NAME_____ F19

OREGON STATE UNIVERSITY DEPARTMENT OF CHEMISTRY

Experiment 2A

Integrated Laboratory Experiment

DETERMINATION OF RIBOFLAVIN: A COMPARISON OF TECHNIQUES

PART A. MOLECULAR ABSORPTION SPECTROPHOTOMETRY

Experiment 2A

MOLECULAR ABSORPTION SPECTROPHOTOMETRY

I. INTRODUCTION

The absorption of ultraviolet **(UV)** and visible **(Vis)** radiation by molecules results in promotion of the outermost or bonding electrons to higher energy levels. This absorption phenomenon is quantitatively described by **Beer's law** (equation 1) when a beam of parallel and monochromatic light is incident on a cell containing a homogeneous sample to be analyzed.

$$A = abc = \varepsilon bc = -\log_{10} T \tag{1}$$

$$T = \frac{f_i \Phi_o 10^{-A}}{f_i \Phi_o} = \frac{\Phi_s}{\Phi_r} \tag{2}$$

where

A = absorbance

a = absorptivity, cm^{-1} $(\mu g/mL)^{-1}$

ε = molar absorptivity, cm^{-1} $(mol/L)^{-1}$

b = pathlength, cm

c = concentration of analyte, mol/L (or μg/mL)

f_i = fraction of incident radiant power lost due to cell walls, etc.

T = transmittance

Φ_o = radiant power incident on the front surface of the cell, watts

Φ_s = radiant power passed by the cell containing the standard or the sample to be analyzed, watts

Φ_r = radiant power passed by the cell containing the reference or blank solution (usually the solvent in which the sample is dissolved), watts

From Beer's law we find that at any given wavelength, the **absorbance (A)** is directly proportional to **concentration (c)** when **molar absorptivity (ε)** and **pathlength (b)** are held constant. The molar absorptivity at a given wavelength is an inherent characteristic of the absorbing species in a particular environment (e.g., solvent) and is a constant. The pathlength is made constant by using carefully matched cells, or better yet by using the same cell for both the sample and blank measurements.

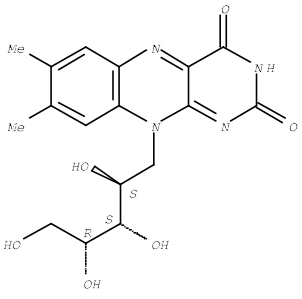
The first step in quantitative analysis by absorption spectrophotometry is the choice of the absorption band for which absorption measurements are to be made. The UV-Vis absorption spectrum of the species to be determined is found either in the literature or is determined experimentally. The profile of an absorption band is controlled by how the absorptivity varies with wavelength. The magnitude of the molar absorptivity of a given molecular species at a given wavelength determines the slope of an analytical curve for absorptivity vs. concentration and influences the useful concentration range over which determinations can be made. Greater values of absorptivity yield greater slopes for absorptivity vs. concentration and generally better detection limits and greater accuracy and precision.

When several absorption bands with suitable peak molar absorptivities are present, the band selected for the analysis should favor wavelength regions that correspond to relatively high output of the light source and high spectral responsivity of the photodetector. This combination provides the most favorable operating conditions. The selected absorption band should not overlap (should be free of interference from) absorption bands of the solvent or other absorbing species that might be present in the sample.

Beer's law predicts a linear calibration curve with a zero intercept. However, for a particular analysis, it is necessary to determine experimentally over what concentration range linearity is observed. This is done by preparing a calibration curve of absorbance versus the concentration of the species of interest. If the plot is linear and goes through the origin, it is said that Beer's law is obeyed.

Deviations from linearity for the analytical curve of absorbance versus concentration are commonly termed **"deviations" from Beer's law**. Such deviations are usually a result of the breakdown of some of the assumptions implicit in Beer's law. They can result from instrumental limitations, improper analytical or instrumental conditions or unwanted chemical equilibria involving the analyte.

In this experiment, the absorption of the vitamin B2 or riboflavin in aqueous solution is studied. First an absorption spectrum is obtained. Next data are gathered to construct a calibration curve (Beer's law plot) and to determine the concentration of the vitamin in two samples: a vitamin pill and a "synthetic unknown," which contains an accurately known amount of riboflavin. Next the limit of detection of riboflavin is determined, and the effect of stray light on the linearity of analytical curves is studied. Finally the effect of spectral bandpass on the linearity and the slope of the Beer's law plot is investigated by studying the absorption characteristics of praseodymium solutions.



Riboflavin

The lab report should be prepared according to the instructions at the end of this experiment.

The data sheet provided at the end of the experiment should be torn off and used to record the data as indicated. This data sheet will be turned in with the report.

II. SOLUTION PREPARATION

In this and later experiments, careful preparation of solutions is required. Solution and sample preparations should be carried out with the utmost care to realize the full potential of any spectrometric technique. The accuracy of the analysis can be no better than the accuracy with which you prepare the standard solutions. **All details of standard and sample preparation (e.g., masses, volumes, other observations) should be recorded in your laboratory notebook.** Recall the following rules for making up solutions:

1. SAFETY GLASSES ARE REQUIRED TO HELP PROTECT YOUR EYES FROM PERMANENT DAMAGE.
2. Do not let solutions stand uncovered for long periods of time because evaporation may change their concentration and contaminants may enter the solution.
3. Pipets should be clean as indicated by absence of droplets adhering to the inside wall.
4. Touch the tip of the pipet to the inside of the container when delivering solutions from the pipet and when adjusting solution level to the mark.
5. Let the pipet drain for a fixed length of time (about 10 seconds) after solution has been delivered. **Do not blow out the last drop.**
6. Rinse out all volumetric flasks a few times with distilled water before use.
7. Use an disposable pipet to bring the solution up the volume mark on the volumetric flask.
8. Mix the final solutions thoroughly by inverting the flask ten times. Since solvent may evaporate from the surface and condense at the top of the flask, mix solutions before using and mix again if solutions stand overnight.
9. For high accuracy work, use volumetric pipets, not graduated pipets. If you do not know the difference, ask.
10. Clean the 125 mL bottles, pipets and vol flasks using water and/or dil acid, rinse very well especially if soapy.

Prepare standard solutions. You will use four standard solutions for this experiment, two of which are already made up and two of which you will prepare.

Solution	Description
100.0 μg/mL riboflavin in water	Stock solutions stored in the refrigerator (TA remove 1 hour before class) and available in the main laboratory
25.00 μg/mL riboflavin in water	
10.00 μg/mL riboflavin in water, 50 mL	Prepared by each group through dilution of stock solutions; see instructions in text.
2.00 μg/mL riboflavin in water, 50 mL	

To prepare the 10.00 μg/mL standard solution of riboflavin in water, pipet exactly 5.00 mL of the 100 μg/mL stock riboflavin solution into a 50-mL volumetric flask and dilute to volume with double deionized water, which will be denoted as **Millipore water or MP water** (house deionized water that has been passed through the Millipore purification system in Gbad 309 and stored in carboys on the bench). In a similar manner, prepare the 2.00 μg/mL standard solution of riboflavin in MP water. Store standard solutions in amber bottles in refrigerator.

Prepare sample solutions. You will analyze two samples: one **commercial B-50 vitamin pill** and **one synthetic vitamin B2 unknown**.

The vitamin pill is a commercial Vitamin B Complex tablet, which contains B vitamins and inert materials (e.g., starch & inorganic binders). You will determine the mass of vitamin B2 in the whole tablet. Weigh one intact vitamin pill(should be approximately 1.00-0.6 g) to +/-0.1 mg on the analytical balance. After grinding the pill with a mortar and pestle, quantitatively weigh out three sub-samples of the resulting powder. The mass of each sub-sample needs to be known exactly but should be approximately 0.16xx-0.17xx g weighed to +/-0.1 mg using the analytical balance. Carefully transfer all of each of the sub-samples into three separate clean labeled medium sized test tubes (obtain from instructor if not in your lockers). **Record all weighing data in your lab notebook.**

Use a 10-mL graduated cylinder to add 20 mL of 80/20 acetonitrile/H_2O solvent to each of the three sub-samples in test tubes and add about 0.5 mL conc phosphoric acid to each tube. CAUTION: acetonitrile is toxic and phosphoric acid is corrosive: wear gloves, wear lab goggles, keep off skin, etc. Warm the test tubes with occasional shaking or swirling at 60 °C in one of the driblock heaters under the hood in rm 313 for 45-60 min (record the time used). Occasionally, carefully swirl each tube. CAUTION: Be especially careful not to point the test tube toward yourself or others when heating. Note that some solid inert materials will not dissolve.

If you do not get to the dilution or transfer steps described below before the end of the lab period today, cover each test tube with aluminum foil and store all 3 solutions in your lab locker. All bottles should be clearly labeled with identification including contents, date, and class and group number.

Cool the contents to room temperature in a cold water bath and quantitatively transfer the entire contents of each test tube into a separate labeled clean 100-mL volumetric flask. To transfer quantitatively means to rinse the test tube carefully with solvent using the wash bottle into the volumetric flask without losing any analyte solution (transfer solids also). Finally, dilute to volume with MP-DI water. These solutions will be designated as X solutions, e.g., X1, X2, & X3. **Put the lid on the flask and mix thoroughly by inverting and shaking flask at least ten times.** After mixing, allow the flasks to stand undisturbed for 1 min to allow the undissolved materials to settle. Transfer each X-solution into a clean, labeled 125-mL amber bottle for storage (include name of solution, your group name, and date); be sure to rinse the bottle with a little of the solution before making the final transfer to condition the storage bottle. Store these X-solution bottles in your lab lockers when not in use.

Next, make a quantitative 1/10 dilution of solution X for each sample in a 50-mL volumetric flask using MP-DI water as the solvent. Transfer these solutions, denoted as the Y solutions (Y1, Y2, Y3), to clean and conditioned 125-mL amber bottles as you will need the flasks. Label the bottles (include name of solution, your group name, and date) and store these bottles in the refrigerator at the end of the balance room when not in use.

Prepare the synthetic-unknown solution according to the following instructions. The synthetic unknown consists of a known volume of a known concentration of riboflavin in a small vial. **Record your synthetic unknown number in your lab notebook and on the 2A data sheet**. Quantitatively transfer the contents of the vial into a 100-mL volumetric flask and dilute to the mark with MP-DI water. Quantitatively transfer means to transfer the total contents of the vial to the volumetric flask and then wash out the vial a few times with the blank (MP water in this case) and transfer these washings into the flask. Dilute to the line and mix thoroughly as above. After thoroughly mixing the solution, transfer each one to another clean conditioned 125 mL amber bottle for storage. Be sure to rinse the bottle first with a portion of the solution to be stored to condition it before transferring the entire solution. Later you will report the concentration of riboflavin after dilution. **As always, all details of the preparation of standards and samples should be recorded in your laboratory notebook as you do the work (not later).**

Waste disposal. When you have completed all of Experiments 2A (absorbance), 2B (fluorescence) and 2C (HPLC), you can dispose of your vitamin sample and standard solutions. Solutions X1, X2, and X3 and any excess 80%/20% acetonitrile/water go in the container marked Flammable Organic Wastes in the hood in Gbad 309. All other vitamin solutions in this experiment are nonflammable and nonhazardous, and waste from these solutions are poured down the drain.

III. INSTRUMENTATION

A. *The Absorption Spectrum for Riboflavin using Commercial Instruments*

A commercial double-beam spectrophotometer (Cary model 219) and a commercial single-beam photo diode array spectrophotometer (HP/Agilent model 8453) will be used in Section IV. A TA will guide one group at a time through the operation of these instruments.

B. *Building a Single-Beam CCD Instrument for Quantitative Measurements*

1. Construction of single-beam instrument

The instrumental setup shown in Figure 1 will be used for Sections V - VII.

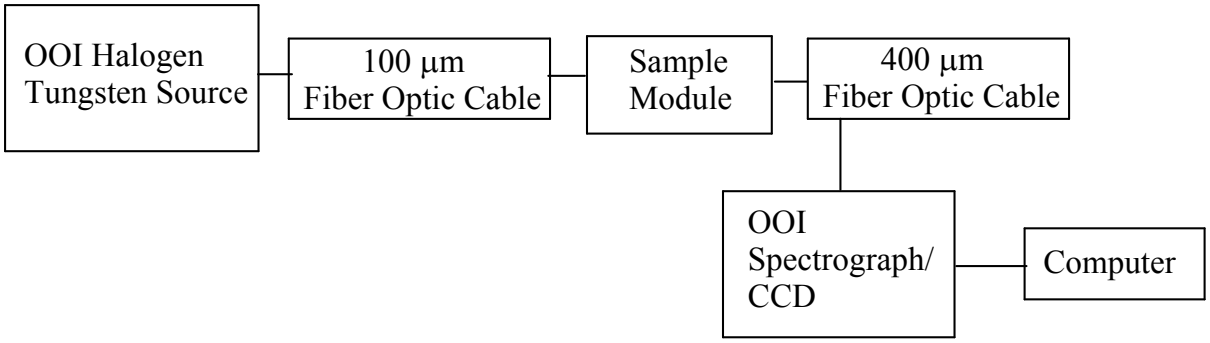


Figure 1. Block Diagram of single-beam OOI spectrophotometer. Fiber optic adapters are required to connect the fiber optics cables to the input and output ports of the sample module. Use a 100-um fiber optic cable (green band) for the input and a 400-um cable (pink band) for the output port (station 7 requires two pink and no greeen). For a dark signal measurement, you can block the tungsten light by placing a thin opaque object in the slot in the lamp housing.

2. Readout modes

The OOI spectrometer can display the signal in several modes. So far you have mostly used the Scope or Signal (S icon) mode, which displays just the raw signal in counts. Other modes include the Transmittance (T icon) and Absorbance (A icon). For these two modes, a dark spectrum (dark bulb icon) and reference spectrum (light bulb icon) must be acquired before these modes are active.

In general, the signal (N) that is displayed as counts for a given pixel is given by

$$N = \Phi K(\lambda)G \tag{3}$$

where

Φ is the radiant power striking a given pixel, photons/s
$K(\lambda)$ is the quantum efficiency for the pixel, electron-hole pairs/photon
G is the electronics and ADC conversion factor, number of counts displayed/ electron-hole pairs generated in a pixel

Transmittance and absorbance readout

As discussed in the introduction, two measurements are needed to obtain the transmittance. For a CCD spectrophotometer, these signals are the number of counts associated with the measurement for the reference (N_r) and the number of counts associated for the sample (N_s):

$$N_r = \Phi_r K(\lambda)G \tag{4}$$

$$N_s = \Phi_s K(\lambda)G \tag{5}$$

The transmittance is calculated as

$$T = N_s / N_r \tag{6}$$

The OOI spectrometer actually saves a complete reference (R), sample (S), and dark (D) spectrum. When the transmittance mode is chosen, the software calculates

$$T(\lambda) = \{N_S(\lambda) - N_D(\lambda)\} / \{N_R(\lambda) - N_D(\lambda)\} \tag{7}$$

where the subscript λ denotes the value for a particular pixel or wavelength. The absorbance spectrum is calculated from equation 6 and equation 1.

If all experimental parameters including the incident radiant power $\Phi_0(\lambda)$, λ, slit width (W) and G remain constant between the reference and sample solution measurements, then equation 5 or 6 gives the same result as equation 2 and the true transmittance can be measured. However, if a change occurs between the sample and reference measurement such as a significant light source intensity drift, moving the optical cables, changing the parameters, then the measured transmittance will be in error.

The OOI spectrometer has a feature that allows partial compensation for changes such as light source intensity drift by allowing the user to obtain absorbance versus time data (often called time based data) for up to six wavelengths which are denoted as "channels" (e.g., A, B, etc). It is also possible to store two combinations which are simple functions involving the difference, ratio, or product for two of these channels. In this experiment you will use a difference function to calculate

a corrected absorbance (A_c) as

$$A_c = A_1 - A_2 \quad (8)$$

where wavelength 1 is set to the normal analysis wavelength maximum and wavelength 2 is set to some other wavelength that is not absorbed by the sample (denoted "corrected wavelength"). The advantage of this **corrected absorbance** is that it partially compensates for factors that change about the same for both wavelengths. For instance, if the source intensity decreases for both wavelengths by 1% between the sample and reference measurements, both A_1 and A_2 would increase even without analyte absorption. However, the difference in absorbance would remain constant if the corrected intensity (counts) associated with each wavelength decreased by 1%. This correction technique can also partially compensate for changes in the position of the sample cell and scattering in solutions.

IV. ABSORPTION SPECTRUM

A. *Introduction*

As stated in the introduction, the first step in quantitative analysis is to obtain an absorbance spectrum to determine the best values for the analysis wavelength. A spectrum of transmittance or absorbance versus wavelength is most easily obtained with a double-beam spectrophotometer or a diode array spectrophotometer. The double-beam spectrophotometer alternately measures the sample signal (E_s) and the reference signal (E_r) as the wavelength is scanned, allowing variations in the source intensity, monochromator efficiency, and detector responsivity with wavelength to be taken into account as the transmittance or absorbance is determined. A single-beam photo diode array spectrophotometer accounts for these variations by storing the entire reference spectrum ($E_\mathbf{r}$ vs. λ) and then the entire sample spectrum ($E_\mathbf{s}$ vs. λ) in computer memory -- then the transmittance or absorbance spectrum is calculated point-by-point.

B. *Experimental*

Obtain an absorbance spectrum of 25 μg/mL riboflavin with the Cary 219 double-beam spectrophotometer in room 314 and the HP 8453 single-beam diode array spectrophotometer in room 318. The TA's will provide the blank (DI water) and some of the 25 μg/mL riboflavin standard for you to measure using a plastic cuvette. Be sure that the instrument has warmed up and that the outside cell walls are clean. Scan the spectrum from 300 to 550 nm on the Cary 219and

from 200 to 600 nm on the HP 8453 (use a fused silica ("quartz") cuvette and obtain a hardcopy or chart of each scan. Be sure to label the output with all instrumental settings and date it. Include the names for all partners on the output.

V. CALIBRATION CURVE AND QUANTITATIVE ANALYSIS

A. *Introduction*

In this section, the absorbance of solutions of different known concentrations of riboflavin will be measured with the CCD spectrophotometer built at your stations. From these data, a calibration plot of absorbance versus known concentration will be made, and the molar absorptivity (ε) of riboflavin will be determined. The absorbance values for several real samples will be measured, and the corresponding riboflavin concentrations then determined from the calibration curve.

B. *Experimental*

Set up spectrometer and acquire signal, transmittance, and absorbance of riboflavin sample. Set up a single beam spectrometer based on Figure 1 and the description in section IIIB1. Remove the PTR tungsten lamp from the baseplate and replace with the OOI Halogen Tungsten source. **Important: coil the optical fibers neatly (no sharp angles) and place them out of the way where they won't be jostled by people, books, wash bottles, or papers during the measurements.** Check that the cuvette holder is square with the light path and that the set screw in the bottom of the cuvette holder is tight to prevent rotation. Also check that the small stop is inserted in the sample compartment right next to the cuvette holder. This stop is adjustable and serves to hold the cuvette holder from moving. Check that the alignment of the light source from the entrance port over to the exit port on the sample compartment is good. Use the following initial instrument settings on the spectrometer:

OOI Spectrometer Acquisition Parameters:

- mode: scope (S)
- integration time: 3 ms
- average: 10
- boxcar: 0
- y scale: default or 0 to 4100
- displayed wavelength range: 400 - 850 nm
- correct for electrical dark: box not checked

With the lamp and the spectrometer on, you should see a source spectrum with a very broad peak

centered around 600 nm (maybe off-scale). If not, check with a TA.

Obtain a plastic cell (cuvette). If one side of the cell is not marked with a triangle symbol at the top, scratch a mark so you can **keep track of the orientation of the cell**. Every time you place the cell in the sample holder, **be sure the marked cell side is facing toward you**. This way the cell will be in the same orientation for all measurements (i.e., the transmission characteristics of the cell would be different if you rotate it 90° or 180°).

Rinse the cell 3 times with 2-3 mL of blank (i.e., reference) solution, which in this case is MP water from a wash bottle. Empty the contents into a waste beaker or aspirate to the trap at your station. Fill the cell with about 3 ± 0.5 mL of reference solution, being careful to avoid bubbles in the cell (solution will be about ½ - 1 cm from top of cell). Tap to remove air bubbles. Always be careful not to touch the walls of the cell through which the light passes (i.e., hold the cell by the other sides). Also be careful not to spill solution across the outside walls. If you do, carefully wipe the outside cell walls with a Kimwipe. **Insert the cell with the blank solution (the reference) into the cell holder in the sample module and put the lid on the sample module.**

Tune-up the spectrophotometer for better precision at the analysis wavelength:

Select the S mode. Block the light at the source (insert piece of yellow cardboard in slot on source) and acquire a dark spectrum (dark bulb icon). Unblock the light source and select the dark spectrum correction (minus dark bulb icon). Consult the absorbance spectrum you obtained in part IVB and set the cursor to the longest wavelength band of riboflavin (peak at approximately 445 nm) and observe the intensity at this wavelength displayed at the bottom of the screen. **To improve precision, increase the integration time so that the counts at the analysis wavelength in the dark-corrected scope mode (S) are between 2000 and 3000 counts** (typically need 5 to 70 ms integration time). **The signal for the light source will be off scale for some of the other regions of the spectrum - that's okay.** To provide further signal averaging, set the average to 10 and the you can increase the boxcar value to 5 if the signal is noisy - otherwise leave it at 0. The boxcar algorithm smooths and dampens the signal. Check with a TA if you have questions, and record all settings you used.

Ready to make some measurements:

1. Once the signal has been optimized, store a new dark spectrum (dark bulb icon) and a new reference spectrum (bright bulb icon). **Apply the new dark spectrum (minus dark bulb icon). Take a new dark and a new reference if you change any of the scan parameters later.**
2. On the data sheet record the analysis wavelength, the integration time, average #, boxcar #, and the dark-current corrected reference signal (number of counts, N_r) at the analysis peak wavelength.
3. Remove the sample cell from its holder, remove the solution with the aid of an aspirator, rinse three times with the 10 μg/mL riboflavin standard, and fill the sample cell with ~3 mL of this riboflavin solution. Replace cell and the lid. We suggest that you pour directly into the cell from either a volumetric flask or a small beaker and not use a pipet to transfer so that you avoid contaminating your reagent solutions. **Reminder: never insert a pipet into a reagent bottle and do not pour used solutions back in any reagent bottle.**
4. Record in the data sheet the number of counts for the dark-current corrected sample signal (N_s), the value for the transmittance T, and the value for the absorbance A at the analysis wavelength by selecting S, then T, and then A from the menu. The absorbance should not change by more than about 2 mAU. If it changes by more than this, adjust the integration and/or the number of averages, or the boxcar, and repeat starting at step 1. If you make changes in the parameters, make sure and record these on the data sheet for reference at the next lab meeting.
5. Later you will calculate the transmittance from the measured number of counts and then use this calculated T to find a calculated absorbance and compare these calculated values to the measured values.
6. The peak maximum for riboflavin with the CCD detector maybe somewhat different than with the commercial diode array since the PDA uses a deuterium UVVis source. Now that you have the 10 ug/mL solution in the cell, locate the peak maximum wavelength and record this updated value for the analysis wavelength on the data sheet and use this next lab meeting.

****Leave your station setup. This is the normal stopping point for the first day of this lab.** **

Tune up spectrometer for "corrected" absorbance and measure solutions:

You have chosen an **analysis wavelength** at the end of the last lab (probably between 445-475 nm) and now you'll choose a **correction wavelength** where riboflavin does not absorb at all (somewhere in the range approximately 750 - 825 nm). Subtraction of the absorbance at the correction wavelength from the absorbance at the analytical wavelength yields a "corrected" absorbance (equation 7), which partially compensates for factors such as drift in intensity of the light source and placement of the cuvette.

1. Turn on the source and load the OOI software. Set the parameters on the spectrometer back to what you found at the end of the last lab.

2. Rinse the cell several times, fill with MP water, and insert in the sample holder and close the lid. Select the S mode, block the light source and obtain a dark spectrum (dark bulb icon), unblock the source and obtain a reference spectrum (bright bulb icon), and apply the dark corrected spectrum. (-dark bulb icon).

3. **Position the cursor at the optimized analysis wavelength (~445 nm) and check the intensity (dark corrected counts should be 2000-3000).** If not, adjust the integration time, obtain a new dark spectrum, unblock the source, and obtain the reference spectrum, apply the dark corrected spectrum. Repeat until the dark corrected counts are 2000-3000. **Recall that it is okay if the light source signal is off scale for some of the other regions of the spectrum.**

4. **Now move the cursor to a point approximately between 750 - 825 nm (where riboflavin doesn't absorb) and where the number of counts is approximately the same as the number of counts at the analysis wavelength (i.e., 2000-3000 as above)**. This will be the **correction wavelength.** Record this correction wavelength on your data sheet and the updated parameters (integration time, etc.). Check with an instructor to make sure that your wavelengths meet the criteria.

Configure the spectrometer for "corrected" absorbance. Once the wavelengths are selected above, configure the spectrometer to calculate the difference between absorbance at the analysis wavelength and absorbance at the correction wavelength.

5. Select Time Acquisition | Configure | Configure Time Channels to open the *Time Acquisition Channel Configuration* screen:
   - **For channel A**, set the **analysis wavelength**
   - **For channel B,** set the **correction wavelength**
   - **For combination 1,** choose A - B.

Be sure the two channels and combination 1 are enabled by checking the box.

6. Open the *Time Acquisition Configuration* screen and set the following:
   - check the boxes for "stream data to disk" "show values in status bar", "continue until manually stopped"
   - do not check the boxes for "save every data acquisition" and "save full spectrum with each data acquisition"
   - write to disk every _____ acquisitions: 20
   - choose your filename in CH461\DATA folder
   - initial delay: 1 s
   - frequency (time between points): 1 s

7. Now select Absorbance mode and start the data acquisition by clicking the **alarm clock icon** to enter time acquisition mode, the **green arrow to starts collection**, and the **red square stops collection**. As data are being acquired you can read the values at the lower left hand corner of the screen: corrected absorbance values (combo 1) should be about zero with the blank in. You should save the data to file and calculate the average of several readings. Stop collecting data by clicking the red square.

Now you are ready to run your standards (2, 10, and 25 μg/mL riboflavin) and samples (your synthetic unknown and your Y pill solutions). Make a copy of Table I of the data sheet in Excel and fill it in the data as you go.

8. ***Measure standards.*** Remove the cell from its holder, remove the solution with the aid of an aspirator, rinse three times with the first solution (2 μg/mL riboflavin standard) and finally fill the sample cell with ~3 mL of the test solution. If necessary, wipe off the outside walls of the cell with a Kimwipe. Place the cell back in the sample module, replace the sample module lid, and record the corrected absorbance (combo 1) in Table I and in a spreadsheet. You should save the data in an Excel file for back-up or future reference.

9. **Repeat step 8 for the rest of your standard solutions**.

10. Before continuing on to the samples, determine that your data look reasonable, i.e., A is proportional to c within at least 5%. Do this by running a linear regression on your data and plotting the calibration curve in the spreadsheet; **and checking that the ratios for A(10-μg/mL) : A(2-μg/mL) and A(25-μg/mL) : A(2-μg/mL) are as expected (10:2 and 25:2)**. You should observe a calibration slope of about 0.03 to 0.04 absorbance units (AU) / (μg/mL). If your standards do not agree within about 5% of expected ratios, do the following two steps first: (1) rerun the standards in question to see if the results are consistent; if they are not, (2) measure a new reference signal and confirm that the absorbance for the blank is still near zero. If it is not, store a new reference spectrum and redo the measurements of the standards that don't agree. If the ratios are still in question, remake the faulty standards and re-measure. You may want to check with a TA if you have questions. Check your calibration standards before moving on!

11. ***Measure samples.*** Measure a new reference signal for the blank if you have not just completed one above. Measure the absorbance (combo 1) of your synthetic unknown riboflavin solution and record the value in Table I. From the absorbance of the unknown and the calibration equation for the standards, determine the concentration of riboflavin in this unknown solution. **Check this value with an instructor now** to see if you are within 5% of the correct value. If your result is not acceptable, you need to address the situation with your instructor and ultimately demonstrate that you can obtain an acceptable value for a synthetic unknown before you continue.

12. Measure the absorbance of all diluted sample vitamin pill solutions (just the Y solutions of the vitamin pill, not the X solutions) and record all data in Table I and the spreadsheet. Check if your results are consistent with the mass of each sub-sample.

After you are sure you have the data for three standards and four unknowns, empty the contents of the waste beaker and/or aspirator trap flask into the sink; the dilute vitamin solutions are not hazardous and are degradable. Store your Y solutions for Experiment 2C HPLC which you will do later.

VI. STRAY LIGHT

A. *Introduction*

Negative deviations from linearity are sometimes observed at high concentrations of analytes (large absorbance, but low transmittance) due to such factors as **stray light** and **polychromatic radiation**. If stray light is significant the observed absorbance, A', is approximately equal to

$$A' = - \log T' = - \log (T + f) \quad (9)$$

where **f is the fraction of the detected light that is due to stray light**, T' is the observed transmittance with stray light (T ' = T + f), and T is the theoretical transmittance in the absence of stray light (f = 0). The theoretical T can be estimated by extrapolation of the linear portion of the calibration curve out to the very high concentration measured here (i.e., 100 μg/mL) . **Note that stray light getting through the exit slit into the detector makes the measured absorbance lower than expected for the analyte alone (and makes the measured transmittance higher than expected), hence the term "negative deviation" from Beer's Law.**

B. *Experimental*

Measure the absorbance of the 100 μg/mL riboflavin solution with the experimental setup and procedure of Section VB. Be sure to block the light source and store a new dark spectrum immediately before the absorbance measurement because a small error in the dark signal has a large effect when the absorbance is high and the transmittance is very low. Record the value of the absorbance (combo 1) for this solution on the data sheet; **it should be between 2 and 3 AU.** If it is not make sure you have stored a new dark spectrum as directed above. All else fails, check with a TA.

VII. PRECISION AND THE DETECTION LIMIT

A. *Introduction*

The **detection limit (DL)** is the smallest concentration that can be determined with a specified level of confidence. It is usually defined as the analyte concentration yielding an analytical signal (S) equal to 3 times the **uncertainty of a blank measurement** (ΔS_{bk}). Thus, the detection limit is three times the blank uncertainty divided by the slope of the calibration curve (m) or

$$DL = 3\ \Delta S_{bk}/m \tag{10}$$

Usually ΔS_{bk} is estimated as the **standard deviation of the blank measurement (s_{bk})**.

For absorption measurements, S = A or

$$DL = 3\ \Delta A_{bk}/m \tag{11}$$

where m is the slope of the calibration curve is expressed in absorbance units (AU) per concentration unit or reciprocal concentration units [e.g., $(\mu g/mL)^{-1}$] and **ΔA_{bk} is the uncertainty in the blank measurement expressed in absorbance units.**

Three factors that may contribute to the uncertainty in the blank signal are readout resolution, noise in the measured signals, and imprecision in positioning of the cell as it is removed and replaced (or emptied and refilled).

If repetitive measurements of the blank all yield identical values, the measurements are **readout resolution** limited. In this case, ΔA_{bk} is estimated to be the resolution displayed on the screen, which is 0.0001 AU for the OOI system. Readout resolution is likely to be limiting only with low resolution readout devices such as analog meters which provide a resolution of 0.2-0.5 % T. Most modern spectrophotometers with high resolution digital readout provide resolution from 0.1 - 0.01 % T or 0.001 - 0.0001 AU.

If values from repetitive measurements of the blank differ, the uncertainty in the blank absorbance is limited by **noise or cell positioning imprecision**. In this case, ΔA_{bk} is taken as the standard deviation of the blank absorbance. The value obtained with a stationary sample cell is just due to noise. The value obtained when the cell is removed and replaced is determined both by noise and the random error due to positioning the cell. You can ascertain if noise or cell positioning imprecision is limiting by comparing the blank standard deviation obtained by making repetitive measurements without moving the cell to those obtained when moving the cell. If ΔA_{bk} is greater

when the cell is moved, then sample cell positioning limits the DL.

B. *Experimental*

To evaluate the detection limit and what determines it, the following measurements are to be made using only the Absorbance mode. **First put the cell filled with blank solution into the sample cell holder and adjust the integration and or the average so that the intensity in the S mode at both the analysis and the correction wavelengths is about the same, and it is between 2000 and 3000 counts.**

Determine standard deviation in signal with stationary cell. Here you'll make 20 independent measurements of the Absorbance (channels A and B and combo 1). First check that Edit | Settings | File Saving | Saved Precision is set to at least 4 so the data in the file will have at least 4 digits to the right of the decimal point. Click the Time Acquisition Configuration dialog box to make sure the frequency is 1 s and specify a file name. Check that the spectrometer is in the Absorbance mode and start the data acquisition (click on green arrow icon). When you observe that 20 or so data points have been taken, stop the data acquisition, **find the file** and confirm the measurements are stored. **Calculate the standard deviation of the data for all three cases, A, B and combo1, in each of the three columns**. Be sure not to overwrite the file. The variation in the signal is due to the inherent noise in the reference signal.

Determine standard deviation in signal with removal and replacement of cell. Often the positioning of the cell or filling a fixed cell can cause additional imprecision because the orientation and transmission of the cell are changed when the cell is moved. Adjust the data acquisition frequency to 10 s and change the file name. Measure absorbance (channels A and B and combo 1) another 20 times, but this time, remove and replace the sample cell (filled with blank solution) in the sample cell holder between each of the 20 measurements. Immediately after you have noted that the first acquisition (which is denoted zero) has occurred, remove the cell and replace it the same orientation before the next acquisition occurs at 10 s. Coordination with your lab partners helps. Repeat moving the cell in/out of the holder until 20 points are taken. **Find the file** and confirm the measurements are stored. **Calculate the standard deviation** between the 20 measurements. How do these three standard deviations compare to those for the stationary cell in the last test?

VIII. EFFECT OF SLIT WIDTH OR SPECTRAL BANDPASS ON ABSORBANCE

A. *Introduction*

The monochromator slit width (W) controls the amount of radiation and the wavelength range incident on the sample or reference solution through determining the spectral bandpass ($s = R_d W$). In some molecular absorption instruments, the slit width is fixed and in others it can be varied and must be chosen for a given situation. In contrast to atomic lines where the natural halfwidth, w_h, or FWHM (full width at half the maximum intensity), is 0.01 nm, w_h for a molecular band is normally 10 - 100 nm and usually much larger than the monochromator spectral bandpass, **s**. When measuring intensity from an atomic line, the slit width must be large enough to pass sufficient radiation to yield reasonable precision (high signal to noise, S/N) yet small enough to insure sufficient spectral resolution (especially if there is interfering non-analyte absorption near by). Adherence to Beer's law, and a large "effective" or "average" molar absorptivity, are also required.

Beer's law is derived with the assumption that the incident radiation is monochromatic, which is never achieved in real spectrophotometers. However, by adjusting the spectral bandpass with the slit width to be significantly smaller than the half-width of the absorption peak, ε is effectively constant over s and Beer's law applies. If s or W is increased too much, then the polychromatic nature of the radiation incident on the sample can manifest itself in two ways. First, by a **decrease in the slope of the calibration curve**, giving a lower "effective" molar absorptivity, and secondly, by **negative deviations at higher absorbance values**, giving an apparent deviation in Beer's law. Basically, the light at different wavelengths is absorbed to different degrees. **To prevent non-linearity due to polychromatic radiation, the rule of thumb is that the monochromator spectral bandpass should be less than 1/10 of the natural half width (i.e., $s/w_h < 0.1$)**.

To illustrate these effects, the dependence of the absorbance of praseodymium on concentration and slit width is studied here. Praseodymium ion (Pr^{3+}) forms a complex with H_2O in aqueous solution, as do most of the other rare earth ions, which displays quite narrow absorption bands. The absorption band at 482 nm studied here has a half-width of about 4 nm.

Measurements will be made with a simple commercial single-beam spectrometer (e.g., a Turner 330), which has a fixed spectral bandpass of 10 nm and compared to absorbance data taken with a high resolution, Cary 219 spectrophotometer (provided in Table III) operated with a spectral bandpass of 0.7 nm and cell pathlength of 1.0 cm.

B. *Experimental*

Three test tube sample cells (pathlength of 1.2 cm) are available in the instrumental laboratory and contain 0.5 M $HClO_4$, 0.08 M Pr^{3+} in 0.5 M $HClO_4$, and 0.02 M Pr^{3+} in 0.5 M $HClO_4$.

Determine transmittance of two Pr^{3+} solutions. Measure T of the two Pr^{3+} solutions with the Turner spectrophotometer at the wavelength of maximum absorption (482 nm). Calculate A later. The TA will review the operation of the Turner spectrophotometer.

IX. LAB REPORT

The lab report should include answers to questions on pp. 20 - 22; data sheets pp. 23 - 24; duplicate lab notebook pages; and hardcopies of spectra. **No abstract is required**. Label answers to questions with the appropriate question number and put these in the order in which the experiment was done. The data sheets are requested as compact summaries of numerical values calculated in the answers to questions or of other key data. The lab notebook pages show information about solution preparation, raw data from the lab, etc. **Save a copy of the report for yourself** as you will need some of the results for the next lab report (e.g., absorbance spectrum, absorptivities, calibration curve equations, detection limits, and amounts of riboflavin in your vitamin pills).

DATA, CALCULATIONS, QUESTIONS

Do not round-off numbers used in calculations. Round-off only when you report your final answers, which should have no more than three significant figures. Always use leading zeros when reporting values less than 1 (write as 0.543 not .543). Use scientific notation where applicable for very large or very small numbers and always give units.

IVB 1. Label both of the hard copies for the absorption spectra taken with the two commercial spectrophotometers for the 25 μg/mL riboflavin solution. This includes giving instrumental parameters.

2. For the absorption band with the longest wavelength, report the wavelength of maximum absorption (λ_m) and the half-width (w_h) in nanometers and indicate both directly on the hardcopies charts. The half-width is an estimate good to no more than 2 significant figures.

VB

1. Use the data for the standard solutions (not the samples). Plot the experimental data for corrected absorbance ("combo 1") for the standards vs. concentration for the standards in units of μg/mL in excel using markers only for the data. Perform linear regression on the data, calculate expected values of absorbance from the regression equation, and plot the regression fit line as line only on the same graph with the experimental data. The linear regression lines or curves **should extend to zero concentration (forecast back to 0)**, not just to the lowest standard, so you can observe the predicted intercept. Give the equation for the regression line (showing slope, intercept, and standard errors in each) directly on the graph in text boxes. Report the spreadsheet data table (C, A, A') plus the graph, and the results of the regression i.e., the calibration equation and standard error for the slope and intercept. This table, graph and regression should be virtually identical in format to those of the spreadsheet proficiency test.

2. Use **the slope of the linear regression line** to calculate the absorptivity in $(\mu g/mL)^{-1}\ cm^{-1}$ and also the *molar* absorptivity of riboflavin ($M^{-1}\ cm^{-1}$). Assume the path length b = 1.00 cm. Report both values for absorptivity including units and proper significant figures.

3. Use the results from the linear regression for the calibration equation based on the standards to calculate the concentration of riboflavin for your test solution of the synthetic unknown. Report this value in μg/mL and provide a sample calculation demonstrating with a formula and numbers exactly how you obtained your result.

4. Calculate the effective spectral bandpass *s* for the measurement based on the range of pixels covered with the boxcar setting that you used. Report this value for *s*.

5. Calculate and report the ratio s/w_h. Use the effective spectral bandpass from question 4 and the observed width at half height (w_h) obtained from the spectrum in Part IVB. From this ratio, do you think polychromatic radiation causes significant non-linearity in your calibration curve for riboflavin. Does this conclusion agree with your results?

6. For the three sub-samples you prepared for the B-complex vitamin pill, calculate the following quantities and place them in a proper data table. **After the table, provide a sample calculation for each quantity, a through h, give the formula, then demonstrate with values how you got the result. Use units and keep one extra significant figure until the final result (which should be 3-4 sig figs).**

(a) concentration of riboflavin in μg/mL in the test solution found from the calibration equation that was determined by linear regression of the standards;

(b) mass (μg) of riboflavin in the total volume of test solution y;

(c) mass (μg) of riboflavin in the total volume of test solution x;

(d) mass (mg) of riboflavin per gram of pill;

(e) mass (mg) of riboflavin per whole pill;

(f) Find the average value for part (e). If one of the values in (e) is significantly different from that of the other two samples, consider eliminating the outlier value from the average by using the appropriate statistical test. Report the mean.

(g) Report the precision based on the standard deviation (SD) and the relative standard deviation (RSD)from the three values you obtained for the milligrams of riboflavin/pill (e above).

(h) Compare the mean experimental value of the amount per pill (f above) to the value listed on the bottle, and calculate and report the percent error. If your results do not agree well with the values listed on the vitamin bottle, first check your calculations, then briefly discuss one or two scientific reasons why this might be the case. Also consider the SD of your trials.

VIB 1. Extrapolate the calibration curve (using only the data for the lower concentrations where linearity is good) to forecast a value for the absorbance for the 100 μg/mL riboflavin solution and report this. Use this projected absorbance to calculate a projected transmittance. From this value and using the absorbance that you actually measured for

the 100 μg/mL test solution, estimate the % stray light from equation 8 and report it. Note that the percent stray light (100 f) should be between about 0.2 and 2%

VIIB 1. Calculate three estimates of the detection limit (DL) from three different estimates of the uncertainty in the blank measurement ($\Delta A_{\mathbf{bk}}$) from the following three cases (a-c). Tabulate values for ΔA_{bk} and DL in μg/mL in a proper table.

a. DL limited by readout resolution of the OOI spectrometer;
b. DL limited by the measured noise in the signal from the stationary cell;
c. DL limited by the reproducibility of making reference measurements with cell re-positioning.

Report values for ΔA_{bk} for channels A, channel B, and for combo 1 but calculate the DL from equation 11 only using ΔA_{bk} for the corrected absorbance from combo 1. Include sample calculations. Discuss the significance of the results. Which value of the three cases (a,b,or c) do you think is the best estimate of the actual detection limit for the experiment and give a brief explanation of why you think this?

2. Compare ΔA_{bk} from channels A and B to that for combo 1, with and without cell positioning. Does it appear that the use of a reference absorbance (combo 1) reduces random error due to either noise or cell re-positioning? Note that with 20 measurements, differences in the standard deviation of less than a factor of two are not significant.

VIIIB 1. Calculate ($s/w_{\boldsymbol{h}}$) for each instrument. Assume w_h = 4 nm. The spectral bandpass of the small Turner spectrophotometer is 10 nm while it is 0.7 nm for the Cary 219 spectrophotometer in room 314.

2. Briefly discuss the performance of the two instruments in terms of the absorbance values for a given solution, the possibility of negative deviation, and the magnitude of (s / w_h) relative to the "rule of thumb" on p 19.

IX. 1. Make sure you have included all the information on the Checklist sheet at the end of the data sheet section.

DATA SHEET FOR EXPERIMENT 2A

Name _____ Station # _____ Date _____

Name of person who has spectra attached to lab report _____

Team Synthetic Unknown # = _____

Section VB: Calibration Curve and Quantitative Analysis

analysis wavelength = _____ nm **(check that your wavelength is near 445 nm**)

Integration = _____ ms Averages = _____ Boxcar = _____

Conc. (μg/mL)	N_r (counts)	N_s (counts)	T (T mode)	A (A mode)
10				

From N_r and N_s in the S mode given above, calculate:

T (from count ratio) = N_s / N_r = _____;

A = - log T (from count ratio) = _____;

A = - log T (from T measured in T mode) = _____.

How well do the two calculated values for A above compare to what you measured for A using the A mode in the software?

Corrected Absorbance Setup:

Updated analysis wavelength = _____nm

Correction wavelength = _____nm

Integration = _____ ms Averages = _____ Boxcar = _____

Table I. Absorbance data for standards and samples - make a table in Excel and fill in as you collect this data. Make a plot and add a trend line and give the slope and intercept to three sig figs. Run Data Regression (or LINEST) and find the SE for the slope and for the intercept. Add these to the plot as you did in the spreadsheet quiz the first week.

Conc. (µg/mL)	A (combo 1)	Ratio for A values for Stds 10:2	Ratio for A values for Stds 25:10	Ratio for A values for Stds 25:2
2				
10				
25				
syn unknown				
Y1				
Y2				
Y3				

Section VIB: Stray Light

measured A (combo 1) = _____ (**check that value is between 2 and 3.5**)

Section VIIB: Detection Limit

Attach the labeled spreadsheets with the 20 repetitive measurements of A with a stationary cell and with cell re-positioning. Record the voltage range used on DMM. _____

Table II. Noise and Cell Positioning Signal Data

channel	quantity	with stationary cell	with cell re-positioning
A	Mean		
A	Std Dev		
B	Mean		
B	Std Dev		
Combo 1	Mean		
Combo 1	Std Dev		

Section VIIIB: Polychromatic radiation

Fill in the shaded cells in the table below with the measured values for the T you took using the Turner spectrometer at both concentrations and then calculate and report the equivalent value for A for both solutions using the Turner spectrometer.

Table III. Polychromatic Radiation Effects

Spectrophotometer	**c (M)**	**T**	**A**
Cary 219	0.02	-	0.085
Cary 219	0.08	-	0.320
Turner	0.02		
Turner	0.08		

SUMMARY SHEET and CHECKLIST FOR EXPERIMENT 2A

Section II: Solution Preparation - Mass data - report grams used to +/- 0.0001 g and report leading zeros where appropriate, (i.e., **0**.1876 not .1876).

mass of whole vitamin pill	
mass of sample for solution X1	
mass of sample for solution X2	
mass of sample for solution X3	

Checklist for the short answers to questions that the grader will be looking for in your report:

Question	Done?	Information Requested
IVB. 2.		wavelength of peak maximum for vit B2
		half width of absorption band used for vit B2 analysis
VB. 1.		regression calibration equation
		standard error slope
		standard error intercept
VB. 2.		absorptivity, a, in (μg/mL)-1 cm-1
		calculated **molar** absorptivity for riboflavin (ε)
VB. 3.		riboflavin concentration in the synthetic unknown
VB. 4.		effective spectral bandpass
VB. 5.		s / wh
VB. 6.		the mean and RSD of riboflavin in a pill (mg B2 / tablet)
VIB. 1.		forecast value of absorbance for
		100 μg/mL riboflavin solution
VIB. 2.		percent stray light, f
VIIB.		values for three methods to find detection limits, based on:
		a) readout resolution for the DMM or ADC
		b) electronic noise (stationary cell)
		c) cell positioning in/out of holder