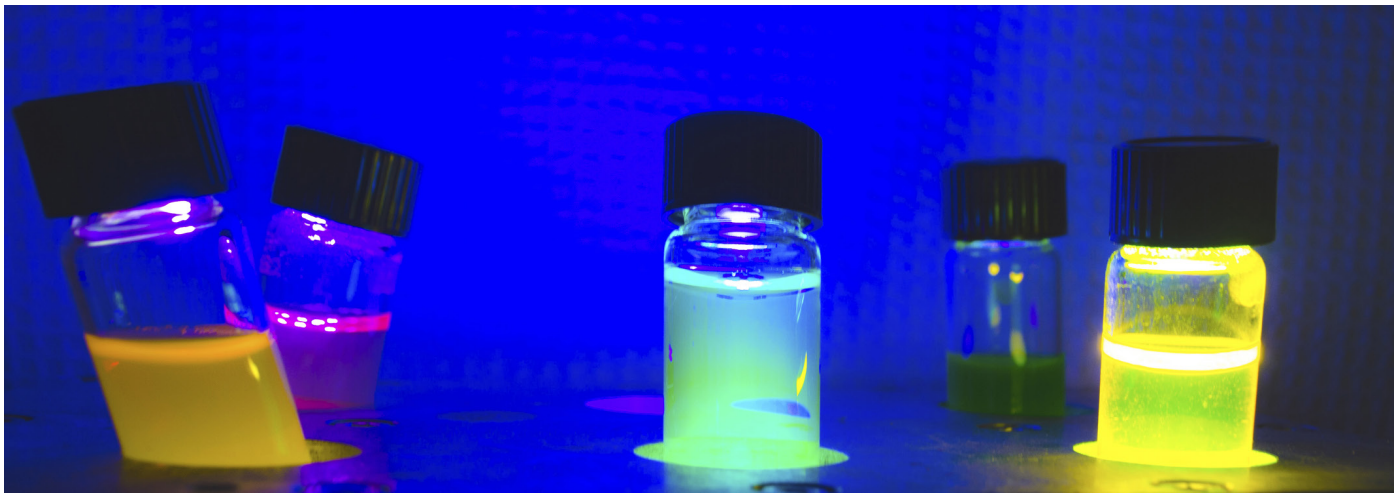
APPLICATION NOTE

MEASURING THE FLUORESCENCE QUANTUM YIELD OF FLUORESCEIN, EOSIN Y AND PHLOXINE B USING THE RELATIVE METHOD



INTRODUCTION

The absorption of a photon brings a fluorophore from the ground state (S_0) to a higher energy state (excited state S_n). If n > 1, the molecule relaxes very rapidly (within a few picoseconds or less) to the first excited state (S_1). What happens next depends on the molecular structure and surroundings. In many cases the deactivation (return to S_0 by loss of energy) occurs either by emission of a photon (fluorescence) or energy transfer to the surroundings (internal conversion or intersystem crossing) [1,2](**Figure 1**).

The fluorescence quantum yield (Φ_F) is an important photophysical property of fluorophores and is defined as the ratio between the number of photons emitted as fluorescence and the number of photons absorbed [1,2]. The magnitude of Φ_F, which is directly related to the intensity of the observed fluorescence, gives the probability of the excited fluorophore being deactivated by fluorescence rather than by nonradiative competing processes (internal conversion and intersystem crossing) [2,3].

Keywords:

Fluorescence Spectroscopy

Monochromatic Light Source

SENSE Spectrometer

Xanthene Dyes

Fluorescence Quantum Yield

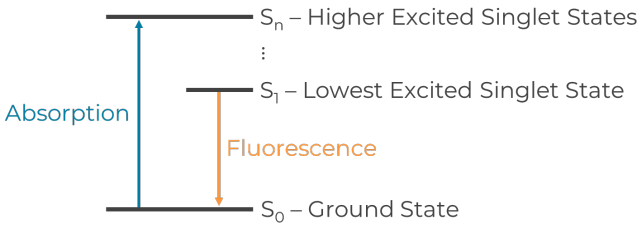


Figure 1 – Simplified Perrin-Jablonski diagram describing the electronic levels of common organic molecules.

Φ_F can be measured using relative or absolute methods. In the relative method, the Φ_F of the fluorophore of interest is measured by comparing its fluorescence intensity to that of a reference compound with known Φ_F and optical properties like those of the fluorophore of interest [1-3].

The relative method also requires knowledge of the absorbance of both the reference and fluorophore at the excitation wavelength. Unlike the absolute method, which requires an integrating sphere and allows measuring the Φ_F of solid and liquid samples, the relative method can be applied using conventional fluorometers with a single cuvette holder [2,4]. Despite its apparent simplicity, the relative method requires careful consideration of several experimental considerations, such as excitation and emission correction factors, Φ_F and spectral range of the reference compound, non-linear effects (inner filter effect), among others.

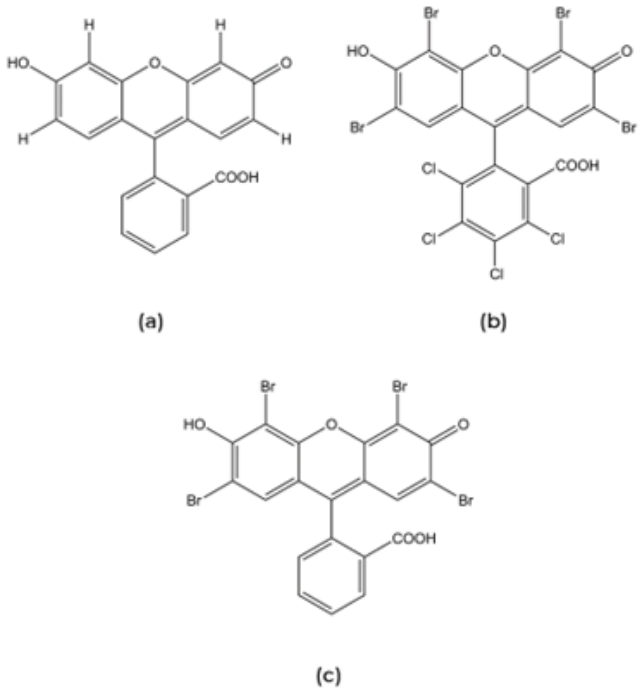


Figure 2 – Chemical structure of the samples used in this application note: **(a)** Fluorescein, **(b)** Phloxine B, and **(c)** Eosin Y.

In this application note, we use the relative method to determine the Φ_F of three well-known xanthene dyes (fluorescein, eosin Y, and phloxine B, see **Figure 2**) by combining a monochromatic 150 W xenon excitation light source with our SENSE spectrometer (non-corrected for the optical and detection components).

In this work, some experimental procedures, such as the neglect of correction factors, narrow excitation and emission range, identical acquisition parameters, and non-linear effect were considered for both our samples and reference compound (the reference selected was rhodamine 6G, which emits in the same wavelength region of our samples and has a Φ_F of 0.95 in ethanol) [5,6]. The results obtained here are then compared with those obtained using a fluorimeter where corrected spectra are used.

MATERIALS & METHODS

Reagents

- Fluorescein ($C_{20}H_{12}O_5$, Mw ≈ 332.12 g/mol, > 95%)
- Eosin Y ($C_{20}H_6Br_4O_5.Na_2$, Mw ≈ 691.86 g/mol, > 90%)
- Phloxine B ($C_{20}H_2Br_4Cl_4O_5.Na_2$, Mw ≈ 829.63 g/mol, > 90%)
- Rhodamine 6G ($C_{28}H_{31}N_2O_3Cl$, Mw ≈ 479.02 g/mol, > 95%)
- Ethanol (C_2H_5OH, Mw ≈ 46,07 g/mol, Spectroscopic Grade)

Instruments and Accessories:

- A 150 W Xenon light source coupled with a single monochromator
- Optical fibers with 1000 µm of diameter
- Multipurpose cuvette holder set into a fluorescence configuration
- SENSE Vis/NIR spectrometer (**Figure 3**)
- FAbS Spectrophotometer (**Figure 4**)
- 10 mm quartz fluorescence cuvettes

(1) (2)

Figure 3 – SENSE spectrometer (1), and multipurpose cuvette holder (2) were used to measure the fluorescence quantum yield of some xanthene dyes.

EXPERIMENTAL PROCEDURE

1. A few mg of each dye was dissolved in 10 mL of ethanol.

2. The absorption spectra of both standard and xanthene dyes were measured using the FAbS spectrophotometer (1 cm pathlength), see **Figure 4**. To avoid errors due to non-linear effects, such as the inner filter effect, the absorption of each dye at the excitation wavelength was set to a maximum of 0.06.

Figure 4 – The FAbS spectrophotometer was used to measure the absorption spectra of both reference and sample xanthene dyes.

3. The fluorescence emission of both reference and sample xanthene dyes was measured using the SENSE spectrometer. A narrow excitation bandwidth was obtained by combining the SENSE spectrometer with a xenon light source coupled with a monochromator.

4. The instrument's parameters used in the LightScan software were changed according to the dye measured. Each time the instrument's parameters were changed, the standard was remeasured.

RESULTS

The absorption spectra of the xanthene dyes whose quantum yield is to be measured are given in **Figure 5**.

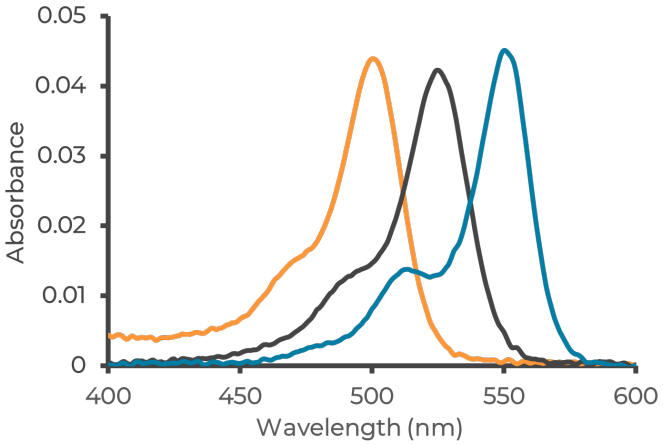


Figure 5 – Absorption spectra of **fluorescein**, **eosin Y**, and **phloxine B** in ethanol, measured with FAbS spectrophotometer.

The absorption spectra of the molecules studied here display typically a shoulder at lower wavelengths followed by a maximum at longer wavelengths. The red shift observed from fluorescein (orange line) to phloxine B (blue line) is consistent with the increase of halogen atoms in the molecular structure [2,5,6]. The fluorescence emission spectra of the molecules given in **Figure 2** is shown in **Figure 6**.

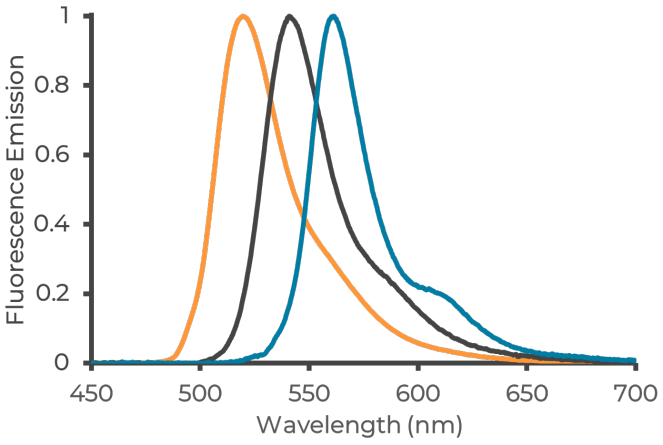


Figure 6 – Fluorescence emission spectra of **fluorescein**, **eosin Y**, and **phloxine B** in ethanol, measured with SENSE spectrometer.

The fluorescence spectra given in **Figure 6** displays a mirror-image relationship with respect to the absorption ones (the maximum is now observed at lower wavelengths while the shoulder is located at longer wavelengths).

The addition of heavy atoms like Cl, Br and I to the structure has an expected effect on the fluorescence efficiency (the well-known Heavy-atom Effect), with the fluorescence being strongly reduced [2,5], hence decreasing the Φ_F [2,5].

The Φ_F of fluorescein, eosin Y, and phloxine B was determined using the relative method, given by **Equation 1** (rhodamine 6G was used as quantum reference),

$$(\Phi_F)_S = (\Phi_F)_R \times (A_R/A_S) \times (E_S/E_R) \times (n_S/n_R)^2 \text{ (eq. 1)}$$

where $(\Phi_F)_S$ and $(\Phi_F)_R$ are the fluorescence quantum yield of the sample and quantum reference, respectively, A_S and A_R are the absorbance of the sample and quantum reference at the excitation wavelength, respectively, E_S and E_R are the integrated emission of the sample and quantum reference, respectively, and n_S and n_R are the refractive indexes of the solvent of the sample and reference, respectively. If the same solvent is used for both sample and quantum reference then **Equation 1** can be simplified to **Equation 2**,

$$(\Phi_F)_S = (\Phi_F)_R \times (A_R/A_S) \times (E_S/E_R) \text{ (eq. 2)}$$

The Φ_F of fluorescein, eosin Y, and phloxine B were obtained using **Equation 2** and are presented in **Table 1** (in this work, ethanol was used for the reference and for all samples).

Table 1 – Φ_F of fluorescein, eosin Y, and phloxine B obtained with **Equation 2**. Absorption and fluorescence emission were recorded using FAbS and SENSE spectrometer, respectively.

Sample	Φ_F (%) Calculated	Φ_F (%) Reference	Excitation Wavelength*
Fluorescein	89 ± 4	-	495 ± 3 nm
Eosin Y	63 ± 4	65 [7]	495 ± 3 nm
Phloxine B	65 ± 5	-	535 ± 3 nm

* for both sample and reference

The results obtained with our SENSE spectrometer were compared with those obtained in a standard fluorimeter with corrected spectra (**Table 2)**.

Table 2 – Φ_F of fluorescein, eosin Y, and phloxine B obtained with **Equation 2**. Absorption and fluorescence emission were recorded using with FAbS and a standard fluorimeter, respectively.

Sample	Φ_F (%) Calculated	Φ_F (%) Reference	Excitation Wavelength*
Fluorescein	87 ± 1	-	510 ± 3 nm
Eosin Y	66 ± 2	65 [7]	510 ± 3 nm
Phloxine B	64 ± 3	-	510 ± 3 nm

* for both sample and reference

The Φ_F values obtained with our SENSE spectrometer are very similar to those obtained with a standard fluorimeter, whose emission spectra were corrected for the response of the gratings, detector, and other optical components. For eosin Y, the Φ_F values calculated in this application note are in agreement with the published value [7].

The results given in both **Table 1** and **2** indicate that, when combined with a monochromatic excitation source, our SENSE spectrometer can be used to measure fluorescence quantum yields. With SENSE spectrometer, the accuracy of the Φ_F value depends on the spectral range and fluorescence intensity of both sample and reference (the spectral range must be similar).

CONCLUSIONS

The work presented in this application note aims to demonstrate how SENSE spectrometer can be used to measure fluorescence quantum yields.

The combination of this equipment with a monochromatic excitation light source allows for an important application when dealing with fluorescence measurements.

Important experimental considerations such as a similar excitation wavelength and spectra range for both sample and quantum reference must be considered to yield more accurate results.

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