190. Correction for Inner Filter Effects in Fluorescence Spectroscopy

by **Hans-Peter Lutz** and **Pier Luigi Luisi***

Technisch-Chemisches Laboratorium, ETH-Zentrum, CH-8092 Zürich

(9.III.83)

Summary

A method is proposed for correcting experimental fluorescence readings for inner filter effects, *i.e.* the absorption of the exciting light and/or the absorption of the emitted radiation, which cause the non-linearity between fluorescence intensity and fluorophor concentration. Basically the method consists of measuring the fluorescence intensity at two different points along the diagonal in the cell. Unlike similar methods proposed in the literature, the two points are corrected simultaneously for both absorption of excitation and of emission radiation without the necessity of reading the optical density of the solution, and with a very simple data elaboration.

Introduction. – Fluorescence spectroscopy has found wide application in analytical chemistry and biochemistry mostly due to its extreme sensitivity and structural specificity. A few experimental and theoretical difficulties, however, still hinder its use as a reliable quantitative tool. One such difficulty is given by the non-linearity of the relationship between fluorescence intensity and fluorophor concentration due to the so-called 'inner filter effect' [1].

Various methods have been proposed to correct for the inner filter effect [2–6]. One of the best known-ways, generally used to correct for absorption of the exciting light in right-angle cell geometry *(Fig. 1A)*, is based upon the equation of *Parker & Barnes* [7]:

$$\frac{F_0}{F} = \frac{2.303 \cdot D_x \cdot \Delta l_x}{10^{-D_x \cdot l_x} \cdot \left(10^{D_x \cdot \frac{\Delta l_x}{2}} - 10^{-D_x \cdot \frac{\Delta l_x}{2}}\right)} \qquad (1)$$

where F_0 is the 'true fluorescence' (absorption-corrected fluorescence), F is the observed fluorescence and D_x is the optical density of the solution at excitation wavelength.

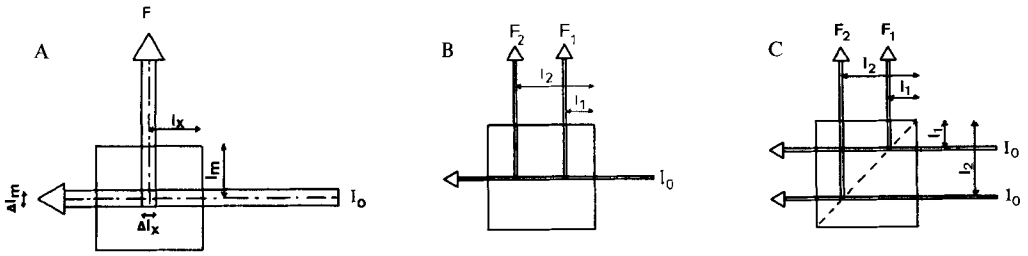

Fig. 1. A: *Radiation geometry in the cell for the right-angle method of illumination and viewing;* B: *pathways of the radiations for the cell-shift method;* C: *pathways of the radiations for the measurement at two points on the cell diagonal*

Eqn. 1 can also be used to correct for inner filter effects due to absorption of the emission radiation by replacing D_x by D_m (optical density at emission wavelength) and l_x, Δl_x by l_m, Δl_m, respectively.

Correction with the equation of *Parker & Barnes* has been found to be effective up to optical densities of 2 [8] [9]; a main disadvantage is that the optical densities D_x and D_m must be measured independently.

A way to obviate this inconvenience was proposed by *Britten et al.* [10] and, also by correcting wall-reflection effects, by *Novak* [11]. These authors showed that the 'true' fluorescence F_0 could be obtained without reference to the optical density, provided that the apparent fluorescence is measured at two different points along the incident beam in the cell (corresponding to two different pathways, l_1 and l_2, and different absorbivities for the incident beam). The measurements are carried out by manually shifting the cell along the excitation pathway to measure at l_1 and l_2. This is the so-called 'cell-shift' method (see *Fig. 1B*). The equations derived by *Britten et al.* [10] are:

$$D_x = \frac{\log\left(\frac{F_1}{F_2}\right)}{(l_2 - l_1)} \tag{2a}$$

$$F_0 = F_1 \cdot \left(\frac{F_1}{F_2}\right)^{\frac{l_1}{(l_2 - l_1)}} \tag{2b}$$

where F_1 and F_2 are the observed fluorescence intensities at the positions l_1 and l_2 (see *Fig. 1B*).

The equations proposed by *Novak* [11] are:

$$A = 10^{-D_x} = t \cdot \left(\frac{1 - \rho \cdot t^{2 \cdot (1 - l_1)}}{1 - \rho \cdot t^{2 \cdot (1 - l_2)}}\right)^{\frac{1}{(l_2 - l_1)}} \tag{3a}$$

with

$$t = \left(\frac{F_2}{F_1}\right)^{\frac{1}{(l_2 - l_1)}} \tag{3b}$$

$$F_0 = F_1 \frac{1 - \rho^2 \cdot A^{2l}}{(1-\rho) \cdot (A^{l_1} - \rho \cdot A^{(2l-l_1)})} \tag{3c}$$

where A is the absorbivity, l is the length of the cell and ρ is the reflection coefficient.

Both equations assume a small Δl_x (see *Fig. 1A*). *Eqn. 2* was developed to calculate the fluorescence efficiency from F_0/D_x without measuring D_x independently. *Eqn. 3* corrects also for wall-reflection effects, and its use has also been demonstrated for the correction of inner filter effects due to absorption of the fluorescence absorption of the fluorescence radiation [11]. *Christmann et al.* [12] describe an instrument which corrects inner filter effects also with the 'cell-shift' method on the basis of *Eqn. 3*. They found good accuracy of 2% for optical densities up to 2.7 [13].

Despite its considerable theoretical validity, *Eqn. 3c* cannot easily be utilized without the use of a computer, and its formal complexity hinders the physical transparency of the correction itself. The use of the above equations is particularly awkward when a long series of routine measurements has to be made, as, for example, in systematic binding studies.

Eqn. 2b is easy to use, but corrects only for inner filter effects due to absorption of excitation radiation.

In the present work, we have set up a procedure in which the advantages of the cell-shift method are combined with formal and instrumental simplicity and an easy elaboration of data.

The main points of our approach are *a*) to mesure F_1 and F_2 along the diagonal of the cell. This has the advantage of the possibility for correcting for both types of inner filter effects (absorption of excitation and of emission radiation) with only two measurements. *b*) Optimization of the geometrical parameters, so that it is possible to work without taking wall-reflection effects into account.

Measuring F_1 and F_2 along the cell diagonal allows for the correcting for inner filter effects due to absorption of excitation radiation *and* of emission radiation, with only two measurements and one parameter, whereas the other methods require at least three measurements and two parameters.

Experimental. – The instrument used in our study was an *Aminco SPF-1000* spectrofluorimeter [15]. We replaced the original cell holder by a home-made cell-shift compartment for 1 × 1 cm cells *(Fig. 2)*. This compartment permits the operator to pneumatically move the cell along its diagonal. The two positions of the cell holder were adjusted to $l_1 = 2.25$ and $l_2 = 7.75$ mm with the help of a laser beam through the monochromators. (Preliminary experiments showed, that $l_1 = 2$ and $l_2 = 8$ mm are the extreme points, where no appreciable wall-radiation effect occurs.) Slit widths of 2 nm for the excitation and the emission monochromators were used to keep Δl_x and Δl_m small (*ca.* 0.5 mm).

Absorption measurements were carried out on a *Beckmann Acta MVI* spectrophotometer. Tryptophan, quinine sulfate and fluorescein were purchased from *Fluka*. NADH was obtained from *Boehringer*.

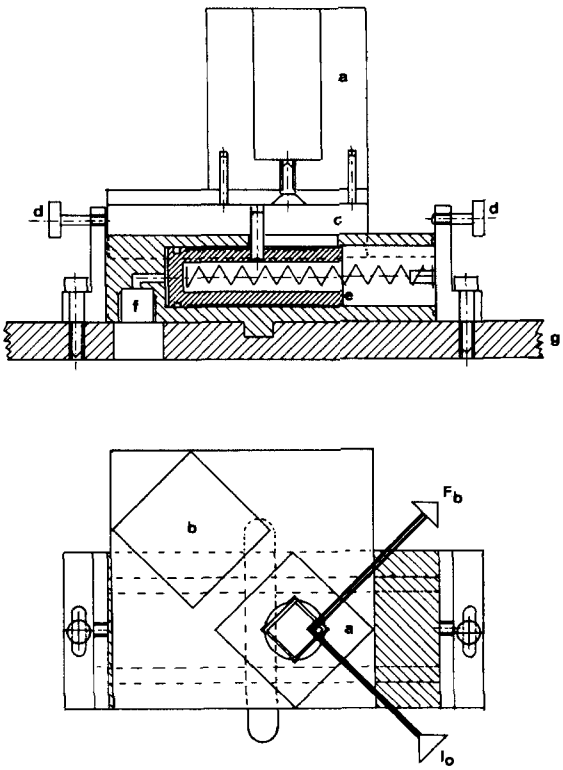

Fig. 2. *Vertical and horizontal projection of the cell-shift compartment.* a) Cell holder; b) collector for the passing excitation radiation; c) cell slider; d) adjustable slider stops; e) piston; f) pressed air inlet; g) instrument ground plate.

Results and Discussion. – *Fig. 1C* illustrates the cell geometry for the measurement at two positions on the cell diagonal for the simultaneous correction for inner filter effects due to absorption of excitation and emission radiation. F_1 and F_2 can be expressed according to *Eqn. 4:*

$$F_1 = F_0 \cdot 10^{-D_x \cdot l_1} \cdot 10^{-D_m \cdot l_1} = F_0 \cdot 10^{-(D_x + D_m) \cdot l_1} \tag{4a}$$

$$F_2 = F_0 \cdot 10^{-(D_x + D_m) \cdot l_2} \tag{4b}$$

From *Eqn. 4a* and *4b*, *Eqn. 5* is deduced

$$D_x + D_m = \log(F_1/F_2)/(l_2 - l_1) \tag{5}$$

which, inserted into *Eqn. 4a*, leads to *Eqn. 6:*

$$F_0 = F_1 \cdot (F_1/F_2)^{l_1/(l_2-l_1)} \tag{6}$$

where l_1 and l_2 are geometry-dependent constants, so that the expression $l_1/(l_2-l_1)$ can be replaced by a single geometry-dependent parameter a. Formally, this equation is the same as *Eqn. 2b.* However, there is an important difference: l_1 and l_2 are measured along the cell diagonal. As already mentioned, this enables one to correct for both types of inner filter effects with only two measurements. *Eqn. 6* then becomes simply:

$$F_0 = F_1 (F_1/F_2)^a \tag{7}$$

The validity of such an elaboration for the correction for the inner filter effect due to absorption of the exciting radiation was tested with a concentration series of tryptophan solutions *(Fig. 3).* The inner filter effect was corrected with *Eqn. 7* and the best fitting value of the parameter a, determined by a non-linear regression, was found to be a = 0.44. This differs slightly from the value fixed at a = 0.41 (for $l_1 = 2.25$ mm and $l_2 = 7.75$ mm) for the instrumental geometrical parameter, which may be due to neglecting the reflection effects on the cell walls. With this adjusted value of a = 0.44, as shown in *Figure 3*, the deviation from linearity for the corrected fluorescence F_0 is less than 3% in the optical density range of 0.05 to 2.

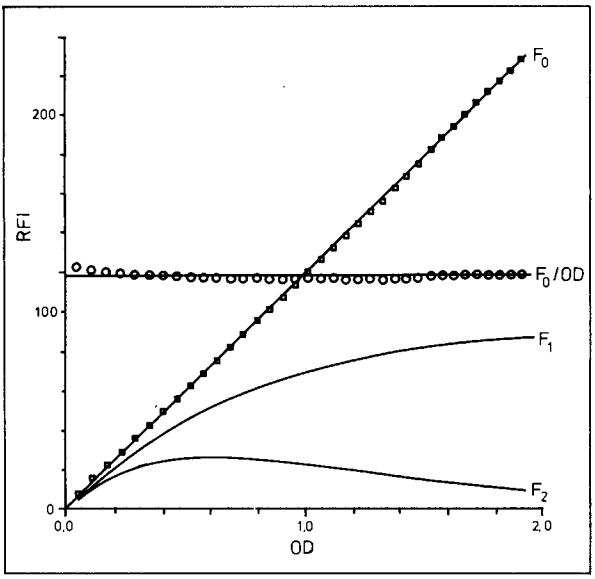

Fig. 3. *Relative fluorescence intensity* (RFI) *at 350 nm* (excitation 280 nm) vs. *optical density* (D) *at 280 nm for tryptophan solution* (in 0.1M phosphate buffer, pH 7.0). F_1 and F_2 are the fluorescence intensities measured at the two points on the cell diagonal, F_0 is the 'true' fluorescence corrected with *Eqn. 7* and a = 0.44.

The correction for the inner filter effect due to absorption of the fluorescence radiation was tested with a series of solutions having a constant concentration of quinine sulfate and varied concentrations of fluorescein, which absorbs at the emission wavelength of quinine sulfate. As shown in *Figure 4*, the corrected fluorescence F_0, corrected with *Eqn. 7* and $a = 0.44$, deviates by 3% at the most from the expected constant value for optical densities up to 2.

More generally, *Figures 3–5* show how drastic are the distortions brought about by inner filter effects on the experimental fluorescence readings. By an optical density of 0.4 and an optical pathway of 0.75 cm, which are close to typical experimental values, the error is increased by a factor of two or more (*Fig. 3* and *4*). Furthermore, marked distortions in the shape of the emission spectrum are also encountered. This is shown in *Figure 5*, which illustrates an experiment where corrections for both types of inner filter effect are applied to an emission spectrum of tryptophan using a 40 μM aqueous tryptophan solution (the actual absorption spectrum is shown in *Figure 5A, a)* and adding 80 μM of NADH (absorption spectrum shown in *Figure 5A, b). Figure 5B* shows the uncorrected emission spectra (F_1 and F_2) of the pure 40 μM tryptophan solution as well as the F_0-spectrum obtained after correction. *Figure 5C* shows the spectra for solutions in the presence of 80 μM NADH (which absorbs at both excitation and emission wavelengths, see *Figure 5A, b).* The uncorrected fluorescence (F_1) simulates a quenching of *ca.* 40% and gives an artifact, a red shift of the emission maximum of *ca.* 8 nm. After correction, the intensity of the emission spectrum is *ca.* 2% smaller than the theoretical value, *i.e.* a difference that is within the experimental accuracy of fluorescence measurements.

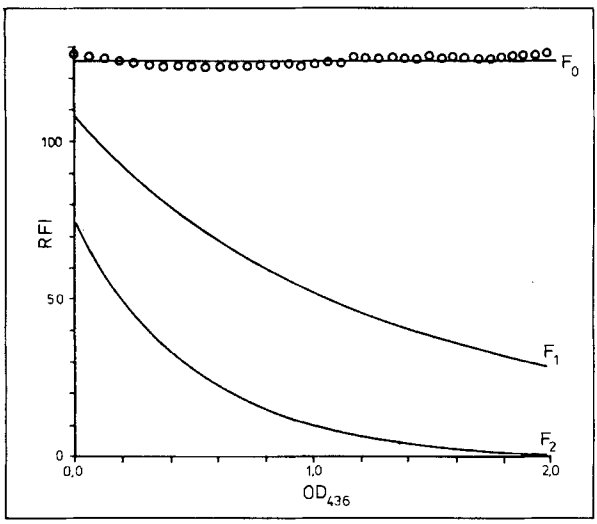

Fig. 4. *Relative fluorescence intensity* (RFI) *at 436 nm* (excitation 365 nm) vs. *optical density* (D) *at 436 nm for a solution with constant quinine sulfate concentration* ($D_{365} = 0.328$) *and increasing fluorescein concentration* (in 0.1 N H_2SO_4). F_0 was calculated using *Eqn. 7* and $a = 0.44$.

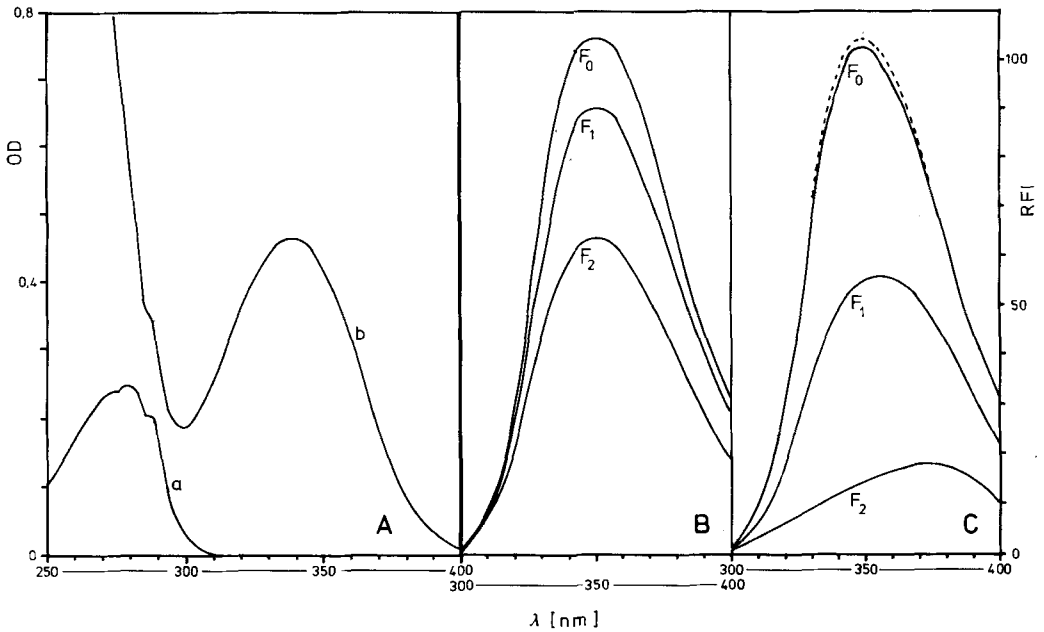

Fig. 5. *Absorption and emission spectra of tryptophan solution* (40 mM) *before and after addition of 80* μM *NADH.* A) Absorption spectra before (a) and after (b) addition of NADH; B) emission spectrum before, and C) after addition of NADH.

The accuracy of 3%, which is illustrated by the above described experiments for our system is only a little worse than the 2% accuracy, reported by *Christmann et al.* [13] for their system. But, in contrast to their system, our system needs only a little expenditure of mechanical and calculating treatments.

REFERENCES

[1] *C.A. Parker & W.T. Rees,* Analyst *87*, 83 (1962).
[2] *J.S. Franzen, J. Kuo & A.E. Chung,* Anal. Biochem. *47*, 426 (1972).
[3] *J.E. Gill,* Appl. Spectrosc. *24*, 588 (1970).
[4] *J.W. Eastman,* Photochem. Photobiol. *6*, 55 (1967).
[5] *R.A. Leese & E.L. Wehry,* Anal. Chem. *50*, 1193 (1978).
[6] *R. van Slageren, G. den Boef & W.E. van der Linden,* Talanta *20*, 501 (1973).
[7] *C.A. Parker & W.J. Barnes,* Analyst *82*, 606 (1957).
[8] *J.F. Holland, R.E. Tests, P.M. Kelly & A. Timnick,* Anal. Chem. *49*, 706 (1977).
[9] *D.R. Christmann, S.R. Crouch, J.F. Holland & A. Timnick,* Anal. Chem. *52*, 291 (1980).
[10] *A. Britten, J. Archer-Hall & G. Lockwood,* Analyst *103*, 928 (1978).
[11] *A. Novak,* Collect. Czech. Chem. Commun. *43*, 2869 (1978).
[12] *D.R. Christmann, S.R. Crouch & A. Timnick,* Anal. Chem. *53*, 276 (1981).
[13] *D.R. Christmann, S.R. Crouch & A. Timnick,* Anal. Chem. *53*, 2040 (1981).