Experimental Correction for the Inner-filter Effect in Fluorescence Spectra

Mikael Kubista, Robert Sjöback, Svante Eriksson and Bo Albinsson*

Department of Biochemistry and Biophysics, Chalmers University of Technology, S-412 96 Gothenburg, Sweden

Recorded fluorescence intensity is in general not proportional to sample concentration owing to absorption of the incident and emitted light passing through the sample to and from the point inside the cell where the emission is detected. This well known inner-filter effect depends on sample absorption and on instrument geometry, and is usually significant even in samples with rather low absorption (the error is about 8% at an absorbance of 0.06 in a 1 cm square cell). In this work we show that a particular experimental set-up can be calibrated for the inner-filter effect from the absorption and fluorescence excitation spectra of a suitable standard. The calibration takes only a few minutes and provides correction with sufficient accuracy for most practical situations.

Keywords: *Fluorescence; inner-filter effect; inner-filter correction*

Introduction

Fluorescence spectroscopy has found wide application in analytical chemistry and biochemistry because of its extreme sensitivity and structural specificity. However, some experimental difficulties hinder its use as a quantitative tool. One such difficulty is that the recorded fluorescence intensity is not proportional to the concentration of the fluorophore owing to the so-called inner-filter effect.[1,2] This effect is due to the absorption of some of the incident light before it reaches the point in the sample at which luminescence is observed ('primary' inner-filter effect), and re-absorption of some of the emitted light before it leaves the cell ('secondary' inner-filter effect). Because of the inner-filter effect, the observed fluorescence intensity, I^{em}_{obs}, depends on the optical density of the sample at both excitation, $D(\lambda_{ex})$, and emission, $D(\lambda_{em})$, wavelengths (D = absorbance cm^{-1}), and is not a linear function of the fluorophore concentration, C:

$$I^{em}_{obs} \propto f\,[D(\lambda_{ex}),D(\lambda_{em})]C \approx f_p[D(\lambda_{ex})]\,f_s[D(\lambda_{em})]C \quad (1)$$

where factorization of the general correction function, $f\,[D(\lambda_{ex}),D(\lambda_{em})]$, assumes that the primary, $f_p[D(\lambda_{ex})]$, and secondary, $f_s[D(\lambda_{em})]$, inner-filter effects can be treated independently. This assumption is generally made, although its validity for strongly absorbing solutions has recently been questioned.[3]

Most corrections for the inner-filter effect for a right-angled cell geometry are based on the equations derived by Parker and Barnes:[4]

$$f_p[D(\lambda_{ex})] = \frac{10^{-D(\lambda_{ex})l_p}(10^{D(\lambda_{ex})\Delta l_p/2} - 10^{-D(\lambda_{ex})\Delta l_p/2})}{2.303D(\lambda_{ex})\Delta l_p} \quad (2)$$

where l_p is the distance from the point inside the cell at which luminescence is observed, Δl_p, the width of the excitation light beam, and $D(\lambda_{ex})$, the optical density of the sample at the wavelength of excitation (Fig. 1). The correction for the secondary inner-filter effect, $f_s[D(\lambda_{em})]$, is obtained by replacing $D(\lambda_{ex})$ with $D(\lambda_{em})$, and l_p and Δl_p, with l_s and Δl_s. Correction with these equations has been found to work well up to absorbances of about 2.[5,6] A disadvantage of this approach, however, is that the instrument parameters l_p, Δl_p, l_s, and Δl_s must be known, and the optical densities, $D(\lambda_{ex})$ and $D(\lambda_{em})$, of the sample must be measured.

An experimental approach to correct for the primary inner-filter effect that avoids some of these difficulties has been developed.[7,8] This so called 'cell-shift' method is based on the measurement of fluorescence intensities at two points in the cell along the incident beam, and gives high accuracy for absorbances up to 2.7.[9] The original approach was later modified to measure fluorescence intensities at two points along the diagonal of the cell, which also allows correction for the secondary inner-filter effect.[10] More sophisticated approaches to correct for the inner-filter effect, based on multiple detectors and fibre optics, have also been developed.[11,12]

The intention of this work was not to provide a new method for very accurate corrections of the inner-filter effect; the precision of those already cited is sufficient for most purposes. Instead, our aim was to provide a simple and easy approach for the calibration of a particular experimental set-up in a few minutes, using standard commercial instruments, which provides an accuracy sufficient for most practical situations. Our study was limited to the primary inner-filter effect, and is therefore only applicable to samples that do not absorb appreciably at the wavelength of emission. This is, however, the common situation.

Theory

If a sample does not absorb at the emission wavelength, the secondary inner-filter effect is negligible. The observed

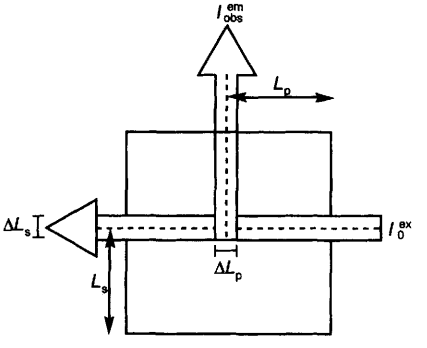

Fig. 1 Cross-section of a right-angled fluorescence cell. I^{ex}_0 = Intensity of the incident beam; I^{em}_{obs} = observed fluorescence intensity; l_p and l_s = distances from the point inside the cell at which luminescence is observed to the entry and exit cell walls, respectively; and Δl_p and Δl_s = widths of the emission and excitation light beams, respectively

* Present address: Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, Colorado 80309-0215, USA.

fluorescence intensity, when normalized with the intensity of the incident beam (quantum correction), is

$$I^{em}_{obs} \propto C\varepsilon(\lambda_{ex})\phi f_p[D(\lambda_{ex})] \quad (3)$$

where $\varepsilon(\lambda_{ex})$ is the molar absorptivity of the fluorophore, ϕ its fluorescence quantum yield, and $f_p[D(\lambda_{ex})]$ is the correction for the primary inner-filter effect, which depends on the absorbance of the sample at the wavelength of excitation. From eqn. (3), it immediately follows that the correction function can be determined experimentally by measuring the fluorescence intensities of a dilution series of a standard sample.[13]

Recalling that $C\varepsilon(\lambda_{ex}) = D(\lambda_{ex})$, eqn. (3) can be rewritten as

$$I^{em}_{obs} = \kappa D(\lambda_{ex}) f_p[D(\lambda_{ex})] \quad (4)$$

where κ is a proportionality constant, containing instrument parameters and also the fluorescence quantum yield of the fluorophore, which is assumed to be wavelength independent. As seen from eqn. (4), the fluorescence intensity does not depend directly on sample concentration, but only on sample absorption. As absorption generally also varies with wavelength, the correction function $f_p[D(\lambda_{ex})]$ can be determined using a single sample. By plotting the fluorescence intensity values recorded at different excitation wavelengths (at a fixed emission wavelength) *versus* the corresponding absorption values, the correction function for the entire interval from zero to maximum sample absorption can be determined. The approach requires that the absorption and fluorescence spectrophotometers are wavelength matched, and that the quantum correction of the spectrofluorimeter is appropriate. Further, the fluorophore used for calibration must have a wavelength-independent fluorescence quantum yield over a reasonable wavelength range. The calibration is then valid for the particular set-up, *i.e.*, for the type of cell used when placed at a certain position in the spectrofluorimeter.

Results

Fig. 2 shows fluorescence excitation spectra of 9,10-diphenylanthracene (DPA) in cyclohexane, measured in a 1 cm square cell, of samples with absorbances of 1.6, 0.2 and 0.02 at 392 nm. These spectra illustrate how serious the inner-filter effect can be. The excitation spectrum of the most dilute sample (C) shows negligible inner-filter effect and its shape is, within experimental error, identical with that of the absorption spectrum (D). This proves that the fluorescence quantum yield of DPA is constant in the wavelength region required for calibration. The spectrum of the sample of intermediate concentration (B) is broadened owing to a greater inner-filter effect at high absorption. For the most concentrated sample (A) the entire excitation spectrum is deformed: instead of a single maximum two maxima are observed, and a local minimum is found at the wavelength of the absorption maxima of the other samples. Here the inner-filter effect is so serious that the observed fluorescence intensity has begun to decrease with increasing absorption.

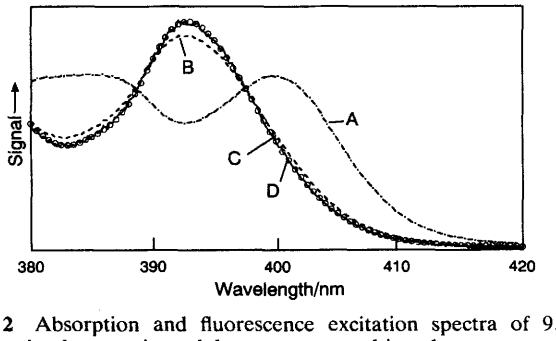

Fig. 2 Absorption and fluorescence excitation spectra of 9,10-diphenylanthracene in cyclohexane measured in a 1 cm square cell. Excitation spectra of samples with absorbances of A, 1.6, B, 0.2 and C, 0.02 at 392 nm. The absorption spectrum, D, was independent of concentration. Spectra are scaled to equal areas

Fig. 3(*a*) shows the fluorescence intensities of the most concentrated DPA sample plotted against the corresponding absorbances in the wavelength region 392–450 nm (the long wavelength side of the low energy absorption band). The non-linear behaviour of the plot is due to the inner-filter effect. The straight solid line (C) represents the fluorescence intensity that would have been observed in the absence of the inner-filter effect, and the plot can be used directly to correct experimental readings for the inner-filter effect: the ratio between the straight line and the curve, at the optical density at the excitation wavelength, is the appropriate correction factor.

For practical use it is desirable to express the correction function in functional form. The broken line (B) is the best fit to the function $D(\lambda_{ex})10^{-D(\lambda_{ex})/l_p}$ with $l_p = 0.56$ cm. Here, $10^{-D(\lambda_{ex})/l_p}$ is an approximation of the correction function described in equation (2) that is valid for small Δl_p. The good agreement between the fitted curve (B) and the experimental data (A) suggests that the approximation is adequate. The size of the emission bandpass affects the volume element from where luminescence is detected, and thus Δl_p. We found, however, that the same correction function is valid when bandpaths between 0.5 and 15 nm are used, suggesting that the value of Δl_p is not crucial, and that the same correction function can be used for all bandpaths.

Using the approximation $f_p[D(\lambda_{ex})] \approx 10^{-D(\lambda_{ex})l_p}$, eqn. (4) can be linearized as follows:

$$\log [D(\lambda_{ex})/I^{em}_{obs}(\lambda_{ex})] = l_p D(\lambda_{ex}) - \log \kappa \quad (5)$$

and l_p and $\log \kappa$ can be determined as the slope and intercept of a plot of $\log [D(\lambda_{ex})/I^{em}_{obs}(\lambda_{ex})]$ *versus* $D(\lambda_{ex})$ [Fig. 3(*b*)].

Once l_p is determined, fluorescence intensities recorded with the particular set-up can readily be corrected for the primary inner-filter effect:

$$I^{em}_{corr} = I^{em}_{obs}\, 10^{D(\lambda_{ex})l_p} \quad (6)$$

Discussion

We have presented a simple and easy way to determine the correction function for the primary inner-filter effect in

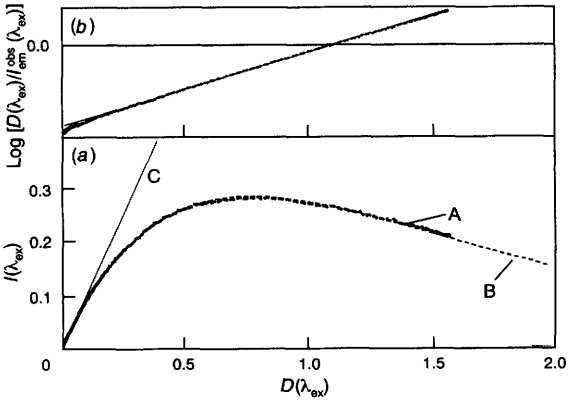

Fig. 3 (*a*) A, Plot of fluorescence intensities recorded at various excitation wavelengths *versus* the corresponding absorption intensities for DPA in cyclohexane; B, function $D(\lambda_{ex})10^{-D(\lambda_{ex})/l_p}$ with $l_p = 0.56$ cm; and C, fluorescence intensities that would be observed in the absence of the inner-filter effect. The fluorescence intensities are scaled to give the proportionality constant $\kappa = 1$. (*b*) Plot of log $[D(\lambda_{ex})/I^{em}_{obs}(\lambda_{ex})]$ *versus* $D(\lambda_{ex})$. The slope and intercept are l_p (0.56 cm) and log κ, respectively. Data are from the wavelength ranges (*a*) 392–450 nm and (*b*) 392–415 nm. Cell length, 1 cm; $D(392) = 1.6$; $\lambda_{em} = 505$ nm

fluorescence spectroscopy. Using a fluorophore that has a wavelength-independent fluorescence quantum yield, the correction for the primary inner-filter effect for a particular experimental set-up can be determined by plotting the recorded fluorescence intensities at different wavelengths *versus* the corresponding absorbances. The correction is found to be well described by the function $10^{D(\lambda_{ex})l_p}$, where l_p is the depth in a right-angled measuring cell from where luminescence is measured, and $D(\lambda_{ex})$ is the total optical density of the sample at the excitation wavelength.

Scale of Error Induced by the Inner-filter Effect

The absolute error induced by the inner-filter effect is the deviation of the recorded fluorescence intensities from the straight line shown in Fig. 3(*a*). The absolute error is small at low absorbances, being essentially negligible below 0.06. It increases rapidly thereafter, becoming very large at high absorbances. However, the small absolute error at low absorbance may be misleading. In most instances, the relative error is more important:

$$(I^{em}_{corr} - I^{em}_{obs})/I^{em}_{obs} \approx 10^{-D(\lambda_{ex})l_p} - 1 \approx -2.303D(\lambda_{ex})l_p \quad (7)$$

where the latter approximation is valid within 10% at optical densities below 0.1 cm^{-1}. For our set-up, $l_p = 0.56$ cm, the relative error is roughly $1.3D(\lambda_{ex})$, therefore also being significant at low absorbance. At an absorbance of 0.06, where the absolute error is negligible, the relative error is about 8%.

In summary, correction for the primary inner-filter effect is, in most instances, desirable. In addition to being necessary for quantitative comparison of fluorescence intensities, it is required for comparison of excitation spectra recorded on different instruments. In the latter case, correction is particularly important because information about sample absorption, which is in general available, is insufficient for comparison purposes as the inner-filter effect also depends on the particular experimental set-up.

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References

1 Parker, C. A., and Rees, W. T., *Analyst*, 1962, **87**, 83.
2 Parker, C. A., *Photoluminescence of Solutions*, Elsevier, Amsterdam, 1968, ch. 1, pp. 15–22; ch. 3, pp. 220–234.
3 Street, K. W., and Tarver, M., *Analyst*, 1988, **113**, 347.
4 Parker, C. A., and Barnes, W. J., *Analyst*, 1957, **82**, 606.
5 Holland, J. F., Tests, R. E., Kelly, P. M., and Timnick, A., *Anal. Chem.*, 1977, **49**, 706.
6 Christmann, D. R., Crouch, S. R., Holland, J. F., and Timnick, A., *Anal. Chem.*, 1977, **49**, 706.
7 Britten, A., Archer-Hall, J., and Lockwood, G., *Analyst*, 1978, **103**, 928.
8 Novak, A., *Collect. Czech. Chem. Commun.*, 1978, **43**, 2869.
9 Christmann, D. R., Crouch, S. R., and Timnick, A., *Anal. Chem.*, 1981, **53**, 2040.
10 Lutz, H.-P., and Luisi, P. L., *Helv. Chim. Acta*, 1983, **66**, 1929.
11 Yappert, H., and Ingle, B., *Anal. Spectrosc.*, 1989, **43**, 759.
12 Ratzlaff, E. H., Harfmann, R. G., and Crouch, S. R., *Anal. Chem.*, 1984, **56**, 342.
13 Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983, ch. 2, pp. 43–47.

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