Fluorescence Spectroscopy
Chemistry 465

I. Overview

Fluorescence spectroscopy is one the most sensitive spectroscopic analytical techniques available. It is applicable only to those analytes which exhibit strong fluorescence signals in accessible portions of the electromagnetic spectrum (i.e. those that have a high ***quantum efficiency*** for fluorescence). The fact that only a select number of species fluoresce can be used to our advantage by using fluorescence as a **selective** technique. For example, the number of compounds in a sample that have strong enough chromophores to absorb UV light may make it quite difficult to quantitate specific analytes. However, since only a few will fluoresce, and each one will have a distinct fluorescence emission spectrum (and lifetime), this spectroscopic tool can be used for both sensitive and selective detection. Alternatively, we can convert non-fluorescent analytes into fluorescent ones by a variety of chemical reactions (e.g. derivatization).

The purpose of this experiment is to gain a better understanding of fluorescence spectroscopy by determining the quantum efficiency of fluorescein. We will use quinine sulfate as a standard since it has a well known quantum efficiency.

Before we can carry out our fluorescence measurements, we need to learn a little bit about the spectrophotometric properties of our analytes. This means measuring the excitation and emission spectra of fluorescein and quinine.

II. Background

UV/VIS absorption measurements use the log of the ratio of the incident (i.e. P_0) and transmitted (i.e. P) light intensity to represent the response (recall $A = -\log[P/P_0]$. Fluorescence spectrophotometers simply measure the fluorescence intensity emitted from the sample. The fluorescence signal is reported in arbitrary "fluorescence units" which is really just the output signal from the detector employed by the fluorometer. As we have found with absorbance signals, the fluorescence signal is proportional to concentration. The fluorescence response follows the following relationship:

$$F = 2.303 I_0 \Phi_F f(\theta) g(\lambda) \varepsilon bc \qquad \text{Equation 1}$$

where

- I_0 is the intensity of the excitation beam
- ϕF is the quantum efficiency = the fraction of photons emitted per photon absorbed,
- $f(\theta)$ is a geometrical factor that takes into account the optical collection efficiency of the instrument.
- $g(\lambda)$ is the wavelength-dependent detector response.
- εbc is the absorbance

In practice, we employ a set of standard solutions to measure the relationship between the fluorescence signal and concentration (i.e. we don't actually measure the parameters in Equation 1. However, in some applications (can you suggest any?) one may want to determine the fluorescence quantum efficiency ϕ_F. In order to determine ϕ_F, a reference fluorophore with known ϕ_F is used, thereby allowing determination of the other factors involved in the fluorescence yield. In this experiment, we will use quinine sulfate as our standard. According to the literature, quinine sulfate has a ϕ_F of 0.51 at a λ_{ex} of 350 nm in 0.1 N H_2SO_4. So, we can use quinine sulfate and Equation 1 to determine the quantum efficiency of another compound (fluorescein).

To determine the quantum efficiency of fluorescein, we first need to determine the molar absorptivities (ε) of the solutions using either the Agilent 8453 or the SII 440. We can then obtain the fluorescence spectrum and determine the fluorescence yield (ϕ_F) by integration of the fluorescent peak area.

II. Procedure

A. Make up a solution of ~100 mg/L quinine sulfate (QS) in 0.1 N H_2SO_4 (density of conc. H_2SO_4 = 1.8305 g/mL) and a separate solution of ~1X10^{-5} M fluorescein (FS) in 0.1 N NaOH. Record the exact concentrations of each solution.

B. Use either the Agilent 8453 OR the SII 440 to obtain the absorbance spectra of the QS and FS solutions (say from 220-600 nm). Use the appropriate acid or base solution for the blank. Make sure that the absorbances at 350 nm for QS and 366 nm for FS are in the appropriate range. Although 366 nm is not the peak absorbing wavelength for fluorescein, it absorbs at this wavelength and it is close to 350 nm, making it safe to assume that the $f(\lambda)$ and $g(\theta)$ terms (Equation 1) are the same for both species. Record the absorbances. These data will be used to determine ε in Equation 1.

C. The concentrated QS and FS solutions used to obtain the absorbance measurements will likely be well off scale on the ISS K2 fluorometer and need to be diluted prior to acquiring the fluorescence measurements. Start with solutions in the μM range. Make dilutions with the original solvent solutions (0.1 N H_2SO_4 or 0.1 N NaOH). Obtain fluorescence spectra of the dilute QS and FS solutions using the following paramters: For QS: λ_{ex} = 350 nm, 10 nm bandpass, λ_{em} = 360 – 800 nm; for FS: λ_{ex} = 366 nm, 10 nm bandpass, λ_{em} = 380-800 nm. Check to make sure that the signals do not go off-scale (if so, further dilution will be necessary - make sure you keep track of all dilutions as they will need to be accounted for in the final calculations). Also, make sure the spectra were successfully saved to a file. Since the fluorometer is very sensitive, take care to wash the cuvet carefully so that no contamination or carry-over from the more concentrated solutions occurs.

D. Obtain background fluorescence spectra using the appropriate solvents with the same settings used to obtain the QS and FS spectra. Save these 2 spectra (you will need to subtract the background spectrum from the QS and FS spectra to eliminate

contributions from water Raman and scattered light. These change over time, so make the background scan close to the same time as the other scans.

E. Use the appropriate software (IIS K2 or Excel) to 1) subtract the background spectra from the sample spectra and 2) integrate the background-corrected fluorescence spectra for both QS and FS. The integration of the background-corrected fluorescence spectra yields a number that is proportional to the total number of photons emitted. These integration values will be used later on to calculate the ϕ_F.

III. Results and Discussion -

A. Include the organic structures in your report.

B. Include absorbance and fluorescence spectra for QS and FS.

C. Put the results into a single table (conc. for UV/VIS, absorbance, molar absorptivity, conc. for fluorescence measurements, fluorescence area, quantum efficiency, anything else deemed appropriate).

D. Using Equation 1 and the results from your lab, determine the quantum efficiency of fluorescein in 0.1 N NaOH.

E. Explain the origin of molecular fluorescence and give reasons why ϕ_F is typically less than 1 and often 0 for many compounds.

F. Discuss possible external (environmental) conditions that can affect fluorescence yields.