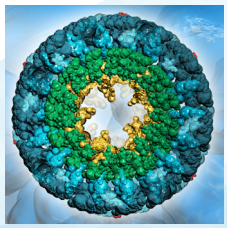
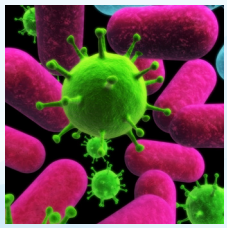
HORIBA Scientific

Fluorescence

Automatic Correction of Fluorescence Spectra for Primary and Secondary Inner-filter Effects (IFE) with Duetta™





Technical Note
Life Sciences

Introduction

Fluorescence is a highly sensitive spectroscopic technique that is used in a wide variety of scientific disciplines such as materials, life science, environmental and food sciences. The fluorescence intensity is linear with the concentration of a given molecule, but only at rather low concentrations. As the concentration increases the absorbance of light within the sample becomes too high, causing a loss of linearity. This absorbance limit depends wholly on the sample being measured, but is typically around 0.1 or 0.2 A.U. If samples in solution are not prepared carefully with this in mind, it is easy to exceed the linear absorbance range, causing measured fluorescence to be inaccurate. In this case, primary and secondary inner-filter effects (IFE) distort the measured fluorescence intensity. The resulting acquired spectra will be lower in intensity than expected and non-linear with concentration. Primary IFE occurs when the excitation light does not properly reach the center of a cuvette due to high absorbance of light from high concentration of the absorbing molecule(s). Secondary IFE also occurs when the emitted photons from the fluorescent sample are reabsorbed before reaching the emission detector, making the fluorescence intensity at the detector measure as lower than expected and also non-linear with the concentration. Secondary IFE is especially predominant in fluorescent molecules that have small Stokes shifts where the absorption and emission spectra have large overlap. The linear limit may be lower or higher than 0.1 A.U., depending on the sample composition (other absorbing species) and the spectral overlap of absorbance and emission spectra of a particular sample, requiring each sample type to be carefully evaluated to empirically determine this concentration limit.

To avoid the lack of linearity at increasing concentrations, scientists are advised to work at concentration ranges that are low enough so as not to experience any IFE. If your sample is sufficiently diluted, you will ensure you are working in a linear concentration range. By eye, however, a low concentration solution of fluorophore and a high concentration solution may look the same, causing some people to make erroneous measurements. This is more likely when working with fluorescent molecules that emit in the UV wavelength regions where absorbed light is not visible. There are methods to correct for IFE. For high concentration samples, both primary and secondary inner-filter effects on the fluorescence spectrum can be corrected using the absorbance spectrum of that sample (Lakowicz, 2006). Equation 1 defines Absorbance, A as:

$$A = -\log\left(\frac{I}{I_0}\right)$$

Equation 1: Absorbance calculated from incident light to the sample (I_0) and light transmitted through the sample (I).

Because Duetta is a 2-in-1 fluorescence and absorbance spectrometer, it can measure both fluorescence and absorbance spectra simultaneously, an IFE correction can be applied to each fluorescence signal and from this, accurate fluorescence spectra are obtained. Using the absorbance spectrum, the inner-filter effect correction is applied to the measured (observed) fluorescence intensity spectrum using Equation 2 below, where F_{corr} is the corrected fluorescence spectrum, F_{obs} is the measured or observed fluorescence intensity, A_{ex} is the absorbance at the excitation wavelength and A_{em} is the absorbance of the sample at the emission wavelength.

$$F_{corr} = F_{obs} * 10^{[(A_{ex}+A_{em})/2]}$$

Equation 2: Inner-filter effect correction to measured (observed) fluorescence intensity using the absorbance.

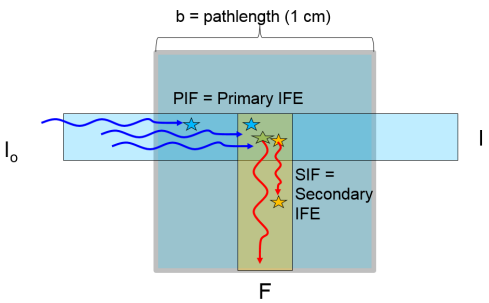


Figure 1: Diagram of a cuvette from the top showing incident light (I0), transmitted light (I) and the path of fluorescence detection at90 degrees (F). Primary and secondary inner-filter effects on the detected fluorescence intensity are shown with blue and yellow stars respectively.

Here, the difference between corrected and uncorrected fluorescence data is demonstrated by measuring quinine sulfate, an aromatic fluorophore found in tonic water, with some overlap between excitation and emission spectra. Figure 2 shows the graph of quinine sulfate absorbance and fluorescence emission measured below concentrations where IFE is an issue. The plots are normalized for spectral shape comparison.

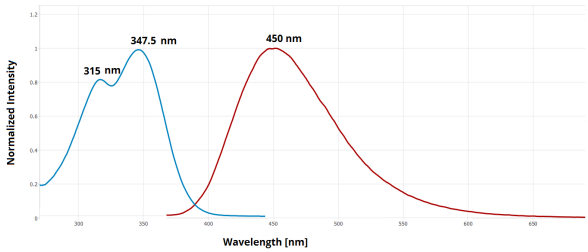


Figure 2: Absorbance (blue) and fluorescence emission (red) of quinine sulfate, normalized spectra. The concentration of quinine here is 14 ppm with absorbance below inner-filter effect requirement (< 0.1).

Quinine sulfate Absorbance-Transmittance Excitation Emission Matrices (A-TEEM™) are measured with, and without the inner-filter effect correction applied. The A-TEEM method measures the fluorescence emission spectrum for each excitation wavelength across the range of absorbance wavelengths, giving a 3D contour plot of excitation wavelength vs. emission wavelength vs. fluorescence intensity. For a traditional scanning fluorometer, this is referred to as an EEM. With the HORIBA A-TEEM method, the absorbance spectrum is also measured in the same experiment as the excitation wavelength scans across this same range, and also importantly, for the same sample volume and geometry. The absorbance spectrum is then used to correct the fluorescence EEM for IFE, resulting in a corrected fluorescence profile (A-TEEM). These A-TEEM contour plots clearly show the difference in shape and contour of the fluorescence of quinine sulfate across different excitation wavelength ranges when inner-filter effect correction is used. Without IFE correction, it is easy for the fluorescence spectrum of mid to high concentration samples to be measured inaccurately.

Even subtle differences in concentration of solutions of quinine sulfate cannot be detected by eye, as the absorbance and fluorescence of quinine sulfate are in the UV wavelength ranges, outside of the visible range. The image in Figure 3 shows two cuvettes with 14 and 86 µM quinine sulfate solutions and demonstrate this point.

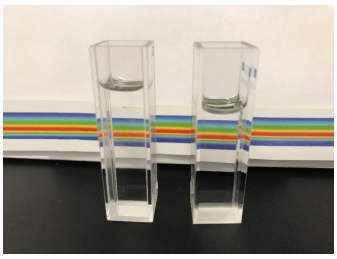


Figure 3: Two cuvettes with solutions of 14 µM quinine sulfate (left) and 86 µM quinine sulfate (right) in 0.1M perchloric acid (aq.) look similar by eye. Absorbance values at 349 nm are 0.08 and 0.49, respectively, and fluorescence spectra are different if not corrected for IFE.

Fluorescence EEMs are often used for molecular component analysis in a wide variety of applications. The EEM is analyzed with chemometrics software such as parallel factor analysis for component identification. A critical requirement for proper chemometric analysis is that a particular EEM molecular fingerprint is independent of concentration. For chemometric analysis, the A-TEEM molecular fingerprint, with its correction for IFE at higher concentrations, can be a much more robust fingerprint than a traditional fluorescence EEM.

Experiment and Results

Absorbance-Transmittance and Excitation Emission Matrices (EEM) were measured both with and without the inner-filter effect equation applied for six solutions of quinine sulfate at different concentrations (5, 14, 20, 38, 63, and 86 µM). These solutions have absorbance values which correspond to 0.03, 0.08, 0.12, 0.22, 0.36, and 0.49 absorbance units, respectively, at 349 nm.

Figure 4 shows absorbance spectra for all six samples. Solutions of quinine sulfate in 0.1M perchloric acid have an extinction coefficient of 5700 $M^{-1}cm^{-1}$ at 349 nm (Mielenz, 1980) (Eaton, 1988). Ultrapure water was used as a blank. Concentrations are obtained by first getting the absorbance spectra and then calculating concentration from those values using the Beer-Lambert law (Equation 3).

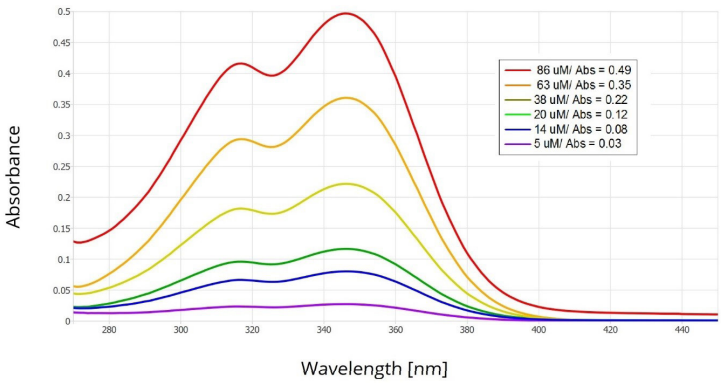


Figure 4: Absorbance spectra of six solutions of quinine sulfate in tonic water measured on Duetta 2-in-1 fluorescence and absorbance spectrometer with reported absorbance values at 349 nm.

$$A = \varepsilon * b * c$$

Equation 3: Beer-Lambert law where A is absorbance, ε is molar extinction coefficient in $M^{-1}cm^{-1}$, b is cell path length in cm and c is concentration in mol/L.

Absorbance-Transmittance Excitation Emission Matrices (A-TEEM) were collected on a Duetta spectrometer with excitation scanning from 270 to 450 nm, 2 nm step increment and 0.1 sec integration time for each emission spectrum ranging from 300 to 700 nm. All spectra are corrected for detector dark noise, spectral correction, and lamp intensity.

In Figure 5, the emission spectrum of quinine sulfate is plotted without correcting for IFE and with IFE correction applied. It is clear that without IFE correction, the spectral

shape is skewed, especially in the blue excitation regions where the absorbance is high and the spectral overlap is affecting the fluorescence intensity. The quinine sulfate EEM contour plots are shown in Figure 6 to show the differences in spectral shape as concentration increases from 14 µM to 86 µM with and without the IFE correction. The spectral shape of quinine sulfate is maintained accurately when the IFE correction is applied and is most obvious in the example of the 86 µM solution where the absorbance at 349 nm is close to 0.5. With IFE correction applied, the A-TEEM contours are similar at all concentrations.

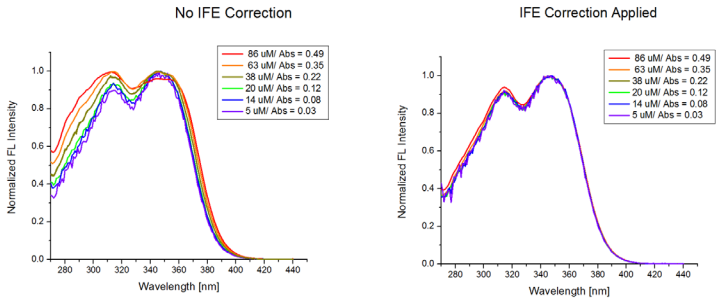


Figure 5: Normalized Excitation spectra of different concentrations of quinine sulfate without inner-filter effect correction (left) and with inner-filter effect correction applied (right). Absorbance values reported at 349nm.

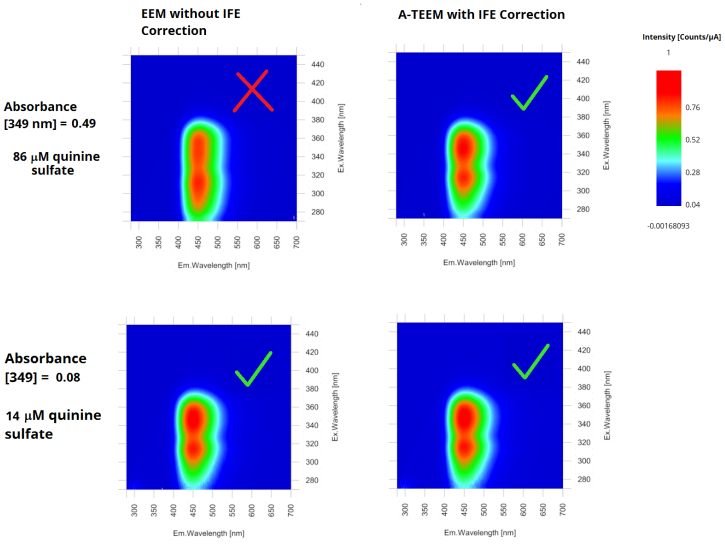


Figure 6: Fluorescence Excitation Emission Matrices of two concentrations of quinine sulfate in tonic water diluted in 0.1 M perchloric acid (aq.) with and without inner-filter effect corrections applied.

Summary

If the concentration of fluorescent molecules in a sample is even slightly too high, a measured fluorescence spectrum can easily be inaccurate without knowing it. Inner-filter effect correction is a useful tool for extending the dynamic range of an instrument to higher concentration solutions so that fluorescence data is collected easily and accurately. The Duetta 2-in-1 fluorescence and absorbance spectrometer is uniquely equipped to measure absorbance as well as fluorescence data on the same instrument and EzSpec software enables any user to apply the inner-filter effect correction so that fluorescence spectral data is correct every time.

References

Berlman, I. (1971). Handbook of Fluorescence Spectra of Aromatic Molecules. New York: Academic Press.

Eaton, D. (1988). Reference materials for fluorescence measurement. Pure Appl. Chem., 60, 1107-1114.

Lakowicz, J. R. (2006). Principles of Fluorescence Spectroscpy (3 ed.). Baltimore, MD: Springer.

Mielenz, R. V. (1980). Standard Reference Materials: A Fluorescence Standard Reference Material: Quinine Sulfate Dihydrate. National Bureau of Standards, National Measurement Laboratory. Washington, D.C. 20234: U.S. Department of Commerce.

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