The Modern Student Laboratory:

Fluorescence Spectroscopy

Primary and Secondary Inner Filtering

Effect of $K_2Cr_2O_7$ on Fluorescence Emission Intensities of Quinine Sulfate

Sheryl A. Tucker, Vicki L. Amszi, and William E. Acree, Jr.
University of North Texas, Denton, TX 76203-5068

Fluorescence spectroscopy is an extremely versatile, sensitive experimental technique used in identification and quantification of many environmentally important compounds: polycyclic aromatic hydrocarbons, polycyclic aromatic nitrogen heterocycles, and polycyclic aromatic sulfur heterocycles. Through judicious selection of excitation and emission wavelengths, one can often analyze a single desired fluorophore in complex mixtures containing several absorbing and fluorescing species.

Over the past few years many laboratory experiments have appeared in *this Journal* (*1–7*) and standard laboratory manuals (e.g., ref *8*) involving determination of analyte concentrations based on spectrofluorometric methods. To our knowledge, only one (ref *7*) discussed primary and secondary inner filtering artifacts (solely from the standpoint of solute self-absorption) associated with accurate determination of fluorescence emission intensity data.

Inner Filtering

Inner filtering is a major problem associated with obtaining correct fluorescence data, which assumes that the sample is optically dilute (A cm^{-1} < 0.01) at all analytical wavelengths. Most commercial instruments use right-angle fluorometry, which reduces stray radiation by placing the emission detector at 90° with respect to the incoming excitation beam (see Fig. 1). Only fluorescence emission originating from the center interrogation zone of the sample cell is actually collected. Attenuation of the excitation beam before reaching the region viewed by the fluorescence detection optics (prefilter region) and through the interrogation volume element is denoted as primary inner filtering.

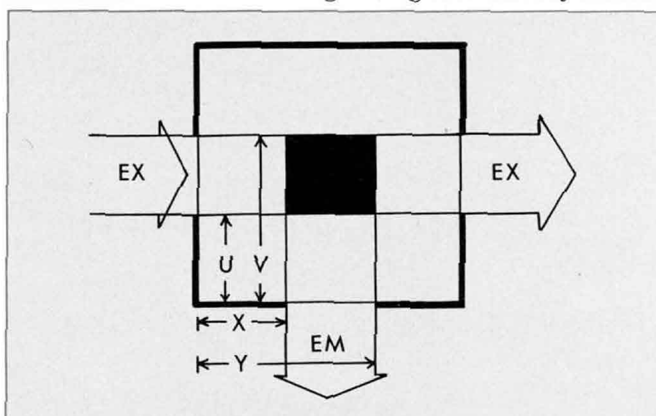

Figure 1. Typical cell configuration for right-angle fluorometry. Window parameters (x, y) and (u, v) are determined by masking apertures or some other limiting aperture in emission and excitation beam, respectively.

The correction factor f_{prim} in primary inner filtering (*9–11*) is given by

$$f_{prim} = \frac{F^{corr}}{F^{obs}} = \frac{2.303\,A\,(y - x)}{10^{-Ax} - 10^{-Ay}} \tag{1}$$

where F^{corr} and F^{obs} are the corrected and observed fluorescence emission signal; A is the absorbance per centimeter of pathlength at the excitation wavelength; and x and y denote distances from the boundaries of the interrogation zone to the excitation plane, as shown in Figure 1.

Equation 1 strictly applies to monochromatic light, which experimentally can not be obtained, even with the finest spectrofluorometers with small spectral bandpasses. Yappert and Ingle (*11*) derived a more rigorous mathematical treatment for nonmonochromatic excitation and emission beams. At the undergraduate level, the assumption of monochromatic beams greatly simplifies the computations and laboratory time needed to perform the required absorbance and fluorescence measurements.

Primary inner filtering can often be ignored in experiments that require the determination of intensity ratios, as the excitation wavelength remains constant (i.e., A in eq 1 remains constant). Emission intensities of both bands are thus affected by the same relative amount. This assumption may not be entirely true where large amounts of inner filtering are involved with the use of large cuvettes or with high absorbances (*12*). In extreme cases, highly absorbing solutions can prevent the excitation beam from ever reaching the interrogation zone.

Secondary inner filtering results from absorption of large quantities of emitted fluorescence. The correction factor f_{sec}

$$f_{sec} = \frac{F^{corr}}{F^{obs}} \approx \frac{(v - u)(1/b)\ln T}{T_{at\ v/b} - T_{at\ u/b}} \tag{2}$$

includes the sample transmittance (T) across the entire cell pathlength (b) at the emission wavelength (*10*). Transmittances at the two interrogation zone boundaries $T_{at\ v/b}$ and $T_{at\ u/b'}$ are calculated from the measured absorbance at the emission wavelength using the Beers–Lambert law. Remember that v/b and u/b now serve as the new cell pathlengths. The corrected fluorescence emission intensity is given by

$$F^{corr} = f_{prim}\,f_{sec}\,F^{obs} \tag{3}$$

(*Continued on page A11*)

Fluorescence Emission Intensity and Absorbance Data for Solutions Containing 4 ppm Quinine and Various Concentrations of $K_2Cr_2O_7$

				x = 0.45; y = 0.55 u = 0.45; v = 0.55			x = 0.40; y = 0.60 u = 0.40; v = 0.60		
$C_{K_2Cr_2O_7}$ (mg/mL)	Emission[a] Intensity	A^b (350 nm)	A^b (450 nm)	f_{prim}	f_{sec}	F^{corr}	f_{prim}	f_{sec}	F^{corr}
0.000	249	0.070	0.000	1.084	1.000	270	1.084	1.000	270
0.025	188	0.260	0.042	1.349	1.050	266	1.348	1.050	266
0.050	156	0.414	0.059	1.610	1.070	269	1.608	1.070	268
0.100	106	0.712	0.090	2.268	1.109	267	2.260	1.109	266
0.150	69	1.042	0.138	3.312	1.172	268	3.288	1.172	266
0.200	44	1.369	0.184	4.817	1.236	262	4.758	1.236	259

[a]Measured on a Shimadzu RF-5000U spectrofluorometer.
[b]Measured on a Bausch and Lomb Spectronic 2000.

assuming that primary and secondary inner filtering are independent processes.

Experimental Measurements

Experimentally, the effect of primary and secondary inner filtering can be easily illustrated by measuring the fluorescence emission signals at 450 nm of 4 ppm quinine solutions containing 0, 0.025, 0.050, 0.100, 0.150, and 0.200 mg/mL of $K_2Cr_2O_7$. Each solution is 0.05 M in sulfuric acid.

As shown in Figure 2, emission signals decrease significantly with increased $K_2Cr_2O_7$ concentration. By also determining absorbances of the six solutions at both 350 nm (excitation wavelength of quinine) and 450 nm (emission wavelength), it can be shown that primary inner filtering is largely responsible for the reduction in observed emission intensities. Secondary inner filtering is easy to show visually. Being orange in color, $K_2Cr_2O_7$ does absorb radiation at 450 nm. Representative values are listed in the table for both fluorescence and absorbance measurements.

Care must be taken to measure all emission intensities at the same wavelength, rather than the peak maximum. Inner filtering is greatest at the shorter wavelengths, thus shifting the "apparent peak maximum" slightly to longer wavelengths.

Correction Calculations

At this point in time, the effect that inner filtering has on fluorescence intensities has been shown. An attempt can be made to calculate f_{prim} and f_{sec} correction factors, but the volume and dimensions of the interrogation zone must be known. Realizing that most instrument manufacturers rarely supply information regarding the slit widths of the apertures of their sample compartments, particularly for the less expensive spectrofluorometers found in undergraduate laboratories, we elected to base f_{prim} and f_{sec} computations on assumed values of x, y, u, and v. Each laboratory group was instructed to select values of x and u from 0.40–0.48 cm, and y and v values from 0.52–0.60 cm to calculate the corrected fluorescence emission intensities of all six solutions.

The first solution without $K_2Cr_2O_7$ poses a problem, until students realize that $A = 0$ gives an indeterminant form for f_{sec}. Alternatively, one could expand the transmittances in the denominator as

$$10^{-A(v/b)} = 1 - 2.303\,A\,(v/b) + \frac{(2.303\,A\,(v/b))^2}{2!} - \frac{(2.303\,A\,(v/b))^3}{3!} + \ldots$$

and

$$10^{-A(u/b)} = 1 - 2.303\,A\,(u/b) + \frac{(2.303\,A\,(u/b))^2}{2!} - \frac{(2.303\,A\,(u/b))^3}{3!} + \ldots$$

remembering that

$$T_{\text{at } v/b} = 10^{-A(v/b)} \text{ and } T_{\text{at } u/b} = 10^{-A(u/b)}$$

Retaining only the first term of each expansion, it is easily shown that

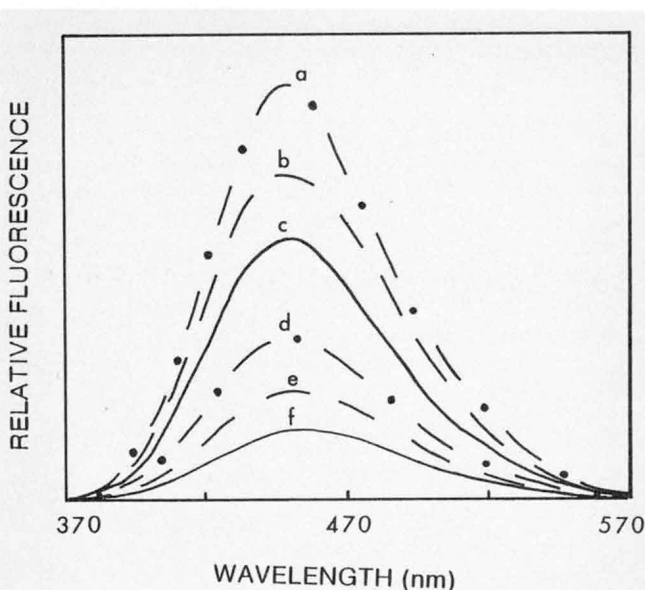

Figure 2. Effect of $K_2Cr_2O_7$ in the fluorescence emission intensities of 4-ppm quinine solutions. Spectra a–f correspond to $K_2Cr_2O_7$ concentrations of 0, 0.025, 0.050, 0.100, 0.150 and 0.200 mg/mL, respectively.

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$$f_{sec} = \frac{(v-u)(1/b) ln\ T}{T_{at\ v/b} - T_{at\ u/b}} = \frac{-(v-u)(1/b)\ 2.303\ A}{1 - 2.303\ A(v/b) - 1 + 2.303\ A\ (u/b)} = 1 \quad (4)$$

where f_{sec} equals unity at $A = 0$. Similarly, it can be shown that f_{prim} also equals unity at $A = 0$, though this latter condition is not really needed for the values given in the table.

Within ±3% or so, students generally obtain roughly the same corrected intensity, regardless of $K_2Cr_2O_7$ concentration. As noted by one reviewer, inner filtering corrections work well for f_{prim} and f_{sec} values that are less than 3. Inherent in this calculation is the assumption that inner-filtering is solely responsible for the reduced emission intensities, and that $K_2Cr_2O_7$ neither alters the peak wavelengths (unlikely based on Fig. 2) nor changes the fluorescence quantum yield through quenching reactions. At the concentrations studied, we observed only a 1-nm shift in the emission peak maximum, perhaps because the peak is fairly broad.

Conclusions

Although the computational method does incorporate several assumptions and is by no means perfect, it does familarize undergraduate students with an important aspect of fluorescence spectroscopy—inner filtering effects. The actual time required for performing the experimental measurements is relatively short.

The inner filtering study can be incorporated into much larger instrumental analysis laboratory experiments involving quantitative determination. For example, it can be done with previously published experiments for the determination of quinine in tonic water and the effect of pH on fluorescence properties of quinine (*4, 6, 8*). One previously published inner filtering study (*7*) is more of a "stand-alone" experiment and involved 2,3-butanedione dissolved in carbon tetrachloride, which is labelled as toxic and a cancer suspect agent. The quinine–$K_2Cr_2O_7$ study discussed above eliminates the potental health hazards imposed by carbon tetrachloride.

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