Magnitude of $s_r(K_q)$ is comparable with $s_r(I_{q1})$ in the region of not very high absorbance values, being double of $s_r(I_{q1})$ first at $A_q > 2$ for the arrangement used. As the used difference is $(l_{q2} - l_{q1}) \approx 0{\cdot}5$ cm, the inner filter effects can successfully be corrected for $I_{q2}/I_{q1} \geqq 0{\cdot}1$. The correction factors K_q can reach at the same time the magnitude of several units.

There are two advantages in the possibility of direct T_x and T_m measurement with fluorescence spectrophotometer as compared with the alternative method of obtaining the transmissivity data by measurement of absorbtion spectrum with a specialized apparatus (UV VIS spectrophotometer). The method avoids possible error due to different spectral composition of light beams in the two spectrometers (various adjustments of monochromators, slit width *etc.*) Furthermore measurement with a single apparatus means acceleration and simplification of the procedure. The method of cell shift is experimentally the simplest one of all the procedures discussed, and it is given preference before the method of two cells which is used in experimental arrangements not allowing to change the sample cell position.

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LIST OF SYMBOLS

c	concentration of the fluorescent component of sample
f_i	the reflected part of light in simple perpendicular reflection on the interphase between the cell wall and medium with the refractive index n_i
F	distance between the axis of excitation beam and centre of the closest lens along the trajectory of the emitted light towards the detector
i_x	luminous flux of the excitation beam at the point of incidence on the cell (number of light quanta per time unit)
I	the part of luminous flux I_F incident on the detector
I_{corr}	the I value corrected for the inner filter effects and refraction

Symbol	Meaning
I_F	luminous flux of the fluorescence radiation emitted by the measured part of the sample in the whole space within the wavelength interval scanned by the emission part of the spectrophotometer (number of quanta per time unit)
I_{mT}, I_{xT}	the part of the flux I_F incident on the detector, if the emitted or the excitation light is reduced by passage through the second sample cell (C_m or C_x, respectively)
I_{m_1}, I_{x_1}	the part of the flux I_F incident on the detector, if the sample cell is in the position P
I_{m_2}, I_{x_2}	the part of the flux I_F incident on the detector, if the sample cell is in the position P_m and P_x, respectively
I_{mI}, I_{xI}	the part of the flux I_F incident on the detector in measurement in the sample cell C
k	the apparatus constant giving the relative part of the flux I_F incident on the detector at $n_s \doteq 1$
K	the correction factor for effects of light absorption and reflection inside the sample cell
l_m^{eff}	effective l_m value for divergent emitted light
l_{m_1}, l_{m_2}	the trajectory of fluorescence light through the sample towards the detector, if the sample cell is in the position P and P_m, respectively
l_{x_1}, l_{x_2}	the trajectory of the excitation beam through the sample to the point where fluorescence is scanned (the sample cell in the position P and P_x, respectively)
l_{mI}, l_{xI}	the trajectory of the fluorescence light through the sample towards the detector and that of the excitation beam through the sample to the point where fluorescence is scanned in the sample cell C, respectively
l_{mII}, l_{xII}	thickness of the cells C_m and C_x inserted in the trajectory of the emitted and the excitation light, respectively
L	thickness of the square cell
n	refractive index of the material of the cell walls
n_a	refractive index of air
n_s	refractive index of sample
R	correction factor for the effect of refractive index of the sample
s	standard deviation
s_r	relative standard deviation
t, t'	the approximative transmissivity of the sample (layer thickness L) calculated with the neglect of reflexions
T	the sample transmissivity (layer thickness L) corrected for the light reflections inside the sample cell
α	the factor correcting the effect of non-negligible thickness Δl_m and Δl_x
δl_m, δl_x	thickness of the idealized beam scanned by the emission part of the spectrophotometer and the idealized excitation beam, respectively
Δl_m, Δl_x	thickness of the real beam scanned by the emission part of the spectrophotometer and the real excitation beam in the measured sample part, respectively
$\Delta\lambda$	the spectral band width transmitted through the monochromator
ε	absorptivity of fluorescent component of the sample
λ	wavelength
λ_{m_1}, λ_{m_2}	limits of the fluorescence spectrum
ϱ_{ij}	summary reflectivity when light passes from medium of refractive index n_i through cell wall in medium of refractive index n_j
τ^*	transmissivity of cell wall not involving effect of reflexions
τ	summary transmissivity of cell wall involving effect of reflexions
φ	normalized spectral intensity of fluorescence
Φ	quantum yield of fluorescence

Indexes

a	air
i, j	general indexes standing for a or s
m	the emitted light
q	general index standing for m or x
s	sample
x	the excitation light
T	measurement with a second inserted cell
0	very dilute solution
1, 2	position with a weaker and a stronger inner filter effect, respectively (in the method of cell shift)
I, II	the sample cell and the inserted cell, respectively (in the method of two cells)

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