Fresenius J Anal Chem (1991) 340:341–344

Fresenius' Journal of Analytical Chemistry



# Assessment of inner filter effect corrections in fluorimetry

**M. M. Puchalski [1], M. J. Morra [1], and R. von Wandruszka [2]**

[1] Division of Soil Science, University of Idaho, Moscow, ID 83843, USA
[2] Department of Chemistry, University of Idaho, Moscow, ID 83843, USA

Received February 6, 1991

**Summary.** The inner filter effect (IFE) in fluorescence spectroscopy is not easily distinguished from dynamic and static quenching phenomena, since IFE rarely occurs without quenching. IFE corrections may be subject to under- or over-compensation effects that are difficult to assess accurately. To evaluate existing IFE correction procedures, it is proposed that the linearity of resulting Stern-Volmer plots and the relative change of their slopes with temperature be adopted as criteria. Three correction methods are assayed in this manner, and the equation described by Gauthier et al. is found to produce the best results.

## Introduction

Measurements of fluorescence quenching are widely used in quantitative chemistry and biochemistry. Quenching is defined as a process in which a chemical species decreases the fluorescence intensity of a fluorophore by direct interaction. It is a useful analytical technique because it is highly sensitive and based on actual molecular contact between fluorophore and quencher [1].

The effect of the quencher on the fluorescence of the fluorophore is described by the Stern-Volmer equation:

$$F_o/F = 1 + K_q\tau_o[Q] = 1 + K[Q]$$

where $F_o$ and F are the fluorescence intensities in the absence and presence of quencher, respectively, $K_q$ is the bimolecular quenching constant, $\tau_o$ is the lifetime of the fluorophore in the absence of quencher, [Q] is the concentration of the quencher, and $K = K_q\tau_o$ is the Stern-Volmer quenching constant. The quenching constant is obtained from the slope of the Stern-Volmer plot ($F_o/F$ vs. [Q]). The two primary mechanisms used to describe fluorescence quenching are: (1) dynamic quenching, resulting from collisional encounters between fluorophore and quencher; and (2) static quenching, resulting from ground state complex formation between fluorophore and quencher. In dynamic quenching the Stern-Volmer quenching constant is related to the accessibility of the fluorophore to the quencher, and in static quenching it describes the degree of association or binding between the two species.

An inherent problem of many fluorimetric procedures is the absorption of exciting and/or emitted radiation by dissolved species, including the fluorophore itself. This is termed the inner filter effect (IFE), and while it also results in a decreased fluorescence intensity, it is not a quenching effect [2]. In order to accurately determine Stern-Volmer quenching constants, IFE must be distinguished from true quenching. Several methods have been proposed to correct for IFE [1–9], but no practical comparison of these methods has been reported. Simple comparison between corrected fluorescence values and those obtained before addition of the IFE compound does not provide an accurate assessment of the adequacy of correction, because of the concomitant quenching component.

One of the procedures developed in recent years [3] corrects instrumentally for IFE by measuring the fluorescence intensity of a solution at two different points along the diagonal of the sample cell. This requires special instrumentation which is not generally available, and the technique therefore has limited application.

IFE is most commonly corrected mathematically, and a number of equations have been developed for this purpose [1, 2, 4–9]. Some corrections involve several instrumental parameters manipulated by complex mathematical procedures [4–8]. The three most practical correction equations are described below.

Parker [2] has described a simple equation for the correction of IFE caused only by the absorption of exciting radiation:

$$\frac{F_{corr}}{F_{obs}} = \frac{2.303\,D\,(d_2 - d_1)}{10^{-Dd_1} - 10^{-Dd_2}} \qquad (1)$$

where D is the total optical density/cm of solution at the wavelength used for excitation, $F_{corr}$ [1] is the corrected fluorescence intensity of the solution, $F_{obs}$ [1] is the observed fluorescence intensity of the solution and $d_2$ and $d_1$ are the cuvet dimensions shown in Fig. 1.

Lakowicz [1] has reported a correction equation for IFE caused by absorption of both exciting and emitted radiation:

$$F_{corr} = F_{obs}\,\text{antilog}\,[(OD_{ex} + OD_{em})/2] \qquad (2)$$

where $OD_{ex}$ is the optical density/cm of the solution at the excitation wavelength used and $OD_{em}$ is the optical density/cm at the emission wavelength.

Gauthier et al. [9] have described a more elaborate correction method for IFE caused by the absorption of exciting

*Offprint requests to:* R. von Wandruszka

[1] $F_{corr}$ is referred to as $F_o$ and $F_{obs}$ as F by Parker. The terminology was changed here to prevent confusion with the Stern-Volmer $F_o$ and F

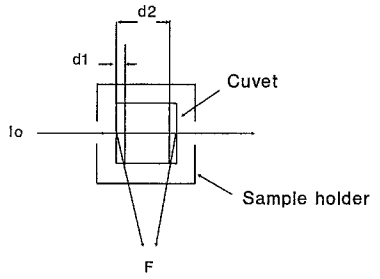


**Fig. 1.** Cuvet dimensions for correction Eq. (1) (*top view*). Io represents the excitation beam, d1 and d2 are distances specific to cuvet geometry, and F represents the observed fluorescence

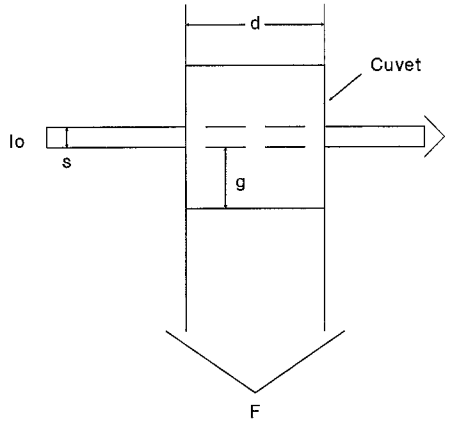


**Fig. 2.** Cuvet dimensions for correction Eq. (3) (*top view*). Io represents the excitation beam of width s, g is the distance from the edge of the excitation beam to the edge of the cuvet, d is the width of the cuvet, and F represents the observed fluorescence

and emitted radiation. It involves a modification of Eq. (1) and the inclusion of a factor for emission absorbance:

$$\frac{F_{corr}}{F_{obs}} = \frac{2.3\,d\,A_{ex}}{1-10^{-dA_{ex}}} \times 10^{g\,A_{em}} \times \frac{2.3\,s\,A_{em}}{1-10^{-s\,A_{em}}} \qquad (3)$$

where $A_{ex}$ is the absorbance/cm of the solution at the excitation wavelength used, $A_{em}$ is the absorbance/cm of the solution at the emission wavelength, and d, g, and s are cuvet dimensions defined in Fig. 2.

It is the objective of the present investigation to establish criteria for comparison and to evaluate the correction Eqs. (1), (2), and (3). Substances were chosen which absorb either the excitation or the emission of the fluorophore quinine sulfate, but not both. In this way, the effects of the absorbance on the measured fluorescence can be assessed separately. Thiourea was chosen for IFE at the excitation wavelength of quinine sulfate, because the absorption spectrum of thiourea overlaps one of the excitation bands of quinine sulfate, but not its emission (Fig. 3). Similarly, 9,10-diphenyl anthracene (DPA) was chosen for IFE at the emission wavelength of quinine sulfate, since the absorption spectrum of DPA overlaps the emission band of quinine sulfate, but not the excitation wavelength used (Fig. 3).

## Experimental

Quinine sulfate (Baker Chemical, Phillipsberg, N.J.) and DPA (Sigma Chemical, St. Louis, MO) were used without

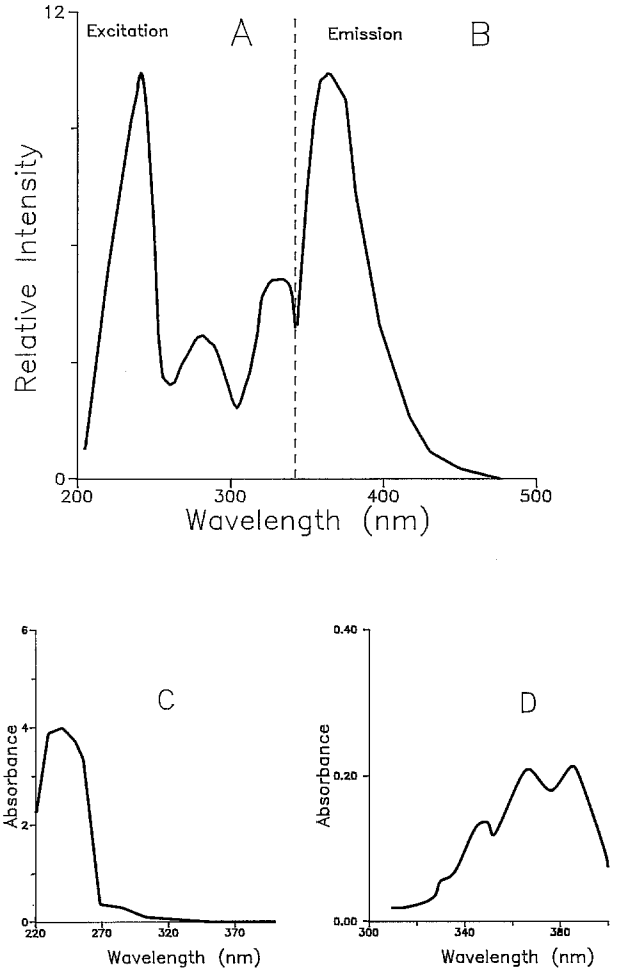


**Fig. 3.** **A** Excitation spectrum of quinine sulfate. **B** Emission spectrum of quinine sulfate. **C** Absorption spectrum of thiourea. **D** Absorption spectrum of DPA

further purification. Thiourea was obtained from U. S. Biochemical Corporation and purified by recrystallization in distilled deionized water (18 MΩcm resistivity) and rinsing with 1-propanol. Dehydrated ethyl alcohol (Quantum Chemical, Tuscola, IL) was used as the solvent in all solutions.

Fluorescence spectra were recorded with a Perkin-Elmer MPF 66 spectrofluorimeter equipped with a thermostated sample compartment. This instrument gives corrected excitation spectra through the use of a built-in Rhodamine 101 quantum counter. Excitation and emission slit widths were set for 1.5 and 1.8 nm bandpass, respectively. The excitation wavelength was 241 nm and the emission wavelength was 364 nm for the quinine sulfate/thiourea experiment. For the quinine sulfate/DPA experiment the excitation and emission wavelengths were 290 nm and 361 nm, respectively. Fluorescence-free quartz cells were used and blank subtraction of all fluorescence spectra was carried out through instrument software. A Beckman DU-50 spectrophotometer was used for absorption measurements.

### *Procedure*

Triplicate samples containing quinine sulfate and either thiourea or DPA were mixed and allowed to equilibrate for

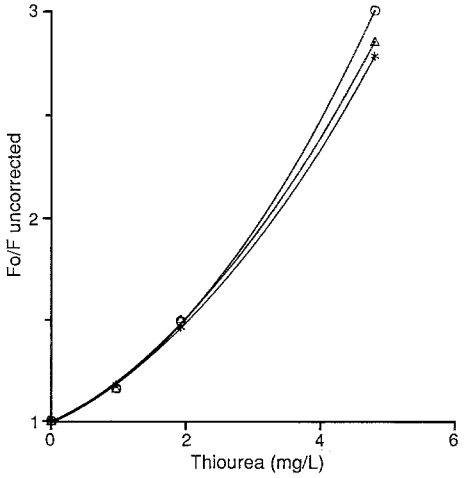


**Fig. 4.** Stern-Volmer plots with uncorrected $F_o/F$ values for quinine sulfate/thiourea at different temperatures. * 4°C; △ 22.5°C; ○ 40°C

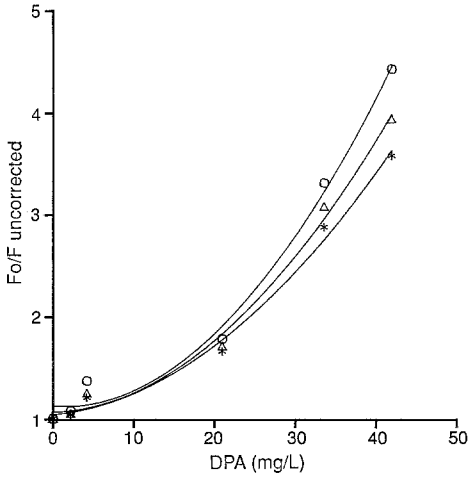


**Fig. 5.** Stern-Volmer plots with uncorrected $F_o/F$ values for quinine sulfate/DPA at different temperatures. * 4°C; △ 22.5°C; ○ 40°C

at least 12 h prior to measuring fluorescence intensities. Quinine sulfate was used at a concentration of $1 \times 10^{-5}$ mol/l for both experiments. Concentrations ranged from 0 to 4.8 mg/l for thiourea and 1 to 41.9 mg/l for DPA. To prevent decomposition, samples were stored in the dark in stoppered glass bottles. Fluorescence measurements were taken of the samples at three different temperatures: 4, 22.5 and 40°C. New samples were similarly prepared before absorptivity measurements were made. Stern-Volmer plots were constructed using uncorrected fluorescence intensity values and corrected intensity values derived from the three correction equations. The slopes from the 40°C and 4°C Stern-Volmer plots were used to calculate the percent decrease of slope with temperature.

## Results and discussion

A solution of a fluorophore which contains a second dissolved component that causes an inner filter effect, but not quenching, may be treated analogously to a quencher solution. Thus a plot of $F_o/F$ ($F_o$ is the fluorescence intensity without inner filter effect) vs. "quencher" concentration will have a finite positive slope K. A correction equation should reduce this slope to zero by making F equal to $F_o$ at all concentrations. In practice, this cannot usually be achieved, because most substances that cause IFE also produce some quenching. In addition, many substances are self-absorbing and thus $F_o$ has an inherent IFE. An accurate assessment of IFE correction equations is therefore not simply a matter of constructing a Stern-Volmer plot with corrected values and establishing that it has a slope of (or near) zero. For a system in which the fluorophore is subject to a certain level of quenching, as well as an inner filter effect, the Stern-Volmer plot will have a slope greater than zero. A correction equation may make adequate amends for IFE, leaving a slope that is truly equal to K. On the other hand, it may over- or under-compensate, producing a slope that is too small or too large. This cannot be deduced a priori from the data.

**Table 1.** Regression statistics for Stern-Volmer plots of quinine sulfate and thiourea using all corrections and temperatures

| Correction | Temp/°C | Regression equation | $P$ | $R^2$ |
|---|---|---|---|---|
| Uncorrected | 4 | $Y = 0.998 + 0.152X + 0.047X^2$ | 0.0001 | 0.95 |
| | 22.5 | $Y = 0.987 + 0.168X + 0.047X^2$ | 0.0001 | 0.97 |
| | 40 | $Y = 0.993 + 0.137X + 0.060X^2$ | 0.0001 | 0.99 |
| Eq. (1) | 4 | $Y = 0.9539 + 0.1173X$ | 0.0001 | 0.82 |
| | 22.5 | $Y = 0.9466 + 0.1255X$ | 0.0001 | 0.88 |
| | 40 | $Y = 0.9314 + 0.1451X$ | 0.0001 | 0.93 |
| Eq. (2) | 4 | $Y = 0.9839 + 0.0409X$ | 0.0139 | 0.47 |
| | 22.5 | $Y = 0.9761 + 0.0473X$ | 0.0030 | 0.60 |
| | 40 | $Y = 0.9646 + 0.0622X$ | 0.0001 | 0.81 |
| Eq. (3) | 4 | $Y = 0.9527 + 0.1223X$ | 0.0001 | 0.82 |
| | 22.5 | $Y = 0.9461 + 0.1310X$ | 0.0001 | 0.88 |
| | 40 | $Y = 0.9288 + 0.1508X$ | 0.0001 | 0.93 |

When uncorrected for IFE, Stern-Volmer plots curve towards the y-axis (Figs. 4 and 5) and can be fitted to a quadratic equation (see Table 1). This can be confused with Stern-Volmer plots of solutions that exhibit both static and dynamic quenching. However, the contributions of static and dynamic quenching to the plots can be graphically determined [1] while IFE cannot. When only one mechanism of quenching causes decreased fluorescence, the Stern-Volmer plot is linear, since the extent of quenching is directly proportional to quencher concentration. Therefore, Stern-Volmer plots completely free of IFE, and subject to only one type of quenching, are linear. A first estimate of the relative adequacy of correction, then, may be gained by comparing the linearities of the Stern-Volmer plots obtained after correcting with different equations. Over- or under-compensation by a correction equation will adversely affect this ultimate linearity.

A second, possibly more determinate, assessment of correction may be obtained from temperature data. Quenching interactions have a well defined temperature dependence, while IFE does not. Dynamic quenching increases with temperature because of an increase in fluorophore-quencher collision. Static quenching, on the other hand, decreases with temperature because of reduced stability of ground state complexes between quencher and fluorophore. In the former case, therefore, the slope of the Stern-Volmer plot increases

**Table 2.** Regression statistics for Stern-Volmer plots of quinine sulfate and DPA using all corrections and temperatures

| Correction | Temp/°C | Regression equation | $P$ | $R^2$ |
|---|---|---|---|---|
| Uncorrected | 4 | $Y = 1.04 + 0.008X + 0.001X^2$ | 0.0001 | 0.99 |
| | 22.5 | $Y = 1.06 + 0.004X + 0.001X^2$ | 0.0001 | 0.98 |
| | 40 | $Y = 1.12 - 0.004X + 0.002X^2$ | 0.0001 | 0.98 |
| Eq. (1) | 4 | $Y = 0.988 + 0.056X + 0.012X^2$ | 0.0001 | 0.83 |
| | 22.5 | $Y = 0.980 + 0.066X + 0.012X^2$ | 0.0001 | 0.89 |
| | 40 | $Y = 0.984 + 0.051X + 0.019X^2$ | 0.0001 | 0.96 |
| Eq. (2) | 4 | $Y = 0.9547 + 0.0152X$ | 0.0001 | 0.86 |
| | 22.5 | $Y = 0.9680 + 0.0170X$ | 0.0001 | 0.85 |
| | 40 | $Y = 0.9921 + 0.0206X$ | 0.0001 | 0.84 |
| Eq. (3) | 4 | $Y = 0.9580 + 0.0152X$ | 0.0001 | 0.86 |
| | 22.5 | $Y = 0.9660 + 0.0174X$ | 0.0001 | 0.86 |
| | 40 | $Y = 0.9915 + 0.0209X$ | 0.0001 | 0.84 |

with increasing temperature, while in the latter case it decreases. A Stern-Volmer plot comprising both quenching and IFE will have a relatively smaller temperature dependence than one that is fully corrected for IFE, because of the temperature independent contribution of IFE to the slope of the former. One may therefore compare the influence of temperature on the Stern-Volmer plots by examining the slopes at each temperatures. The largest variation of slope with temperature is indicative of the best correction.

The effect of temperature on corrected and uncorrected fluorescence intensity values may be complicated by temperature dependence of the absorptivities used in the correction equations. The absorptivity of thiourea was found to decrease with temperature, while that of DPA increased. In contrast, the absorptivity of quinine sulfate was found to be temperature independent. Absorptivities of solutions containing quinine sulfate in addition to one of the other two compounds, did not show measurable temperature dependence at the wavelengths of interest. Since these total absorptivities are the ones used in the correction equations, no temperature-related adjustments had to be made.

*Inner filter effect at the excitation wavelength*

Parker reports that IFE correction becomes inaccurate when concentrations of quencher result in $F_{corr}/F_{obs}$ values greater than 3.0. This was the case for thiourea, in which concentrations greater than 4.8 mg/l resulted in curvature of the Stern-Volmer plots towards the x-axis. Consequently, these concentrations were not included in regression equations for Stern-Volmer plots of quinine sulfate quenching.

Regression equations for Stern-Volmer plots of thiourea and quinine sulfate for all temperatures and corrections equations are listed in Table 1.

Stern-Volmer plots using uncorrected F values were curved (Fig. 4) while corrected plots were linear (Table 1). Corrections with Eqs. (1) and (3) resulted in the most linear Stern-Volmer plots (as determined by P and $R^2$ values). Temperature trends indicate an increase in slope (K) with increase in temperature for all corrections. This type of temperature dependence indicates a dynamic quenching component. The percent decrease in slope from 40° C to 4° C was calculated from the slopes of the Stern-Volmer plots with Eqs. (3) and (1) only, since these corrections resulted in more linear plots. Both temperature plots resulted in similar percent slope decreases: 18.9% for Eq. (3) and 19.2% for Eq. (1). This, along with similar linearities, indicates that they produce comparable corrections for IFE at the excitation wavelength.

*Inner filter effects at the emission wavelength*

Regression equations for Stern-Volmer plots of DPA and quinine sulfate for all temperatures and corrections are listed in Table 2. Stern-Volmer plots for uncorrected values were curved (Fig. 5), and plots using values corrected by Eq. (1) retained this curvature (Table 2). This indicates that Eq. (1) does not correct for IFE at the emission wavelength, which is expected since it does not contain a correction factor for emission absorbance. Stern-Volmer plots using values corrected by Eq. (3) and Eq. (2) exhibited similar linearities (as determined by P and $R^2$ values). Temperature trends indicate an increase in slope (K) with increase in temperature for all corrections. This type of temperature dependence again indicates a dynamic quenching component. The percent slope decrease from 40° C to 4° C for plots using values corrected with Eqs. (3) and (2) were 27.3% and 26.2%, respectively. Since both equations have similar linearities and percent slope change, both can be considered to correct effectively at the emission wavelength.

## Conclusion

Considerations of linearity and temperature response can be used to differentiate between IFE correction methods. They show that only Eq. (3) corrects adequately for IFE at both the excitation and emission wavelength of quinine sulfate, in a case where these can be considered separately. This should be equally true for situations where IFE occurs at both wavelengths. Equation (1) performs as well as Eq. (3) for excitation IFE, but it does not provide for emission IFE. Although Eq. (2) results in similar corrections as Gauthier's for emission IFE, it yields relatively poor Stern-Volmer linearity for excitation IFE.

*Acknowledgements.* Funding for this work was provided by Idaho State Board of Education and State and Federal funds appropriated to the University of Idaho and the Idaho Agric. Exp. Sta.

## References

1. Lakowicz JR (1983) Principles of fluorescence spectroscopy. Plenum Press, New York
2. Parker CA (1968) Photoluminescence of solutions. Elsevier, New York
3. Lutz HP, Luisi PL (1983) Helv Chim Acta 66:1929–1935
4. Leese RA, Wehry EL (1978) Anal Chem 50:1193–1197
5. van Slageren R, den Boef G, van der Linden WE (1973) Talanta 20:501–512
6. Franzen JS, Kuo I, Chung AE (1972) Anal Biochem 47:426–441
7. Gill JE (1970) Appl Spectrosc 24:588–590
8. Eastman JW (1967) Photochem Photobiol 6:55–72
9. Gauthier TD, Shane EC, Guerin WF, Seitz WR, Grant CL (1986) Environ Sci Technol 20:1162–1166