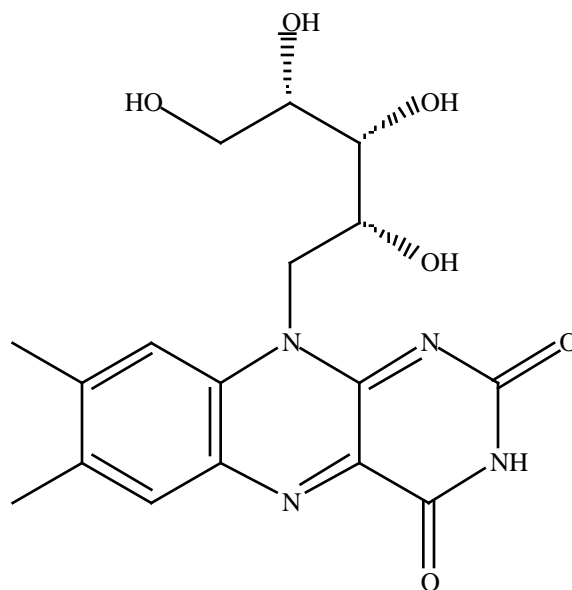


Analysis of Riboflavin in a Vitamin Pill by Fluorescence Spectroscopy

Objectives

In this lab, you will use fluorescence spectroscopy to determine the mass of riboflavin in a vitamin pill. Riboflavin fluorescence is extremely sensitive to its environment. It is sensitive to pH, presence of oxidizing species, and exposure to light. These “matrix effects” can be difficult to correct for in quantitative analyses by direct measurement of their influences on fluorescence intensity. A method of sample preparation known as the standard addition method will be used in this analysis. This method corrects for matrix effects.

Background



riboflavin

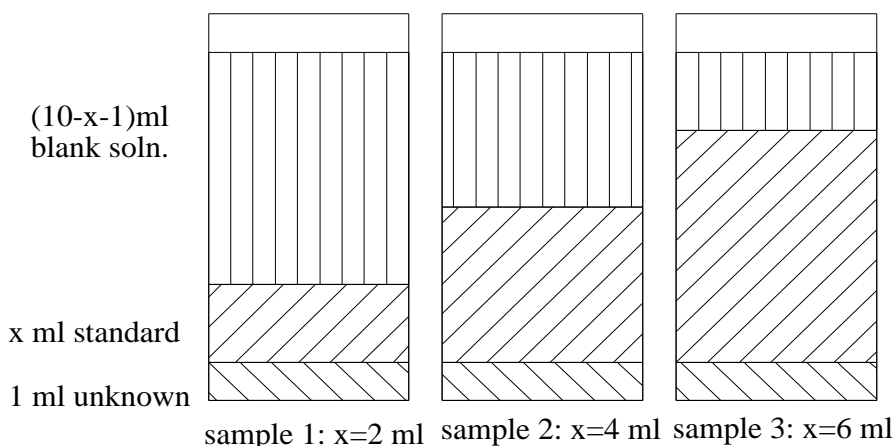
At room temperature, Riboflavin (Vitamin B₂) can be promoted to various excited vibrational levels within the first electronic excited state by absorption of UV and blue light. The absorption process occurs in $\sim 10^{-15}$ seconds (femtoseconds). Absorption is followed by vibrational relaxation, whereby a thermal population distribution of excited molecules is re-established such that they accumulate in the lowest vibrational levels of the electronic excited state. Vibrational relaxation is nearly complete within $\sim 10^{-11}$ sec (~ 10 picoseconds) following absorption. The electronically excited molecules “relax” to the various vibrational levels of the ground state through a spontaneous emission process in $\sim 10^{-7}$ - 10^{-9} sec (~ 1 -100 nanoseconds), resulting in a green fluorescence.

Fluorescence spectroscopy is widely used in biomedical analyses because it has several advantages over absorption spectrometry. Some of these advantages include:

- (1) It is more selective since only a small subset of absorbing molecules fluoresce, and it has two spectral variables; the excitation and emission wavelength,

- (2) It is more sensitive since the detector has only to sense the fluorescence radiation, whereas in absorption measurement the detector must sense the small absorption difference between blank and the sample. Detection methods that have a signal level of “zero” when the analyte concentration is zero are known as “zero background” techniques.

Fluorescence is generally sensitive to environmental factors, such as solvent polarity, hydrogen bonding, temperature, pH, oxidation, and reduction. Riboflavin, for example, shows nearly constant fluorescence from pH 4-8, but is nearly 100% quenched if the pH is raised to 10, or if the molecule is reduced. Consequently, analytical fluorescence measurements are prone to errors resulting from environmental influences on fluorescence intensity. In this lab, you will perform an analysis of riboflavin in a vitamin pill using the **STANDARD ADDITION METHOD**. This is a method of standard preparation that is extremely useful for overcoming complicated environmental influences on analytical signals. To use the standard addition method several samples are prepared, each containing the same volume (V_{unk}) of the analyte solution having concentration C_{unk} of the analyte of interest. A known, but varying volume of a standard solution (V_{std}) is added to each of these, and then each is diluted to the same final volume using the blank for the standard solution.



Consider the composition of each sample to be measured. Each contains an identical volume of the unknown solution diluted with standard and blank, so each solution contains all of the constituents of the unknown sample matrix in the same concentration!! Therefore, the effect of any matrix component will be the same for each sample that is measured. Each sample also contains the analyte of interest, though in different concentrations. The concentration of the analyte of interest is the sum of the concentrations due to the unknown and the standard additions to each sample, and can be calculated as follows:

$$[\text{analyte}] = \frac{C_{unk} V_{unk}}{V_t} + \frac{C_{std} V_{std}}{V_t} \quad (1)$$

where C_{unk} and V_{unk} are the unknown analyte concentration and volume, C_{std} and V_{std} are the standard analyte concentration and volume, and V_t is the total volume of the sample.

The standard addition method will allow us to make measurements of standards in a solution having the same matrix composition as the unknown sample. **This method works for any analytical signal** (i.e., measured quantity or function of a measured quantity) **that is linearly proportional to the concentration of the analyte of interest.** The fluorescence intensity (F) of an optically dilute (Absorbance < 0.05) fluorescent sample is

$$F = 2.3 K \phi P_0 \epsilon bc = Kc \quad (2)$$

where K is an instrument constant, ϕ is the fluorescence quantum yield, P_0 is the incident excitation power, ϵ is the molar extinction coefficient, and c is the analyte concentration. Because all of these factors are constant for a particular sample and a particular set of instrumental conditions, they can be condensed into a single constant, K . Thus, **the standard addition method can be applied to fluorescence spectrometry.** Substituting equation 1 into equation 2 (i.e., $c = [\text{analyte}]$) gives

$$F = K \frac{C_{unk} V_{unk}}{V_t} + \frac{C_{std} V_{std}}{V_t} \quad (3)$$

Note that we can write F as a linear function of V_{std}

$$F = b + m V_{std} \quad \text{where} \quad m = \frac{K C_{std}}{V_t} \quad \text{and} \quad b = \frac{K C_{unk} V_{unk}}{V_t} \quad (4)$$

C_{unk} can now be calculated as

$$C_{unk} = \frac{b C_{std}}{m V_{unk}} \quad (5)$$

Equation 5 is our final result. We are now in a position to establish a procedure for using fluorescence with the standard addition method using the following steps:

1. Prepare a series of unknowns with addition of varying volumes of a standard of known concentration C_{std} . (See illustration on previous page)
2. Measure the fluorescence intensity of each of the solutions prepared in step 1.
3. Plot F versus V_{std} , the volume of standard added to each solution. Find the slope and y-intercept of the plot, and use these values to calculate C_{unk} from equation (5).

Procedure

I. Excitation and Emission Spectra of Riboflavin

- A. Volumetric glassware must be used for preparation of a standard solution. Prepare a 0.10 ppm riboflavin standard solution by the following procedure. First, prepare 50 ml of a 1 ppm riboflavin solution in 0.02 M acetic acid from the 100 ppm stock solution. Next, prepare 100 ml of a 0.10 ppm standard solution for use in the quantitative analysis of a vitamin pill by diluting 10 ml of the 1 ppm solution to 100 ml with 0.02 M acetic acid.
- B. Fill a quartz cuvette 2/3 full with the 1 ppm riboflavin solution, and place this sample in the cuvette holder. Set the scan rate to 20 nm/div. Set both excitation and emission slits to 8 nm and the SAMPLE SENSITIVITY to about 30. With the excitation wavelength set at 340 nm, manually scan the emission grating starting at 360 nm to locate the emission band. (i.e. Watch for a needle deflection.) Adjust the SAMPLE SENSITIVITY or the 100% SAMPLE ADJUST so that near maximum deflection is obtained at that emission maximum.
- C. Make the following measurements
 - 1a. **Examine the absorbance spectrum of riboflavin on page 8 of the procedure. What excitation wavelength would you choose to perform an analysis of riboflavin based on this spectrum?**
 - 1b. *Measure three emission spectra; use excitation wavelengths of 340, 320, and 300 nm and start emission scans at 360, 340 and 320 nm, respectively. Excite at the longest wavelength first. End the emission scan at 600 nm. Record all three spectra on one spectrum paper by rewinding the chart paper, matching up the corresponding wavelengths, to overlap the scans.*
 2. *Measure and record the wavelength of maximum emission intensity of each spectrum. Identify the Raman scattering peak of each spectrum.*
 3. *Fix the emission wavelength to the emission maximum, and obtain an excitation spectrum. Scan from 290 nm toward longer wavelengths until you reach the end of the band (~525 nm) with a SAMPLE SENSITIVITY of 10. Record the excitation maximum.*
- D. *4. Using the results of part C.3, choose appropriate excitation (use the excitation maximum) and emission (use the emission maximum) wavelengths for riboflavin analysis of the vitamin pill.*

II. Quantitative Vitamin Pill Analysis

- A. *Blank measurement:* UV sensitive contaminants may get into your sample from several sources: the plasticizers in plastic and rubber tubing; impurities in “reagent grade” chemicals; and material leached by the solvent from apparently clean glassware. You must always run a solvent blank in order to make sure that impurities are not contributing to the total signal. Place the filter selector in the “S” position and insert the 0.02 M acetic acid blank solution in the spectrometer. Close the lid and move to the “.” position. Measure the emission spectrum

using the excitation wavelength determined in section I.D to search for fluorescence in the blank. Scan from the (excitation wavelength + 10 nm) to 600 nm.

5. **Is there observable fluorescence in the blank solution?** If not then you can assume that the solvent does not contribute to the total fluorescence intensity of the solutions to be analyzed.

B. Weigh several vitamin pills to determine the average mass of a pill. Select one pill for analysis and weigh it.

6. *Record the average mass and standard deviation of the vitamin pills. Record the mass of the pill that will be analyzed.* Crush the vitamin pill using a mortar and pestle, and weigh the resulting powder.

7. *Record the mass of the powder, and deliver it quantitatively to a 1 liter volumetric flask.* **Is the total mass of the original pill recovered? If not be sure to account for the loss in your final calculation of concentration.**

The following procedure has been developed for a vitamin pill that is reported to contain 5 mg riboflavin. A final concentration of 0.5 ppm is desired. Adjust the procedure as necessary to arrive at 0.5 ppm. Dissolve the powder in 1 liter 0.02 M acetic acid. Allow it to sit for a while to be certain that all of the riboflavin is dissolved. (Solids including fillers used in manufacturing the pill may not completely dissolve.) Dilute 10 ml of this sample to 100 ml with 0.02 M acetic acid so that its concentration is around 0.5 ppm.

8. *Record the dilution factor so you can calculate the measured riboflavin concentration in the vitamin pill from your results.*

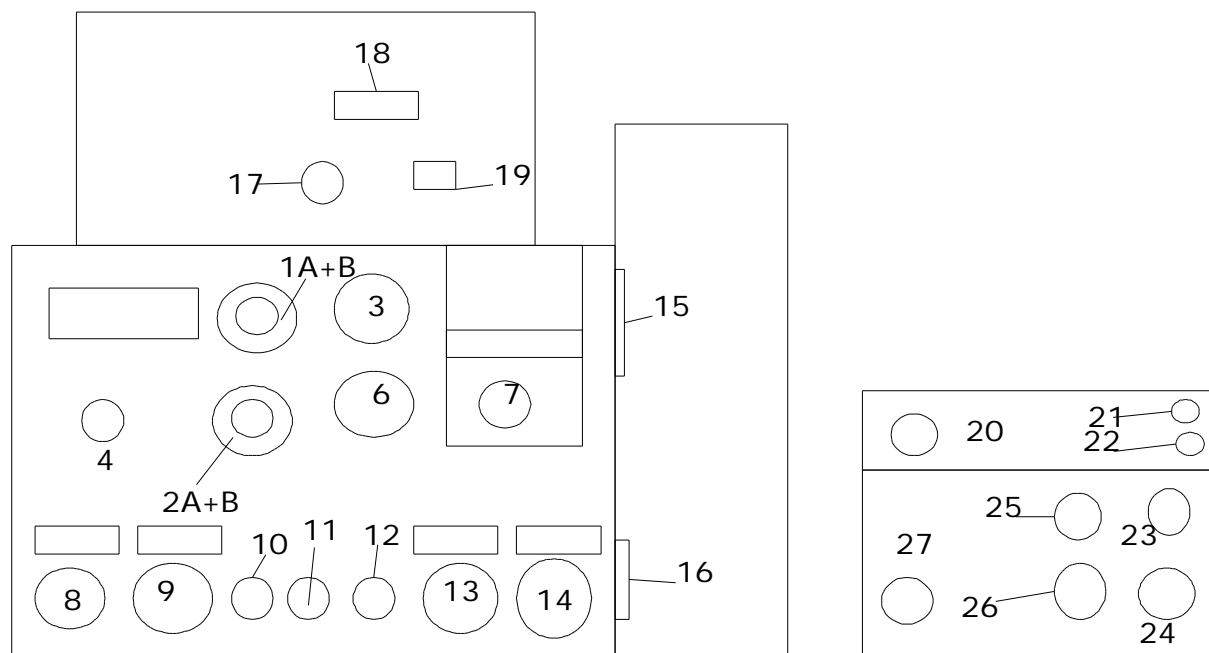
C. Prepare standard addition solutions by the following procedure:

- (1) label six 10 ml volumetric flasks with labels 0 ml, 1 ml, 3 ml, 5 ml, 7ml and 9 ml,
- (2) deliver 1 ml of unknown to each of the six 10 ml volumetric flasks,
- (3) add 0 ml, 1 ml, 3 ml, 5 ml, 7ml and 9 ml of the 0.10 ppm standard solution to the properly labeled flask,
- (4) dilute each solution to the mark with the same 0.02 M acetic acid solution used to prepare the 0.10 ppm standard.

D. Using 4 or 5 nm slits, measure the fluorescence of each solution. **YOU DO NOT NEED TO MEASURE THE ENTIRE SPECTRUM.** You only need to measure the fluorescence intensity at the optimum excitation and emission wavelengths determined from ID. Don't forget to optimize the sensitivity so that the highest concentration sample is on scale!) There is no need to correct for background fluorescence because the standard addition method accounts for any background which might be present.

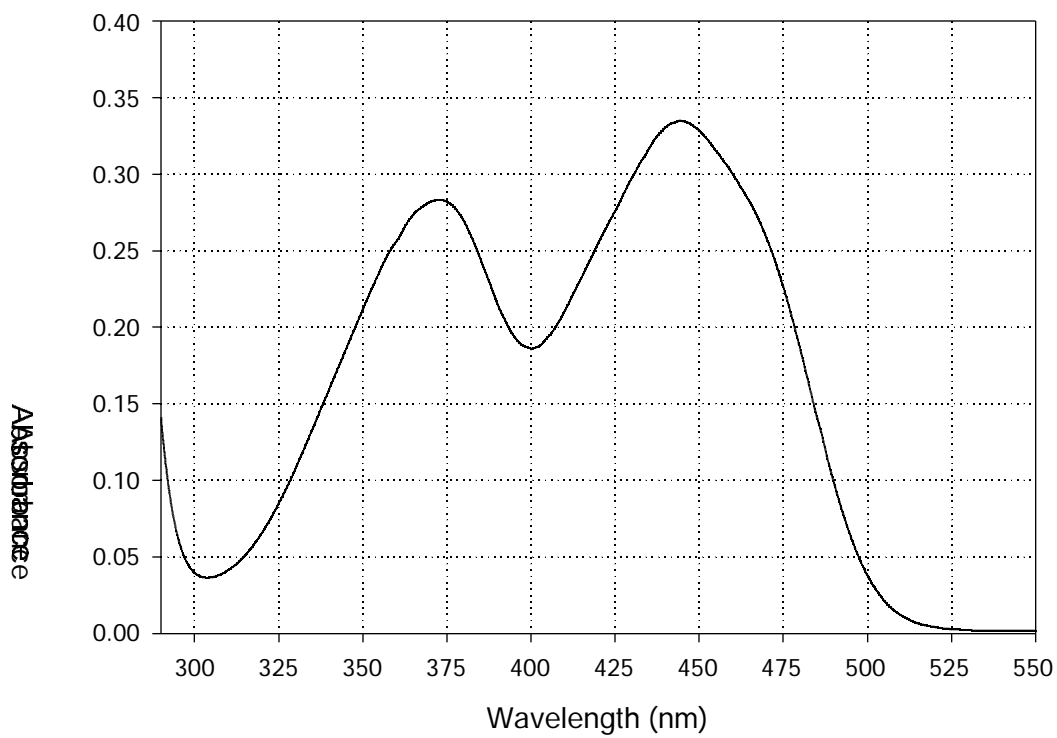
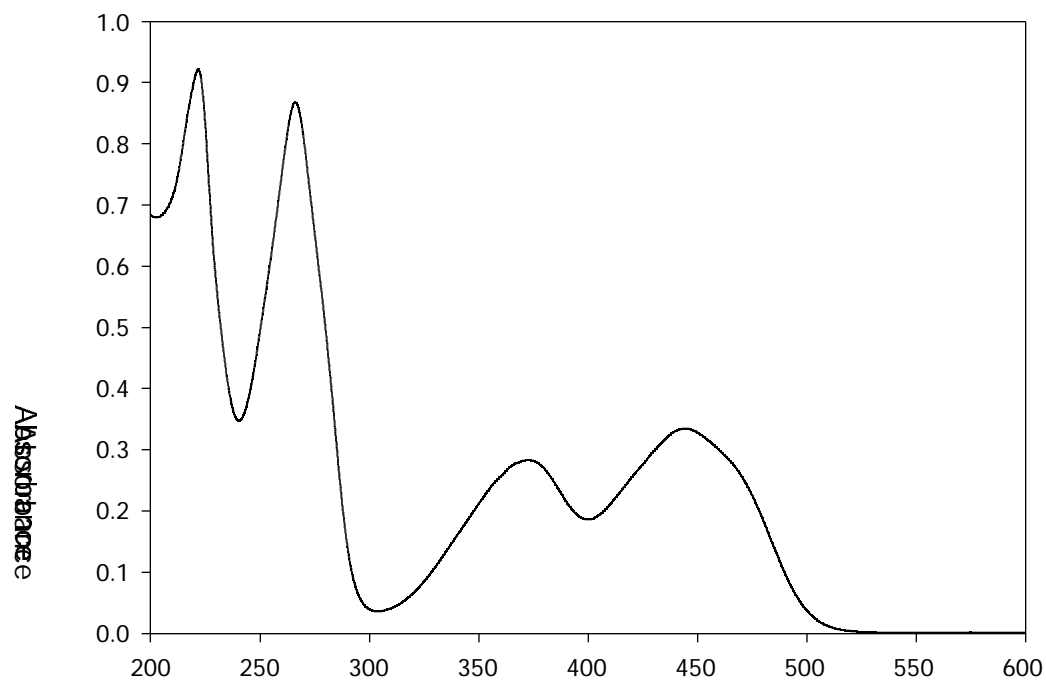
9. *Plot fluorescence intensity vs. volume of standard in each solution. Perform a linear regression analysis to find the slope (m) and y-intercept (b) for the results. Use equation 5 to determine the riboflavin concentration of the sample solution.*

- E. 10. *Using the dilution factors in part A above, calculate the total mass of riboflavin in the vitamin pill. Discuss any observed deviation from the riboflavin mass given on the pill bottle label. Include a discussion of possible source of error in the analysis.*



- | | |
|-------------------------------|--------------------------|
| 1A Sample 100% Adjust Knob | 14 Excitation Slit Dial |
| 1B Sample 0% Adjust Knob | 15 External Shutter |
| 2A Reference 100% Adjust Knob | 16 Scan Mode Knob |
| 2B Reference 0% Adjust Knob | 17 Lamp Start |
| 3 Meter Circuit Selector | 18 Xenon Power Supply On |
| 4 Meter Shunt Switch | 19 Power Pressure |
| 5 Meter | 20 Recorder Power |
| 6 Filter Selector | 21 Pen Up |
| 7 Cell Positioning Knob | 22 Chart Speed |
| 8 Emission Slit Dial | 23 Sample Sensitivity |
| 9 Emission Wavelength Dial | 24 Reference Sensitivity |
| 10 Wavelength Drive Knob | 25 Zero Suppression |
| 11 Scan Speed Knob | 26 Dark Current |
| 12 Excitation Shutter | 27 Power |
| 13 Excitation Wavelength Dial | |

Absorbance Spectrum of
Riboflavin in Water



Fluorescence Report Format

A formal report format will be used for this lab. While the reports that each student turns in should be typed, **ALL OBSERVATIONS SHOULD BE RECORDED IN YOUR LAB NOTEBOOKS!** We will require you to provide photocopies of all lab pages including **spectra and calibration plots with your lab report.** Make sure that your lab notebook contains the standard sections expected in a proper entry, including Title of Experiment, Date of Experiment, Purpose of Experiment, Brief Procedural Outline of Experiment in Checklist format. All instrument settings, readings, and pertinent information should be written down in the Notebook at the time the lab is performed. Sample calculations should also be performed in the lab Notebook including the calculations used for preparation of standards.

For this lab, the student should imagine that he or she is an analytical chemist working for a company, and is approached by a supervisor to use fluorescence spectroscopy to develop a method for the determination of the amount of some species. When the report is turned in, it should follow a format which would be suitable to hand to a supervisor for review of the process and results. This should be neatly typed, double spaced, brief and to the point. **The main purpose of this type of report is to present the results of the analysis to a customer. It must be complete enough for the customer to have confidence in the results, and to understand the limitations of the analysis.** The reports should be formatted using the following outline.

Title Page: This should include the name of the lab, the student's name, date the lab was performed, the expected mass of riboflavin in the vitamin pill and the determined mass of riboflavin in the vitamin pill **with an uncertainty assignment**, and the date the paper is submitted for grading.

Introduction: This section should be brief and should explain the scope of the project (general purpose) as well as some of the methodology used in the lab. This should be no more than 200 words, but should be thorough enough to give some idea as to what the rest of the paper will cover. This lab covers analysis of a vitamin pill with a known mass of riboflavin. You also need to perform some additional measurements to develop the method.

Experimental Section: This should give a more detailed description of the actual procedural steps taken during the lab. This should be in enough detail (but in the student's own words) that someone reading the paper would be able to exactly reproduce the experiment that YOU performed.

Results/Raw Data: This section should contain the actual raw data obtained in the analysis including spectra. Numbers should be compiled in to tables where appropriate (labeled with "Table 1", "Table 2", etc), and tables should be neatly organized for someone who might read your paper (namely the one grading it). This section should not contain explanations or interpretations of the data.

Data Analysis: This is the main text of the report. This should contain any figures, graphs, calibration plots and explanations that might help the reader in interpreting the data presented in the Results/Raw Data section. This is where you will present your calibration plots and your

error analysis. Any calculations required to complete the analysis should be described in detail in this section.

Questions: Answer the following questions in this section:

1. Why is the shape of the fluorescence emission spectrum unaffected by a change in excitation wavelength?
2. How does the emission intensity vary as the excitation wavelength is changed? Why?
3. Compare the fluorescence excitation spectrum with the absorption spectrum of riboflavin.
4. What assumptions are involved in step IID. of the vitamin pill analysis?
5. How can you distinguish a solvent Raman scattering peak from a solute fluorescence peak?

Discussion: This section will contain a discussion of the results including explanation of any unusual data, circumstances that might affect interpretation of the data, or comments on observations that you may consider important. It does not need to be very long. A paragraph or two will usually be enough unless you have significant problems performing that analysis.

It is not necessary to include every piece of information from the lab notebook in the report.