Chem 131A: Absorbance of Riboflavin

Purpose: The purpose of this experiment is to: 1) Familiarize the student with the use of the HP 8452 diode array spectrophotometer, 2) examine the limitations of the photometer, 3) familiarize the student with the Beer-Lambert law and its use, and 4) learn how to properly prepare a dilution series.

Absorbance Measurements and Adherence to the Beer-Lambert Law

The Beer-Lambert law is only valid for low solute concentrations. Violation of the law can be attributed to solute-solute interactions and/or the amount of light that can be accurately measured by the spectrophotometer itself. Typically, the latter defines the limit. The low light levels reaching the photomultiplier at high solute concentrations cannot be precisely measured which usually results in an underestimation of the absorbance. It is important, therefore, that you determine the "absorbance limit" (highest absorbance that follows the Beer-Lambert law) for any spectrophotometer that you plan to use for analytical purposes.

Measurement of this limit is readily determined by measuring a series of solutions in which the solute concentration is varied. A plot of absorbance versus concentration of the solute will reveal a linear portion at the lower solute concentrations and a downward curvature at high solute concentrations where the law is violated. Placing a ruler along the data will readily show where the plot becomes non-linear and hence show the absorbance limit for the spectrophotometer.

The particular absorbance limit is a function of the spectrophotometer design, wavelength of the light, and the age of the excitation lamp. The manufacturer provides an estimation of such limits, but bases this estimation on the performance of the spectrophotometer when it was new. As the spectrophotometer ages, the performance of the optics and electronics can fall below the manufacturer's specifications resulting in a lower absorbance limit. The wavelength dependence is due to the fact that the intensity of light put out by the excitation lamp is not the same for all wavelengths. Typically, the intensity is lower at high and low wavelengths than within the midrange of wavelengths. You may readily observe this phenomenon with the HP8452A spectrophotometer when you employ the lamp test function. In light of this, you need to evaluate the absorbance limit at or near the wavelength that you plan to use in your measurements. As a lamp ages, the intensity at all wavelengths decreases. This necessarily results in a reduced absorbance limit. Periodic examination of the absorbance limit is required for good quality control.

The molar absorptivity or molar extinction coefficient is a useful parameter, as it allows one to determine the concentration of a solute from a measurement of the absorbance of a solution containing the solute. One may obtain molar extinction coefficients from the literature. However, the molar extinction is a function of experimental conditions (e.g. solvent composition, temperature, etc.). Hence, it is prudent to determine the value for this parameter under your experimental conditions whenever possible.

One can determine the value for a molar extinction coefficient by measuring the absorbance of a solution of known solute concentration followed by application of the Beer-Lambert law. It is wise to determine a statistically averaged value for the molar extinction by measuring the absorbance of several solutions that have been independently prepared at the same concentration. The independent preparation will take into account any random experimental error. One can also determine the value for a molar extinction from the absorbance of a series of solutions that vary in solute concentration. A plot of absorbance versus concentration will yield a straight line if the Beer-Lambert law is not violated. The slope of this line is the molar absorptivity divided by the pathlength. If the pathlength is known, the molar absorptivity calculated from it will be a statistically averaged value. Although this method is more time consuming that measuring the absorbance at a single concentration, it has a major advantage in that the data readily shows if the Beer-Lambert law is valid for the absorbance employed in the computation. You will use this second method in the experiment outlined below.

General Notes:

a. The HP spectrophotometer uses special rectangular 3 mL silica cuvets. The spectrophotometer cuvets may be checked out from the stockroom; the instructor must sign the pad for this item. These cuvets are expensive (~$60). BE CAREFUL with them.

b. Always clean the cuvets with the special cuvet washer; NEVER use a brush to clean them.

c. Always inspect the cuvets before using to insure that they are clean.

d. Never wipe the cuvet faces with paper towels or kimwipes. ALWAYS use lens paper.

e. Never touch the clear faces of the cuvet. Hold them by the frosted faces. If you touch one of the faces, wipe it with lens paper.

f. If you are the last person to use a particular photometer, make sure that it is shut off properly.

g. Misuse of photometers will not be tolerated! Clean up any spills around the photometers. Notify the instructor immediately if any solution has been spilled into any of the photometers.

Procedure:

A. Solution Preparation

1. Prepare a dilution series of riboflavin from 8.8 μM to 88 μM in increments of 8.8 μM.
   a. Determine the number of mL of stock 88 μM riboflavin that, when diluted to 10.00 mL, will result in the desired solution concentrations (e.g. 8.8 μM, 17.6 μM, etc.). Prepare a table in your lab notebook that outlines this.
   b. Rinse out a 10 mL ultramax buret and then fill it with the stock 88 μM riboflavin solution.
   c. Deliver the required amount of the stock solution for one of the solutions to a clean 10 mL volumetric flask.
   d. Dilute to the mark with 0.1 M acetate buffer, pH 5.5, and invert to mix thoroughly.
   e. Pour about 8 mL of the solution into a clean and dry 13 x 100 mm test tube, stopper the tube with a polypropylene stopper and discard the remaining solution. NOTE: make sure that you label each tube to denote the riboflavin concentration held within.
   f. Rinse out the flask with distilled water and repeat steps c-e until all solutions have been prepared.
   g. Try to minimize the exposure of these solutions to light. Photooxidation can occur. Keep them in your locker or in a rack that is covered with aluminum foil.

B. Spectrophotometer Procedure

1. Set up a Spec 20, set the wavelength to 444 nm, and read the absorbance of each of these solutions to the nearest 0.2 of a division. Record the values in a table in your lab notebook.

a. Turn on the Spec 20, set the wavelength to 444 nm, and allow it to warm up for 30 minutes.
b. Place about 3 mL of each solution into individual Spec 20 cuvettes. NOTE: if a tube is not dry, rinse it first with a small portion of the solution prior to adding the 3 mL. Make sure that the tube is dry on the outside before placing it into the Spec 20.
c. Place about 3 mL of 0.1 M acetate buffer at pH 5.5 into a clean cuvet. This serves as your blank.
d. Adjust the dark current so that the meter needle points to 0% T.
e. Place the blank into the Spec 20 and adjust the absorbance to zero.
f. Replace the blank with one of your samples and then measure (interpolate between adjacent lines to the nearest 0.2 division) and record the absorbance for the solution in your notebook. NOTE: make sure that the line on the tube is aligned with the mark on the Spec 20 sample holder before reading the absorbance.
g. Repeat steps d-f until all riboflavin solutions have been measured.

2. Scan the entire spectrum of each solution using the HP8452 spectrophotometer and annotate the absorbance at 268, 372, and 444 nm. Record these in your notebook. Print out a plot for your notebook.
a. Check out a set of silica cuvets from the stockroom.
b. Place 2.5 to 3 mL of 0.1 mL acetate buffer into one of the cuvets, place it in the spectrophotometer and scan it as a blank.
c. Place 2.5 to 3 mL of the MOST DILUTE riboflavin solution of your set into the other cell, place it in the spectrophotometer, and scan it.
d. Be sure to annotate the absorbance at 268, 372, and 444 nm.
e. Carefully remove the riboflavin solution from the cuvet and place it back into its test tube.
f. Add the next most concentrated riboflavin solution directly to the cuvet.
g. Repeat steps d-f until all solutions have been measured.
h. Clean and dry both cells using the cell washer.

NOTE: The method employed to change samples will result in some cross-contamination of the samples. If, however, you are careful to remove as much of the preceding solution as possible and always measure the solutions from most dilute to most concentrated, the experimental error will be negligible. Laboratory work always involves a careful weighing of time versus accuracy.

C. Treatment of Spectrophotometric Data

1. Plot the absorbance at 444 nm versus concentration of riboflavin taken from both the HP and the Spec 20 on the same piece of graph paper. Are the graphs linear?
2. Use a ruler to determine the linear portion of the plots and note the absorbance limit (remember the plot curves down at the higher concentrations).
3. Perform a linear regression analysis for the linear portion of each of the two data sets to determine the slope and intercept of the plots. Draw the linear regression lines for each data set. In addition, determine the standard deviations for the slopes.
4. Use the results and a pathlength of 1.000 cm to determine the molar absorptivity for riboflavin at 444 nm and the associated standard deviation for each data set. Remember, the molar absorptivity is equal to the slope of the plot divided by the pathlength.
5. Plot the absorbance at 372 and 268 nm vs. concentration of riboflavin on the same piece of graph paper. Are the graphs linear?
6. Use a ruler to determine the linear portion of the plots.
7. Perform a linear regression analysis of the linear portion of the data for each line to determine the slope and intercept. Draw the linear regression lines on the plot. In addition, determine the standard deviation for each slope.
8. Use the results and a pathlength of 1.000 cm to determine the molar extinction for riboflavin at 372 and 268 nm and the associated standard deviations.