Ultraviolet-Visible Absorption and Fluorescence Spectroscopy

Objectives:

This is a two-week experiment on the simultaneous analysis of a two-component mixture and limits of detection (LOD) comparison between UV-visible absorption and fluorescence spectroscopy.

Fluorescence is an inherently more sensitive method relative to absorption and as a result yields significantly lower LOD. By measuring the noise in the instrument's signal, it is possible to determine the lowest detectable signal associated with an analyte (LOD), which is an important figure of merit of any technique. Another important figure of merit is the limit of quantitation (LOQ).

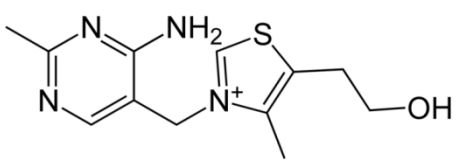
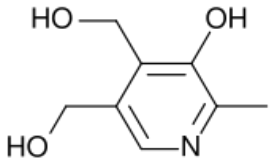
Pre-Lab Questions:

Questions to be answered before doing the experiment. **The answers are due at the beginning of each experiment without exception (the questions are for credit and some may appear on your final exam).**

1). Describe how Beer`s Law can be used to quantify absorbing species. Name the parameters and variables involved. What is the relationship between Absorbance and percent Transmittance?
2). Explain how the absorbance of mixtures is additive. Describe the "two unknowns, two equations" system used to find the concentrations of two absorbing species in mixtures.
3). Name the principal components of a spectrometer and fluorimeter. What function do each component play? Why is detection in fluorimeter performed at 90^o relative to the incident light?
4). How do you measure LOD? Give equations, etc.

Week 1 - Simultaneous Analysis of a Two-Component Mixture:

This experiment involves the determination of the concentrations of vitamin B_1 (thiamine) and B_6 (pyridoxine), in vitamin supplements. Both compounds absorb in the ultraviolet. First it will be verified that the absorbances of a mixture of these compounds are additive over the wavelength range to be used. Then two wavelengths will be chosen for the simultaneous equations method and the necessary data will be obtained. Spreadsheet calculations will be used to determine the concentrations of the two compounds.

Thiamine	**pyridoxine**
	

In week 2 – You will determine the concentration curve of B_6 and the limit of detection (LOD) of B_6 for UV-vis and Fluorescence.

This experiment involves the determination of the sensitivity of the fluorometer. The limit of detection, which is defined as the lowest concentration that can be measured above the noise in the background signal, is compared between the spectrophotometer and fluorometer. The LOD is strictly defined as the concentration that will yield a signal that is equal to 3 times the standard deviation of the background signal. For the UV-vis experiment, you will be required to determine the standard deviation in the blank signal at the wavelength used to determine the concentration of B6 and then using the calibration curve from day 1, you should be able to determine the LOD concentration. For the fluorescence measurement, you will be required to determine the optimum excitation and emission wavelengths and then prepare a calibration curve for concentrations less than 1 mg/L. Again, you should be able to measure the standard deviation in the background for the fluorescence measurement and from the variation in zero reading determine the LOD.

Procedures: Week 1:

You will need to make the following in lab:
- 1 L of 0.01 M HCl from concentrated HCl. This will be your solvent blank and will be used for **all** solutions and dilutions (***remember*** *to include descriptions of how all solutions and dilutions are made along with calculations to determine volumes or weights required and report the actual values measured out in your lab notebook*).

- 100 mL of vitamin B_1 and B_6 stock solutions are supplied at concentrations of 100mg/100 ml or 1 mg/ml.

A. Additivity of UV-vis Spectrum

1. Make two standard solutions: 40 mg/L (40 ug/ml) of B_1 and B_6 in 25 mL volumetric flasks.
2. Make a third solution contains 40 mg/L of each vitamin together in a single 25 mL volumetric flask.
3. Obtain spectrum of the 3 solutions and save each with a file name. Export the data in CSV format for spreadsheet work later. (*the sum of the spectra of the individual solutions should be close to the mixture*)
4. Looking at the spectra of B_1 and B_6, choose two wavelengths (where the vitamins absorb) that you think will be best for the quantitation of the two compounds.

B. Beer's Law Plot of B_1 and B_6

1. Prepare a series of four dilutions 1, 5, 10, 20 mg/L from the 40 mg/L standard of B_1 in the previous section and use the 40 mg/L standard as a fifth sample.
2. Using the Cary **Simple Read** software to determine the absorbance of each standard at both chosen wavelengths. (***note****: specify two wavelengths in the setup window by placing a `;`between the two values. Be sure to zero the instrument with the blank after the settings have been made*). Be sure to record all values in your lab book.
3. Repeat the same process for vitamin B_6. (***suggestions****: 1. It is always good to plot your data as you go to make sure that you are getting a linear response; 2. It also a good idea to run your samples from low concentration to high concentrations to reduce contamination errors*)
4. In preparation for Section F below, with the blank in the instrument after it has been zeroed, make five absorption measurements, waiting 15 seconds between measurements. You will be interested in the variations at the wavelength corresponding to the maximum absorbance of B_6.
5. Keep the 40 mg/L standard of B_6 for the step F.

C. QC with Known Mixture; Sample Analysis

1. You can use the solution from A2 as a quality control (QC) sample. Since you know the concentrations of both components, you can make the same measurement used in B and perform the calculations using the calibration values in B to determine the concentration experimentally. If you get values that don't make sense, you have done something wrong.
2. Obtain the absorbance data for the unknown provided.

D. Operation of the Varian Cary 50 UV-VIS Spectrophotometer:

1. Start-up

1.1 Turn on the computer and monitor if they are not already on. Note that the Cary 50 spectrophotometer is powered from the computer.

2. Zeroing the Instrument – All modes

2.1 Click the "zero" button to zero the system. The program is ready to load the blank.

2.2 Load your blank. Always use a quartz cuvette, and use one cuvette for all measurements since the cuvettes are not matched. Click on "OK" to complete the zeroing of the instrument.

2.3 With the blank solution in the cell holder, click the "zero" button several times until the absorbance is near 0.0000.

3. Method Set-up – Scan Mode

3.1 From the desktop click on the icon **Cary WinUV** to open the Cary WinUV window.

3.2 Click on the icon "**Scan**" to open the scan application.

3.2 Set up the instrument parameters by clicking on the icon "**setup…**". This will display the Setup Dialog/Cary tab:

Start: 200 (or any wavelength)
Stop: 600 (or any wavelength)
Mode: Abs

3.3 Once you are satisfied with your method setup click on "**OK**" to confirm any changes you have made and close the Setup dialog.

3.4 Take a 1 cm quartz cuvette and rinse it well with milli-Q water. Carefully wipe the transparent sides with Kimwipes. Do not scratch the cuvette or touch the surfaces with your fingers. Dust free gloves are good.

3.5 Fill the cuvette with your sample solution, no more than ¾ full. Check to see whether there are air bubbles on the optical surfaces. Refill the cuvette if necessary.

3.6 Carefully install the cuvette in the cell holder. It should slide in with the clear faces parallel to the light path.

3.7 Click on "**Start**" to start the scanning.

4. Method Set-up – Simple Read Mode

4.1 From the Cary WinUV window, click on "**Simple Read**" to open the application.

4.2 Set up the instrument parameters by clicking on the icon "**setup…**". This will display the Setup to make readings at multiple wavelengths, type them in separated by a "**;**" and leave the other settings at their default values (*however, you should always record them in your notebook*)

4.3 Zero the instrument as in previous cases with a blank

4.4 Press Start to make absorbance readings.

5. Data Storage

5.1 After you have completed all the analysis, be sure to save the report in RTF format.

5.2 Spectra can be saved by "saving data as"

5.3 You can print out spectra or reports as a pdf to take with you

6. Shutting Down

6.1 Remove the cuvette from the cell holder, empty the solution, rinse it with MQ water, gently rinse out all liquid remaining from the outside, and place it back into the storage case.

6.2 Close the Cary Win UV menu, but you can leave the computer on.

Week 2:

E. Determine optimum excitation and emission wavelengths for B_6

1. Review your UV-vis spectra for B_6 obtained the previous week. Keep in mind that in order for a molecule to emit, it must first be excited by absorbing light. The wavelengthf corresponding to the peak absorbance is typically the best excitation wavelength.
2. Using this excitation wavelength, obtain an emission spectrum for a 1 mg/L (1 ug/ml.) solution of the B_6. When selecting a range of wavelengths, remember that the molecule will not emit at wavelengths lower than the excitation wavelength (*conservation of energy – you cannot get more energy out that you put in*)
3. Based on your spectra choose what you believe is the optimum emission wavelength.

F. Calibration plot for B_6 and LOD determination

1. Using the Simple Read program, setup the experimental parameters to make measurements using the optimum values you found. You can leave the other settings at their default values (*but you must record what the values are*).
2. Prepare a series of four dilutions spanning the range 0.01 to 1 mg/L from the 40 mg/L standard of B_6
3. Zero the instrument with a blank and take emission readings. In preparation for LOD calculations, while the blank is in place take 5 readings, one every 15 seconds. Remove the cuvette and turn in 90 degrees and take another 5 readings.
4. Using your cuvette in the original orientation used for zeroing, take readings for 5 concentrations between 0.01 and 1 mg/L.

G. Operation of the Varian Eclipse Fluorescence Spectrophotometer

1. Start-up and zeroing the instrument is similar to the UV-vis instrument

1.1 The Eclipse instrument has its own power supply, so be sure to make sure it is on if it isn't already and allow it to warm up for a couple of minutes before starting the software. The LED on the side should turn green when it is ready.

2. Method Set-up – Scan Mode

2.1 From the desktop click on the icon **Cary icon**.

2.2 Click on the icon "**Scan**" to open the scan application.

2.2 Set up the instrument parameters by clicking on the icon "**setup…**". This will display the Setup Dialog/Cary tab. Make sure the emission button is activated. This will produce and emission spectrum. Specify excitation wavelength. Specify beginning and ending wavelengths for to record emission spectrum. The remaining parameters can remain at their default values (*but record the values in your lab notebook*).

2.3 Once you are satisfied with your method setup click on "**OK**" to confirm any changes you have made and close the Setup dialog.

2.4 Take a 1 cm quartz cuvette and rinse it well with milli-Q water. Carefully wipe all four transparent sides with Kimwipes. Do not scratch the cuvette or touch the surfaces with your fingers. Use gloves.

2.5 Fill the cuvette with your sample solution, no more than ¾ full and handle the cuvette only at the top ¼ portion. Check to see whether there are air bubbles on the optical surfaces. Refill the cuvette if necessary.

2.6 Carefully install the cuvette in the cell holder making sure to face the cuvette the same way each time (***hint****: there is a small Q etched onto one side. You can face this Q the same direction each time*).

2.7 Click on "**Start**" to start the scanning.

3. Method Set-up – Simple Read Mode

3.1 From the Cary window, click on "**Simple Read**" to open the application.
3.2 Set up the instrument parameters by clicking on the icon "**setup…**". This will display the Setup Enter the optimum wavelengths and leave the other settings at their default values (*however, you should always record them in your notebook*)
3.3 Zero the instrument as in previous cases with a blank
3.4 Press Start to make emission readings.

4. Data Storage

4.1 After you have completed all the analysis, be sure to save the report in RTF format.
4.2 Spectra can be saved by "saving data as"
4.3 You can print out spectra or reports as a pdf to take with you

5. Shutting Down

5.1 Remove the cuvette from the cell holder, empty the solution, rinse it with MQ water, gently wipe out all liquid remaining, and place it back into the storage case.
5.2 Exit the software and close the Cary window. Leave the computer on, but turn off the instrument (on off switch on the bottom right hand side)

Data Analysis:

1. Justify the choice of the two wavelengths to be used in the simultaneous equations method.
2. Prepare Beer's Law plots for pure B_1 and B_6 at each of the chosen wavelengths. Express the concentration axis in units of mg/L and make sure to include 0,0 as a point in the linear least squares analysis. The two B_1 plots may be presented in one graph, and the two B_6 plots in a second graph. Be sure to give the equations for all the fits along with the correlation coefficients.
3. From the Beer's Law plots calculate the concentrations of the two vitamins in the unknown and the QC mixture measured in Section C
4. Calculate the LOD for the UV-vis determination of B_6
5. Justify the choice of the excitation and emission wavelengths used for the fluorescence measurement
6. Prepare a calibration curve from the fluorescence data and provide the equation for the best fit line
7. Calculate the LOD for the fluorescence measurement and express the value in both mg/L and ppb (parts-per-billion)
8. Calculate the systematic error that could be generated if you accidentally changed the orientation of your cuvette during analysis.

Conclusion:

State the results and describe pertinent major sources of random and systematic errors. In your error analysis, reflect on the assumptions that are made in the simultaneous analysis of a multi-component system. If these assumptions do not hold, how will the results be affected? Discuss the advantages and limitations of the simultaneous equations method. Discuss the advantages and limitation of both fluorescence and UV-vis techniques.

QUESTIONS

1. What are other possible cuvettes one can use and what would be the purpose of using the different types of cuvettes?
2. Which type of transtions are UV-vis absorbance and fluorescence spectroscopy based on? What is the wavelength range corresponding to these transitions?
3. Describe the main differences in configuration between a scanning UV-vis spectrometer (as in the Cary 50) and a diode array instrument (as used on the HPLC).
4. Name some advantages and disadvantages of using UV-vis *vs.* fluorescence spectroscopy in quantitative analysis.