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Technical Notes

Correction of Inner Filter Effect in Mirror Coating Cells for Trace Level Fluorescence Measurements

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The sensitivity of the spectrofluorometric technique can be improved by a factor of about 3.6 using a mirror coating cell. In the case of a large working range, the nonlinear relationship due to the absorbance of solutions between concentration of the analyte of interest and fluorescence intensity (called inner filter effect) must be corrected. This paper suggests a universal inner filter correction equation based on the physical absorbance phenomenon of a mirror coating cell that only depends on the solution absorbance spectra and the cell parameters. These parameters are determined with rhodamine b standard solutions and a simplex method-based mathematical fitting.The methodology has been successfully applied to the correction of classical and synchronous spectra in absorbent media. The partial least squares (PLS) quantification of a mixture of trace levels (∼1−10 μg L^{-1}) of six polycyclic aromatic hydrocarbons (PAHs) by synchronous fluorescence is possible, even in an absorbent matrix. This simple method allows extension of the analytical field of fluorescence quantification to a large working range in absorbent solutions.

Fluorescence spectroscopy is an analytical method widely used in chemical and biochemical research laboratories. This sensitive technique is not as easy to use as one may expect.[1,2] In fact, fluorescence spectroscopy is subject to instrumental and photophysic variations. Many studies have been performed to correct these artifacts. In addition, one of the most important variations highlighted is the inner filter effect due to the solutions absorbance. Few formula corrections have been established in order to linearize the electric signal produced by the spectrophometer versus solution concentration.

The fluorescent components, at trace levels in natural or biological samples, are often present in a complex matrix that can strongly absorb incident light. When available sample volumes are small and when dilution may change molecules' conformation, bonding, solvatation, and degree of association, the use of a reduced fluorescent cell volume is needed. The decrease in optical path length restricts the number of excited molecules and then the fluorescence intensity. The use of mirror coating cells allows limitation of this constraint and an increase in the fluorescence intensity by a factor close to 3.6. The increase in fluorescence intensity leads to a decrease of the detection limit with the same signal-to-noise ratio. Unfortunately with mirror coating cells, inner filter effect is enhanced (10% of attenuation for 0.032 both excitation and emission absorbance).

Nowadays, two kinds of inner filter corrections are available in the literature: (1) an equation developed from physical phenomena[3−7] and (2) empirical equations.[8−11] The major disadvantage in the use of empirical equations is their lack of flexibility. These equations are well adapted to the fluorescence intensity correction at a fixed wavelength and for a given matrix. The inner filter correction of a classical or synchronous fluorescence spectrum is difficult to do and needs numerous experimentations.

The primary goal of this work is to propose a universal equation of inner filter correction for fluorescence measurement. This equation is an extension of the results of McDonald et al.[5] and has been developed from the physical phenomena. The suggested correction factor only depends on the absorbance values for excitation and emission wavelengths after determination of the cell geometrical parameters. A methodology for the determination of cell parameters using rhodamine b as standard

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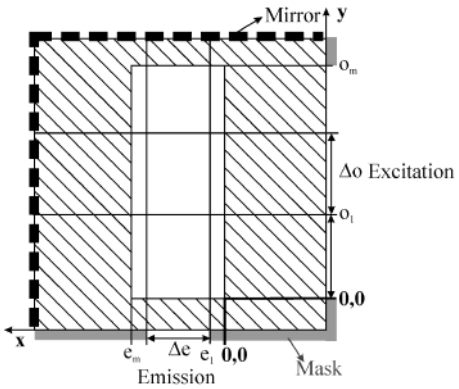

Figure 1. Cell parameters for the mirror coating cell.

component is then proposed. Two applications of this correction illustrate the efficiency of this equation.

EXPERIMENTAL SECTION

Reagents. Quinine sulfate dihydrate purum was purchased from Fluka, and rhodamine b, from Sigma-Aldrich. These two standards were used without any further purification.

UHQ water was prepared from a Maxima system (Elga). Genapol X-80 was purchased from Fluka.

Polyaromatic compounds were purchased from Supelco, and methanol (analytical grade), from Merck. Stock standard solutions of benzo[*a*]pyrene (BaP), benzo[*k*]fluoranthene (BkF), benzo[*ghi*]-perylene (BghiP), benzo[*b*]fluoranthene (BbF), fluoranthene (Fla), and indeno[1,2,3-*cd*]pyrene (IP) were prepared by dissolving the pure solid in methanol. Dilute solutions were prepared from the stock solutions.

Instrumentation and Software. Absorbance spectra were recorded on a HP 8453 spectrometer.

A homemade spectrofluorometer using a computerized system for spectral acquisition and data processing was developed.[12] Spectra of the samples were obtained with a 1000 W Hg/Xe lamp using 2-mm excitation slit widths and 1.5-mm emission slit widths for 4 and 0.6 nm band-pass, respectively.

The detector used was a 9789 QB (Thorn EMI) photomultiplier with a voltage adjustable from 500 to 1000 V, allowing the analysis of a wide concentration range.

Figure 1 shows the shape of the cell support, the mask, and the mathematical notations used in this work. The cell support consisted of an *X*, *Y* combination positioner table.

A mirror coating cell[13] (Hellma: Mullheim, $e_m = 0.4$ cm $o_m = 1$ cm) presented in Figure 1 and three excitation beam positions, P_1, P_2, P_3, were used for both optimization and test.

The simplex method used for correction factor calculations was developed in Visual Basic.

Procedure and Fluorescence Conditions for Standard Solutions Measurements. The rhodamine b solutions were prepared in a 0.01 M NaOH solution and used in a 0.001 to 20 mg L^{-1} concentration range. Excitation was fixed at 546 nm, and emission measurements were recorded between 560 and 600 nm in 0.5-nm steps. Single fluorescence spectra were recorded at 571 nm.

For parameter optimization, only the 571 nm fluorescence intensity was used. Quinine sulfate solutions were prepared in 0.5 mol L^{-1} H_2SO_4 and used in a 0.1−300 mg L^{-1} concentration range. Excitation spectra were recorded at 345.2 nm, and emission spectra were recorded at 456.0 nm.

Preparation and Analysis of PAH Mixture. Each PAH mixture was prepared in an aqueous solution of UHQ water containing 1% (v/v) of methanol, 0.1% (w/w) of Genapol X-80, and an adequate concentration of PAH. Potassium dichromate was added to obtain the inner filter effect. The synchronous spectrum of each mixture was recorded at a wavelength increment ($\Delta\lambda$) of 105 nm between 380 and 480 nm using 1-nm steps.

CORRECTION FORMULA

Technical and Chemical Considerations. (1) The photomultiplier response is directly proportional to the number of photons emitted by the sample. This property was verified, and the photomultiplier response was corrected for all high voltages used by observation of the diffusion beam from a frosted suprasil quartz piece. (2) The working range was the Beer Lambert law range validity in order to check the exponential absorption of incident photons in the whole irradiated cell volume. For rhodamine b, the working range was experimentally determined, and the proportionality between absorbance values ($\lambda = 546$ nm) and concentration was considered for concentrations lower than 9.50 mg L^{-1} ($\epsilon_{546} = 90\,000 \pm 1500$ mol^{-1} L cm^{-1} and $\epsilon_{571} = 41\,000 \pm 1500$ mol^{-1} L cm^{-1}). (3) The cell parameters were determined with fluorescent components that are not sensitive to photochemical reactions. In addition, for the duration of any observation period, the quanta fluoresced by any absorbing fluorophore species were linearly related to the quanta absorbed by this species. These constraints justify the selection of rhodamine b as an autoabsorbance standard, acting as primary and secondary inner filter with a quantum efficiency of $Ø = 0.65$.[14]

Correction Formula. Absorbance is negligible for very dilute solutions. The fluorescence intensity I_F of any point of the cell is related to the exitation radiation intensity, I_{ex}, according to the following relation,

$$I_F = kc\phi I_{ex} \tag{1}$$

where k is a proportionality constant, c is the fluorescent component concentration, and ϕ is the fluorescence quantum efficiency.

Figure 1 shows the cell geometry and the related parameters. The total fluorescence intensity of the irradiated cell volume can be expressed as

$$I_F = kc\phi I_{ex}\Delta h\Delta e\Delta o \tag{2}$$

where I_{ex} is the exitation radiation intensity, I_F is the fluorescence intensity of any point of the cell, ϕ is the fluorescence quantum efficiency, Δh is the beam height, and Δe and Δo are the widths of excitation and emission beams, respectively.

For a mirror coating cell, the incident excitation and emission beams can be partially reflected in the cell and then affect

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fluorophores. The incident radiation intensity is linked to the reflected radiation intensity by the following expression,

$$I_{\text{reflected}} = I_{\text{incident}}\, f_\lambda \tag{3}$$

where f_λ is the reflectance for a given wavelength.

The excitation and emission intensities, I_{ex} and $I_{\text{F}_{\text{obs}}}$, are the sums of the direct and reflected radiations and are defined as follows:

$$I_{\text{ex}} = I_0(1 + f_{\lambda_{\text{ex}}}) \tag{4}$$

$$I_{\text{F}_{\text{obs}}} = I_{\text{F}}(1 + f_{\lambda_{\text{em}}}) \tag{5}$$

Thus, eq 2 + eq 4 + eq 5:

$$I_{F_{\text{obs}}} = kc\phi I_0(1 + f_{\lambda_{\text{ex}}})(1 + f_{\lambda_{\text{em}}})\Delta h \Delta e \Delta o \tag{6}$$

Equation 6 is valid for a very dilute fluorophore solution, without any significant absorption at the emission and excitation wavelengths.

For a more concentrated solution, the excitation and emission radiations are attenuated by the solution absorption. This attenuation is called primary inner filter effect for the excitation radiation absorption and secondary inner filter effect for the emission radiation absorption.

The absorption of a monochromatic radiation by a solution with a thickness x follows the Beer−Lambert law:

$$kxc = \ln\left(\frac{I_{\text{incident}}}{I_x}\right) \tag{7}$$

If $\alpha = kc$ (constant for a given solution),

$$\alpha x = \ln\left(\frac{I_{\text{incident}}}{I_x}\right) \tag{8}$$

and then

$$I_x = I_{\text{incident}} e^{-\alpha x} \tag{9}$$

This equation shows that for both a fluorophore and a chromophore, the probability of absorbing a photon depends on x. Equally, for a photon emitted by the fluorophore, the probability of exiting the cell measurement depends on the y position of the fluorophore (Figure 1).

In a mirror coating cell, the fluorophore is excited both by the direct excitation beam and by the reflection of the excitation beam. Then, the total intensity absorbed by the molecule is the sum of the direct intensity, I'_{ex}, and the reflected intensity, I''_{ex}.

Before hitting the molecule, the beam goes trough x cm of solution, and the direct intensity is expressed as

$$I'_{\text{ex}} = I_0 e^{(-\alpha_{\text{ex}} x)} \tag{10}$$

For the reflected light, the incident beam goes through e_{m} cm of solution before reaching the mirror. This beam is partially reflected, and the reflected light I_{R} is

$$I_{\text{R}} = f_{\lambda_{\text{ex}}} I_{\text{ex}_{\text{m}}} = f_{\lambda_{\text{em}}} I_0 e^{-\alpha_{\text{ex}} e_{\text{m}}} \tag{11}$$

where $I_{\text{ex}_{\text{m}}}$ is the intensity hitting the mirror.

The reflected light goes through $(e_{\text{m}} - x)$ cm of solution before hitting the fluorophore. The total intensity hitting the molecule is then equal to

$$I_{\text{ex}} = I'_{\text{ex}} + I''_{\text{ex}} = I_0(e^{-\alpha_{\text{ex}} x} + f_{\lambda_{\text{ex}}} e^{-\alpha_{\text{ex}} e_{\text{m}}} e^{-\alpha_{\text{ex}}(e_{\text{m}} - x)}) \tag{12}$$

By deductive reasoning, the total fluorescence intensity $I_{\text{F}_{\text{obs}}}$ emitted by a single molecule is

$$I_{\text{F}_{\text{obs}}} = I'_{\text{em}} + I''_{\text{em}} = I_{\text{F}}(e^{-\alpha_{\text{em}} y} + f_{\lambda_{\text{em}}} e^{-\alpha_{\text{em}} o_{\text{m}}} e^{-\alpha_{\text{em}}(o_{\text{m}} - y)}) \tag{13}$$

By combining eqs 1, 12, and 13, the real fluorescence of one point of a cell is

$$I_{\text{F}_{\text{obs}}} = kc\phi I_0(e^{-\alpha_{\text{ex}} x} + f_{\lambda_{\text{ex}}} e^{-\alpha_{\text{ex}} e_{\text{m}}} \text{e}^{-\alpha_{\text{ex}}(e_{\text{m}} - x)}) \times (e^{-\alpha_{\text{em}} y} + f_{\lambda_{\text{em}}} e^{-\alpha_{\text{ex}} o_{\text{m}}} e^{-\alpha_{\text{em}}(o_{\text{m}} - y)}) \tag{14}$$

For the irradiated volume cell, the total fluorescence intensity $I_{\text{F}_{\text{obs}}}$ is

$$I_{\text{F}_{\text{obs}}} = kc\phi I_0 \Delta h \int_{e_1}^{e_1 + \Delta e} [(e^{-\alpha_{\text{ex}} x} + f_{\lambda_{\text{ex}}} e^{-\alpha_{\text{ex}} e_{\text{m}}} e^{-\alpha_{\text{ex}}(e_{\text{m}} - x)})\, \text{d}x] \times \int_{o_1}^{o_1 + \Delta o} [(e^{-\alpha_{\text{em}} y} + f_{\lambda_{\text{em}}} e^{-\alpha_{\text{em}} o_{\text{m}}} e^{-\alpha_{\text{em}}(o_{\text{m}} - y)})\, \text{d}y] \tag{15}$$

A correction factor Cf is derived from the physical model and is expressed as the ratio between the observed fluorescence and the ideal fluorescence.

After mathematical conversions, the correction coefficient can be written as

$$\text{Cf} = \frac{I_{\text{F}_{\text{ideal}}}}{I_{\text{F}_{\text{obs}}}} = \frac{2{,}3^2 A_{\text{ex}} A_{\text{em}} \Delta e \Delta o (1 + f_{\lambda_{\text{ex}}})(1 + f_{\lambda_{\text{em}}})}{AB} \tag{16}$$

with

$$A = (10^{-A_{\text{ex}} e_1}(1 - 10^{-A_{\text{ex}} \Delta e}) - f_{\text{ex}}(10^{-A_{\text{ex}} e_{\text{m}}})^2 10^{A_{\text{ex}} e_1}(1 - 10^{A_{\text{ex}} \Delta e})) \tag{17}$$

$$B = (10^{-A e_{\text{em}} o_1}(1 - 10^{-A_{\text{em}} \Delta o}) - f_{\text{em}}(10^{-A_{\text{em}} o_{\text{m}}})^2 10^{A e_{\text{m}} o_1}(1 - 10^{A_{\text{em}} \Delta o})) \tag{18}$$

where A_{ex} and A_{em} are the absorption values at the excitation and the emission wavelengths, respectively, for a 1-cm cell. This

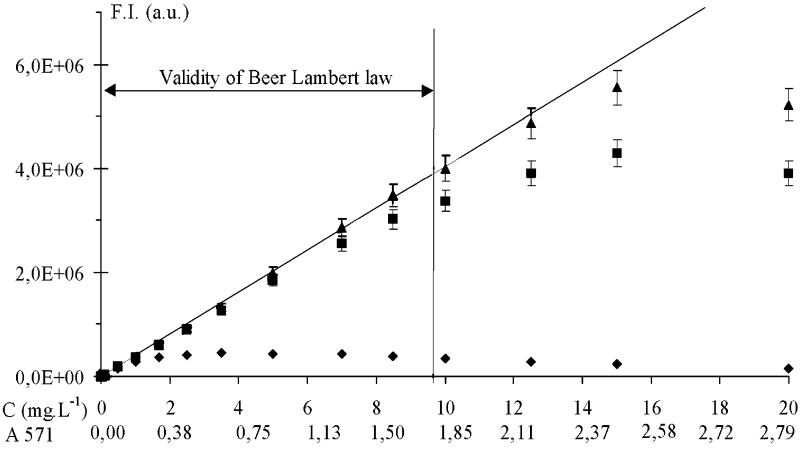

Figure 2. Fluorescence Intensity (λ_{ex} 546 nm, λ_{em} 571 nm, position P1) versus rhodamine b concentration and absorbance at 571 nm (A571) without inner filter correction (◆), with inner-filter correction for measured parameters (■), or optimized parameters (▲).

Table 1. Measured and Calculated Cell Parameters for Three Excitation Beam Positions (P1, P2, P3)[a]

	meas values ± 0.03 cm			calcd values ± 0.005 cm		
parameter	P1	P2	P3	P1	P2	P3
Δe	0.34	0.34	0.34	0.345	0.340	0.330
e_1	0.04	0.04	0.04	0.070	0.055	0.035
Δo	0.32	0.32	0.32	0.320	0.315	0.315
o_1	0.10	0.32	0.55	0.125	0.315	0.515
S	6.9×10^{11}	4.6×10^{11}	6.9×10^{11}	2.5×10^{10}	1.0×10^{10}	1.4×10^{10}

[a] Cell: semimicro, $e_m = 0.4$ cm, $o_m = 1.0$ cm, 2 mirrors

correction factor also depends on the cell parameters e_1, e_m, Δe, o_1, o_m, Δo, $f_{\lambda_{ex}}$, and $f_{\lambda_{em}}$.

For a nonmirror coating cell, $f_{\lambda_{ex}}$ and $f_{\lambda_{em}}$ are equal to 0. Equation 16 is then the same as the one proposed by MacDonald et al. under the same conditions.[5]

RESULTS AND DISCUSSION

Determination of the Analytical Parameters. Rhodamine b was used for the analytical parameters optimization. The ideal fluorescence F_{ideal} (no filter effect) was determined by linear regression with five very dilute solutions in a concentration range of 0.001−0.05 mg L^{-1}, without inner filter effect and for an absorbance <0.010 at 546 nm. Seventeen additional solutions with higher rhodamine b concentrations were used to underscore the inner filter effect. The fluorescence measurements of these 22 solutions were carried out at three excitation beam positions: o_1 = 0.10, 0.32, and 0.55 cm, named P1, P2, and P3 ,respectively).

The other geometrical parameters of the analytical system were measured with a precision of 0.03 cm: $\Delta e = 0.34$ cm, $e_1 = 0.04$ cm, $e_m = 0.40$ cm, $\Delta o = 0.32$ cm, and $o_m = 1.00$ cm, with e_m and o_m given by the manufacturer, Hellma, Mullheim.

Figure 2 shows the raw and corrected fluorescence intensities of rhodamine b solutions with measured parameters. The confidence interval for the corrected fluorescence value was determined from the standard deviation of three measurements. The use of measured parameters does not perfectly fit the ideal fluorescence and does not give the optimum correction. To mathematically optimize the analytical parameters of the solutions respecting the Beer Lambert law, the error sum of square S was used and calculated as follows,

$$S = \sum_{i=1}^{n} (F_{ideal(i)} - F_{corr(i)})^2 \qquad (19)$$

where i is the number of samples, $F_{ideal(i)}$ is the ideal fluorescence calculated for the sample i by linear regression, and $F_{corr(i)}$ is the observed fluorescence intensity corrected with the eq 16.

The graphical representations of S versus the geometrical parameters Δe, e_1, Δo, and o_1 show a strong S variability, even for a weak variation. The S function cannot be modeled using a simple polynomial function, but the simplex method permits easily achieving an accuracy <0.01 cm for the geometrical parameters.

The optimization is based on a simplex method similar to the method outlined by Deming and Morgan.[15] The simplex method minimizes the S function by varying e_1, Δe, o_1, and Δo. The following constraints are then requested.

$$0 \text{ cm} < \Delta e + e_1 < e_m + 0.05 \text{ cm}$$

$$0 \text{ cm} < \Delta o + o_1 < o_m + 0.05 \text{ cm}$$

The mathematical space studied is slightly superior to the real cell surface in order to analyze the response obtained near the edges.

Table 1 shows the measured and the simplex optimized parameters for P1, P2, and P3 excitation beam positions. The

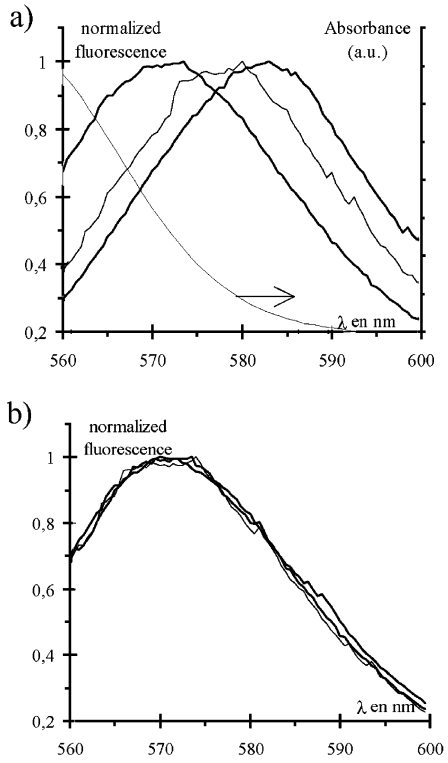

Figure 3. Rhodamine b spectra (excitation 546 nm) for (gray line) 0.01, (solid line) 3.5, and (bold, solid black line) 9.0 mg L^{-1} of rhodamine b (a) without inner filter correction (b) with inner filter correction using optimized parameters.

calculated dimensions of the fluorescence window are in good agreement with the measured values. However, the use of optimized parameters instead of measured parameters leads to an *S* average decrease of 98% and an improved inner filter correction (Figure 2). The deviation observed above 9.50 mg L^{-1} shows the limits of this correction that are valid only in an absorbance range in agreement with the Beer−Lambert law.[16] The same results are obtained using nonmirror coating and semimirror cells.

Furthermore, the cell parameters are determined with quinine sulfate in the UV domain at the P1 position. Because of the absorbance spectrum of this molecule, only the primary filter effect is observed ($\epsilon_{345} = 9500 \pm 100$ mol^{-1} L cm^{-1} and $\epsilon_{455} = 0$ mol^{-1} L cm^{-1}). The optimized parameters with quinine sulfate, $\Delta e = 0.345$ cm, $e_1 = 0.080$ cm, $\Delta o = 0.315$ cm, and $o_1 = 0.120$ cm, are in excellent agreement with those obtained with rhodamine b in the same conditions. The cell parameters are independent of the optimization standards. However, the o_1-optimized value obtained with compounds such as quinine sulfate shows only primary inner filter effect and can be imprecise. The use of a fluorescence standard such as rhodamine b, showing both primary and secondary inner filter effect, leads to a better determination of the whole parameters.

When the cell parameters are determined at a given position using the previous methodology, the correction factor only depends on sample absorbance values.

Spectral Correction. Equation 16 allows the correction of both a single fluorescence measurement for excitation or emission at a single wavelength and a whole spectrum. Figure 3 shows the spectra obtained for rhodamine b with and without correction. The raw spectra show a wavelength shift of the maximum fluorescence intensity. This phenomenon is attributed to the rhodamine b absorbance in this wavelength region. This shift is well corrected for rhodamine b concentrations up to 9.50 mg L^{-1}.

Analysis of 6 PAH Samples in Strong Absorbing Matrix. Analysis of trace level mixtures of 6 PAH compounds (BaP, BbF, BkF, BghiP, IP, Fla) were carried out by synchronous fluorescence ($\Delta\lambda = 105$ nm) in micellar media using multivariate analysis (PLS). Fluorescence measurements and multivariate analysis were performed according to procedures previously described.[17] Inner filter effect was then obtained by the use of potassium dichromate as external chromophore model. This compound was chosen for its spectral absorbance properties, allowing for primary and secondary inner filter effects (Figure 4). Furthermore, potassium dichromate does not interfere with the experimental micellar media and does not produce dynamic and static quenchings with PAHs. This compound was added just before the analysis to avoid potential slow oxidation reactions with PAHs or surfactant. Six mixtures with different amounts of potassium dichromate (absorbance range at 350 nm, 0−1.82) were then prepared.

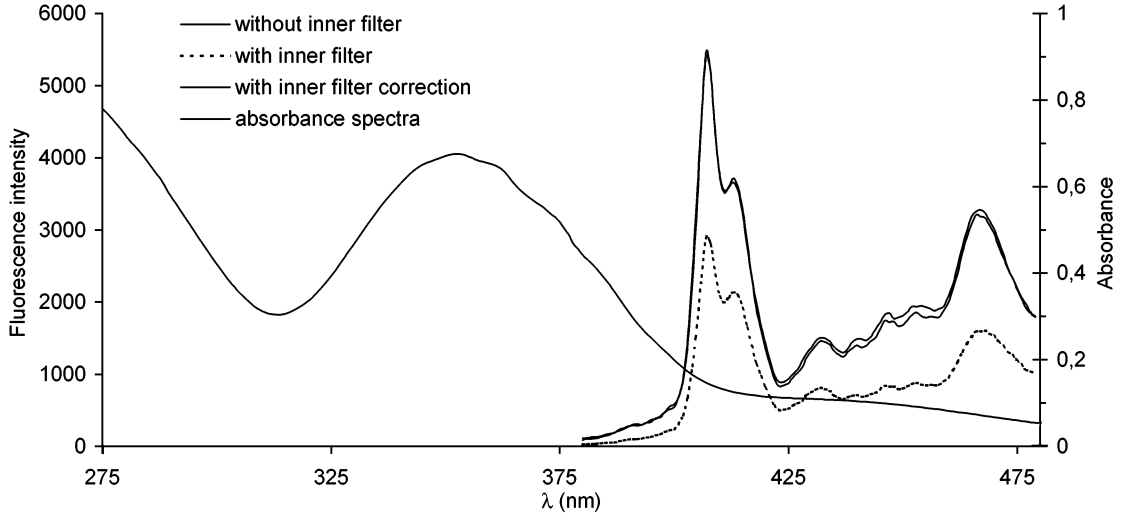

Figure 4. Fluorescence and absorbance spectra of PAH mixture with 0.16 mmol L^{-1} potassium dichromate.

Table 2. PAH Concentrations at Different Potassium Dichromate Concentrations Determined after Spectra Correction

	$[K_2Cr_2O_7]$, mmol L^{-1}					
[PAH], μg L^{-1}	0	0.083	0.125	0.16	0.25	0.5
BaP, ±0.05	0.7	0.69	0.68	0.64	0.61	0.69
BkF, ±0.02	0.3	0.29	0.30	0.30	0.28	0.27
BbF, ±0.19	1.9	2.04	1.88	1.75	1.24	0.9
BghiP, ±0.27	3.2	3.20	3.43	3.35	3.24	3.16
IP, ±1.62	16.1	14.72	14.36	17.44	15.63	14.04
Fla, ±1.05	16.3	15.11	16.92	15.98	16.03	13.11

The calibration was performed with pure compound mixtures in nonabsorbing solutions. For absorbent solutions, the concentration cannot be determined without inner filter correction. Figure 4 shows the spectra obtained with and without $K_2Cr_2O_7$ before and after correction. The corrected synchronous fluorescence spectra superimpose on the ideal spectra, and the quantification is possible. Table 2 summarizes the PAH calculated concentrations for the 6 mixtures after inner filter correction of fluorescence spectra. The confidence interval was determined with a single measurement on the basis of a linear regression of predicted and real concentrations of calibration mixtures.

For a potassium dichromate concentration lower than 0.25 mmol L^{-1} (absorbance at 350 nm < 0.95), predicted concentration values of the 6 HAP mixtures are included in the confidence interval.

For higher potassium dichromate concentrations, although concentrations are still in the Beer Lambert law range validity, considerable deviations are observed. In this case, observed fluorescence intensity is low with regard to equipment background noise. The correction factor increases both the fluorescence signal and the background noise, distorting the baseline. The PLS calibration method used with nonabsorbing solutions only takes into account the constant background level. Noise amplification on the spectrum leads then to a progressive analysis deterioration.

A universal inner filter correction equation has been validated for parallel side cells with and without mirror coating. After determination of cell parameters using rhodamine b as standard solution this correction only depends on the samples' absorbance spectra.

Maximum absorbance, from which quantification becomes imprecise, depends on the Beer Lambert law validity and, especially, on the signal/noise ratio.

The correction can be used for trace level fluorescent compounds' determination in absorbent matrix from environmental and biological samples, while considering photochemical reactions that are occurring. In this specific analysis, the increase in dynamic range and sensitivity can be useful and justifies the use of mirror coating cells. Finally, the work described in this paper proposes an improvement in fluorescence for trace level quantification in absorbent matrix by the use of mirror coated cell and inner filter correction.

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