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A new approach to the old problem: Inner filter effect type I and II in fluorescence

Adam Kasparek *, Bogdan Smyk

Department of Physics and Biophysics, University of Warmia and Mazury in Olsztyn, Oczapowskiego 4, 10-719 Olsztyn, Poland

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ABSTRACT

The fluorescence technique is very popular and has been used in many fields of research. It is simple in its assumptions but not very easy to use. One of the main problems is the inner filter effect (IF) I and II which takes place in the cuvette. IF type I is permanently present, but IF type II occurs only when absorption and fluorescence spectra overlap. To avoid IF type I, absorbencies in the cuvette should be smaller than 0.05, which is however very difficult to obtain in many experiments. In this work we propose a new method to solve these problems in the case of a Cary Eclipse fluorimeter, having horizontally-oriented slits, based on old equations developed in the middle of the last century. This method can be applied for other instruments, even these with vertically-oriented beams, because we share scripts written in MATLAB and GRAMS/AI environment. Calculations in our method enable specifying beam geometry parameters in the cuvette, which is necessary to obtain the correct shape and fluorescence intensity of emission and excitation spectra. Such a specific fluorescence intensity dependence on absorbance can, in many cases, afford possibilities to determine the quantum yield (QY) using slopes of the straight-lines, which was demonstrated with the use of Tryptophan (Trp), Tyrosine (Tyr), and Rhodamine B (RhB) solutions. For example, assuming that QY = 0.14 for Tyr, the QY determined for RhB reached QY = 0.71 ± 0.05, although the measurement for Tyr and RhB was performed at a completely different spectral range.

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1. Introduction

Fluorescence is one of the best-known techniques. It has been used in many experiments, from standard assays to very advanced research. It is used very often but is not very simple for quantitative uses. Both IF effects are caused by absorption. The first one, called type one, is due to the Lambert-Beer law. Intensity transmitted through a given path length decreases exponentially. Fluorescence is proportional to the light transmitted through the cuvette and, therefore, is proportional to (1–10^(−A)). This is not a linear dependence, and this is a normal phenomenon. IF type I correction is based on the assumption that the intensity of the transmitted light should be the same alongside the path length. For this reason, fluorescence intensity must be a linear function of absorbance at a given excitation wavelength. IF type II is also referred as reabsorption. If absorption and fluorescence spectra of a sample overlap, then part of the emitted light will be again absorbed in the overlapped region by the sample, causing a change in the shape of a fluorescence spectrum. It has some restrictions which make it difficult to follow in practice. One of them is the necessity of using samples of very low absorbencies (lower than 0.05) to avoid inner filter (IF) effects. This restriction limits the use of this technique but may be avoided by applying the QY instead of intensities. By using QY, we are indeed able to correct spectra for IF type I, but we need to make additional measurements of the standard. Standard samples should be measured in the same circumstances as the sample, e.g. with the same slits, wavelength of excitation, region of emission, etc. These are next difficulties to overcome. When the QY is unnecessary, for example: investigations of protein-ligand complexes do not need QY, fluorescence intensities of the samples can be used instead to obtain such parameters as stoichiometry, total number of binding sites, different kinds of binding sites or cooperativity (Hill's coefficient) [1], which can be evaluated from the Addair [2] or Scatchard [3] theory. When absorption overlaps fluorescence spectrum, the IF type II (reabsorption) cannot be avoided especially when we use total fluorescence (area under the curve) and not intensity for a given wavelength out of the absorption spectrum range. However, before one can do correction for IF effects, they should apply their own fluorescence calibration curves, because each apparatus has different instrument response correction function. Therefore, it is important to use such a curve, to obtain a true emission spectrum. Many attempts have been undertaken to correct spectra for IF effects. First of them was made by Parker and Barnes [4] who introduced the equation for correction factor f_p for primary IF:

$$f_p = \frac{F_0}{F} = \frac{2.303\,A\,(x_2 - x_1)}{10^{-A_{x_1}} - 10^{-A_{x_2}}},$$

where: F_0 and F – fluorescence intensities after and before correction, A – absorbance of the sample at the excitation wavelength, x_2 and x_1 –

* Corresponding author.
E-mail address: adam.kasparek@uwm.edu.pl (A. Kasparek).

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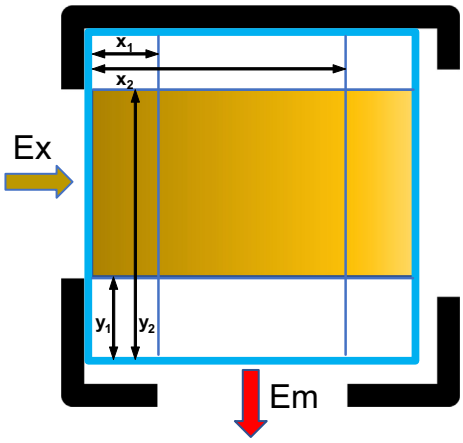

Scheme 1. Top view on the cuvette and the cell holder.

parameters of the beam in the excitation direction (Scheme 1). Holland et al. [5] independently derived the expression for f_p which was the same as the equation given by Parker and Barnes [4]. They also developed a similar equation for IF type 2 (f_s),

$$f_s = \frac{F_0}{F} = \frac{2.303\,A\,(y_2 - y_1)}{10^{-A_{y_1}} - 10^{-A_{y_2}}},$$

where: y_2 and y_1 – are parameters of the beam in the emission direction (Scheme 1). Many trials have been made to evaluate correction factors including Yappert and Ingle [6], Lutz and Luisi [7], Christmann et al. [8], Lees and Wehry [9], Credi and Prodi [10] and Riesz et al. [11]. For highly absorbing samples, an interesting paper was published by Krimer et al. [12] and for Cary Eclipse fluorimeter by Fonin et al. [13]. The latter authors tested the correction factors for absorbencies of at least up to 60, but they failed to obtain a linear dependence between fluorescence intensity and absorbance. The Parker and Barnes [4] equation applied directly to the spectra did not give a strictly linear dependence without any curvature of fluorescence intensity against absorbance for the Cary Eclipse fluorimeter. The main problem connected with IF type I and II was beam geometry in the cuvette. This was very difficult to establish, especially when the beam has vertical geometry.The goal of this work was to develop a simple method for the correction of both IF type I and II in a Cary Eclipse fluorimeter, based on the Parker and Barnes [4] and Holland et al. [5] equations. The very good circumstance was geometry of the excitation beam which was horizontal in the cell due to the horizontally oriented slits and was wider than the input window in the cell holder. This has greatly simplified our attempt, because we did not have to perform geometry calculation for each slit set. We developed computer scripts in MATLAB to optimize input and output widths of the cuvette in the cell holder in right-angle geometry. Effective widths were calculated for 10 × 10 mm and 10 × 4 mm cuvette. All computer scripts and programs can be used as freeware and shared for all, because everyone must calculate beam geometry in their holder. For example, input and output windows in thermostated and not thermostated cell holders have different widths. The scripts were written in GRAMS/AI v.11 and in MATLAB v. R2014a environment. This method has been applied without detailed description in our recent study [14].

2. Experimental

2.1. Materials

Rhodamine B (RhB) was purchased from Sigma-Aldrich (Poland). Tyrosine (Tyr), Tryptophan (Trp), and 1-Anilino-8-Naphthalene Sulfonate (ANS) were purchased from Fluka Chemie AG (Germany). The concentration of Trp ranged from 5 to 350 μM, Tyr from 70 to 1000 μM, and ANS from 3 to 380 μM. In all measurements, except for Stern-Volmer research, Trp, Tyr, and ANS were diluted with a phosphate buffer (0.1 M). During Stern-Volmer measurement, phosphate buffer concentration ranged from 0.005 to 0.18 M. The concentration of RhB in basic ethanol (POCH, Poland) was from 0.8 to 12.5 μM. The acidity of the solutions was established at pH = 6 and was measured with a pH-meter (Jenway 3030, UK) at 24 °C.

3. Methods

Absorption spectra were measured with a Cary 5000 (Agilent, Australia) spectrometer in 10 mm, 5 mm, and 2 mm quartz cells. Fluorescence was measured with a Cary Eclipse (Agilent, Australia) fluorimeter in 10 × 10 mm and 10 × 4 mm quartz cells using right angle geometry. Emission slit was set at 5 nm and excitation slit was set at 2.5 nm. PMT Detector Voltage was in the range between 500 and 600 V. Appropriate correction factors for the voltage other than 600 V was applied. Both instruments were equipped with a Peltier accessory. Temperature was stabilized at 24 °C. Horizontally-oriented slits in Cary fluorimeter greatly simplified the calculation. Area under the spectrum (total fluorescence (TF)) was used as a fluorescence intensity. The script was written in MATLAB program v. R2014a (MathWorks, USA). All programs used for calculations of IF effects also in GRAMS/AI Spectroscopy Software (*.ab scripts) (Thermo Fisher Scientific, USA), attached to this article as a supplement, can be used for free. The goal of this calculation was to obtain f_p and f_s correction functions therefore, x_1, x_2, y_1, and y_2 parameters must be determined. Scheme 1 shows top view on the geometry of the cuvette (blue line) and the cell holder (thick black line).

On this scheme $y_2 - y_1$ is the width of the incident beam and $x_2 - x_1$ is the width of the detection beam. It is possible to acquire f_p and f_s by fitting to one linear equation, but much more precise calculation can be achieved if we divide the calculation into two parts: one for x_1 and x_2, and the second for y_1 and y_2. For the first part it is essential to choose a sample without or with very small reabsorption and for the second part - with high reabsorption. The script in MATLAB requires absorption and fluorescence spectra and wavelength-dependent instrument sensitivity curve for the Cary fluorimeter. This curve is connected to the instrument. Firstly, the algorithm corrects fluorescence spectra for instrument sensitivity. Then, it enters absorbance of excitation wavelength (A_{ex}) and a set of parameters x_1 and x_2. Further, it multiplies the entire fluorescence spectra by factors derived from f_p for IF type I correction. Then, the script corrects fluorescence intensity for each wavelength for IF type II using f_s function for the initial and constant y_1 and y_2 values. Then, the process repeats for another set of parameters x_1 and x_2. Parameters which have the highest coefficient of goodness of fit (R^2) for the linear dependence between TF and A_{ex}, are taken as the final set of parameters. The procedure for y_1 and y_2 determination is very similar to the first script, but the mean squared error (MSE) is calculated instead of R^2 for normalized corrected fluorescence spectra which should give the same shape of each spectrum. Parameters which have the lowest MSE are taken as the final set of parameters. We did not make geometry calculation for a different slit set because

Table 1
Beams geometry in the cuvette: x_2 and x_1 are parameters in excitation direction, y_2 and y_1 in emission direction. For details see the text and Scheme 1.

Cuvette	x_1 [cm]	x_2 [cm]	y_1 [cm]	y_2 [cm]
10 × 10 mm	0.207 ± 0.001	1.085 ± 0.007	0.258 ± 0.029	0.727 ± 0.018
10 × 4 mm	0.037 ± 0.006	0.425 ± 0.021	0.274 ± 0.020	0.701 ± 0.031

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