Comparison of the Performance Characteristics of UV Absorbance and Molecular Fluorescence Spectrophotometry.

We have discussed the relative merits of absorbance and fluorescence techniques, particularly in the context of detection for separations. In this lab experiment, you will do a direct comparison of the two methods by investigating several figures of merit for the pair of methods. For this exercise, you will collect spectra of a fluorescent compound at a wide range of concentrations and explore the nature of fluorescence emission and UV absorbance spectra and the analytical scope of each method.

A Brief Introduction to Fluorescence:

Fluorescence is the emission of radiation from an atom or polyatomic species after the substance has been exposed to electromagnetic radiation. The incident radiation results in excitation of the substance. Fluorescent radiation is emitted during de-excitation of the substance

A fluorometer is a device which measures fluorescence. Radiation from a source passes through either a filter (the primary filter) or a monochromator (the excitation monochromator) which limits the width of the wavelength band. The radiation subsequently travels through the cuvette which contains the sample solution and causes excitation of the sample. Although the resulting fluorescent radiation is emitted in all directions from the cuvette, customarily only that portion of the radiation which is emitted perpendicularly to the incident radiation is measured. By measuring the fluorescent radiation at 90° from the incident radiation, interference from the incident radiation is minimized.
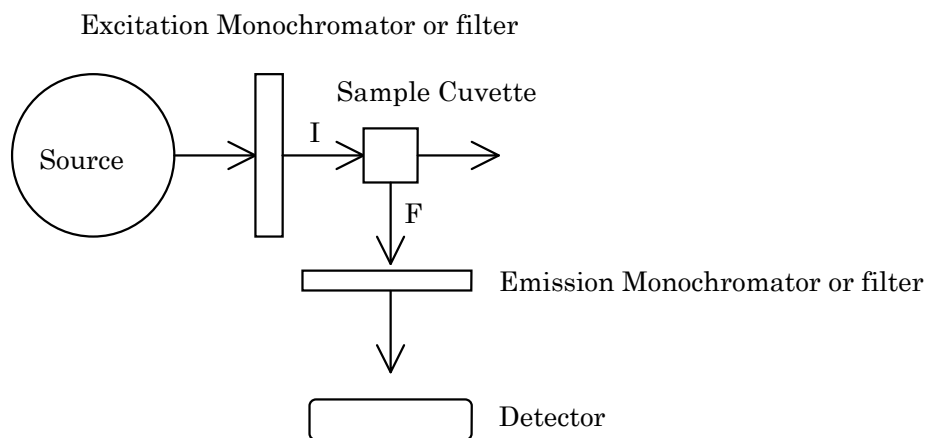

Figure 1. Diagram of the major parts of a fluorometer. The arrows indicate the paths of the EMR. I, incident radiation; F, fluorescent radiation.

The fluorescent radiation passes through a second filter (the secondary filter) or monochromator (the emission monochromator) and strikes a detector. The filter or monochromator serves to allow the fluorescent radiation to strike the detector while preventing scattered, exciting radiation from reaching the detector. The detector is a device which measures the intensity of the fluorescent radiation. If the fluorometer contains filters it is called a filter fluorometer. If it contains monochromators, it is called a spectrofluorometer.

Spectrofluorometers can be used to obtain fluorescent spectra, whereas filter fluorometers usually cannot be used to obtain spectra. A fluorescent spectrum which is obtained by holding the emission wavelength constant while varying the excitation wavelength is an <u>excitation spectrum.</u> A spectrum which is obtained by holding excitation wavelength constant while varying the emission wavelength is an <u>emission spectrum.</u>

In dilute solutions the intensity of fluorescent radiation is directly proportional to the concentration of the fluorescing substance. Since relative fluorescent intensities rather than absolute fluorescent intensities are usually measured, an equation, such as Beer's law for absorbance measurements, normally is not used to directly calculate the concentration of a fluorescent species from its fluorescent intensity. Either the working curve method or the standard addition technique generally is used to determine the concentration of a sample solution from its relative fluorescent intensity.

Prior to Lab:
- Read the standard operating procedures on chemlab.truman.edu corresponding to the Cary50 UV-Vis Spectrophotometer and the Horiba Spectrofluorimeter.
- Set up calculations to determine the concentrations of solutions A-M as they are described below.

Solution Preparation:

Your instructor will assign you an analyte. Dissolve approximately 0.050 g (measured to the nearest 0.1 mg) of your analyte in 100.0 mL of 18 $M\Omega$ water. Label this solution **A**. You will use solution A to prepare several additional solutions via serial dilution by mass as shown in the table below. Prepare solutions B-M using plastic vials and a top-loading balance. For example, to prepare solution B, dilute approximately 10 g of solution A to approximately 20 g total mass (use a top-loading balance). Record the actual masses used and calculate the concentration of the resulting solutions. You may assume a density of 1.00 g/mL for these dilute aqueous solutions.

Dilute Solution	Mass of Concentrated Solution	Concentrated Solution	Dilute to
	(g)		(g)
B	10	A	20
C	2	A	20
D	5	C	10
E	2	C	20
F	5	E	10
G	2	E	20
H	2	G	20
I	2	H	20
J	2	I	20
K	2	J	20
L	2	K	20
M	2	L	20

Measurements:

A. Determination of Optimum Excitation and Emission Wavelengths
1. Using solution G, collect an excitation scan from 200-320 nm, while monitoring the emission at 400 nm. (Adjust the slit settings so that the maximum detector signal is ~10^5 counts.)
2. From the excitation spectrum, determine the wavelength for maximum excitation efficiency, $\lambda_{max,ex}$.
3. Collect an emission spectrum by scanning the emission monochromator from 350-600 nm while holding the excitation wavelength at the $\lambda_{max,ex}$ chosen above. (Again adjust the slit settings so that the maximum detector signal is ~10^5 counts.)
4. From the emission spectrum, determine the wavelength of maximum emission, $\lambda_{max,em}$.
5. Verify with your instructor that you have chosen reasonable wavelengths.

B. Determination of Wavelength of Maximum Absorbance
1. Using the Cary50 spectrophotometer, collect an absorbance spectrum of solution G from 200-600 nm. Use 18 MΩ water as your blank.
2. Record the wavelength of maximum absorbance for this spectrum (λ_{max}). Verify with your instructor that you have chosen a reasonable wavelength.

C. Collection of Emission and Absorbance Spectra of Sample Solutions and Blanks
:
1. For each solution A-M, collect an emission spectrum in the range from 350-600 nm, using the slit settings and optimum excitation wavelength determined in part A. Record the emission intensity at $\lambda_{max,em}$.
2. For each solution A-M, collect an absorbance spectrum in the range of 200-600 nm. Record the absorbance at the λ_{max} you determined in part B.
3. Rinse the cuvette well with water and collect three emission spectra and three absorbance spectra of a water blank. These will be used to determine the precision of each measurement.
4. Export the emission and absorbance spectra to files that can be read using Excel. For fluorescence data, this will involve copying and pasting the raw spectral data into an Excel worksheet. For absorbance spectra, the Cary software will export the spectra as text files.

Data Analysis:

1. Prepare plots of the data such that the linear dynamic range, sensitivity and detection limit can be determined.
2. Prepare a plot that shows emission spectra for solutions A, D, G, J and M on the same axes, with the data offset for clarity.
3. Prepare a plot that shows absorbabce spectra for solutions A, D, G, J and M on the same axes, with the data offset for clarity.
4. Identify the linear dynamic range for each technique. How do the LDRs compare for the two techniques?
5. Calculate the sensitivity of the linear portion and evaluate the detection limit for each technique. How do the sensitivities and LOD's compare for the two techniques?
6. Describe the principles that limit the linearity (and general behavior) of each technique at high and low concentrations.