DETERMINATION OF RIBOFLAVIN CONTENT IN A MULTIVITAMIN

Background

Riboflavin, which is also known as vitamin B_2, is named for the sugar alcohol (ribitol) that is responsible for its hydroxylated tail and its yellow color (*flavin* is from the Latin word for yellow). It has two systematic names: 7,8-dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl) isoalloxazine and 7,8-dimethyl-10-ribitylisoalloxazine. The molecule is heat-stable but easily degraded by light.

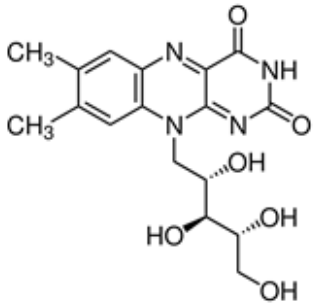

Figure 1. Molecular structure of riboflavin, $C_{17}H_{20}N_4O_6$.

Riboflavin is found in many foods, such as eggs, nuts, grains, dairy products, organ meats, and dark green vegetables. Biochemically, it is vital for proper utilization of carbohydrates, fats, and proteins as energy sources. Specifically, riboflavin is a component of two coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). (Coenzymes are molecules added to certain polypeptides to make them functional enzymes.) FAD and FMN easily lose or gain electrons and hydrogen atoms, aiding certain enzymes in the oxidation or reduction reactions in electron transport chains and a wide array of metabolic pathways.

As indicated by the title of this experiment, the objective is to determine the concentration of riboflavin in a multivitamin. Also noted above, riboflavin is yellow, and, therefore, absorbs strongly in the blue to green spectral regions. *Then, can absorption spectroscopy be used to measure riboflavin's concentration in a multivitamin?* **No**, many other constituents of vitamin pills absorb in the visible spectral region, overlapping with the riboflavin's absorbance. Fluorescence, however, can be used because riboflavin is one of the few vitamin molecules that fluoresces. This selectivity makes the detection of riboflavin possible in a mixture of other vitamins without significant interference.

Fluorescence refers to a process in which a molecule in an electronically excited state loses its electronic energy by a emitting a photon. Only a small fraction of molecules can fluoresce; most electronically excited molecules either lose their energy by heating the environment or breaking apart without emitting any photons. Therefore, if molecules do fluoresce they become very easy to identify in a mixture. Indeed, fluorescence has developed into one of the most sensitive analytical techniques, capable of detecting *single molecules*. Some of the most remarkable scientific advances, including human genome sequencing and in vivo cell imaging, have been made possible by fluorescence spectroscopy.

Furthermore, fluorescence spectroscopy is considerably more sensitive than absorption spectroscopy, making the detection of much smaller concentrations of riboflavin possible. *Why?* The detector in absorption spectroscopy is always illuminated by the light source. Insertion of an absorbing molecule between the light source and the detector leads to a small reduction in the transmitted light intensity. For small absorbances, like those we typically use in analytical measurements, the absorbance is approximately proportional to a small difference between two large values: the signal with the absorber present, I, and the signal with the absorber removed, I_0. *In contrast*, the fluorescence signal without the compound emitting the light, known as the fluorophore, is very small or zero. A small increase in the fluorescence signal on top of this near-zero background is easily detected. Think in terms of the following analogy: a change is more observable if one candle out of a 100 is lit than if one candle out of 100 lit candles is extinguished. In other words, the detection limit for fluorescence spectroscopy is usually much lower than that for absorption spectroscopy.

You will be using a SpectroVis Plus Spectrophotometer (please read the full description on the Vernier website: http://www.vernier.com/products/sensors/spectrometers/svis-pl/). It can record optical absorption spectra in the 380 – 950 nm range, and fluorescence spectra excited by either 405 nm or 500 nm radiation.

Safety

Safety goggles and aprons must be worn at all times. All chemicals used could be contaminated, and, are, therefore, hazardous if ingested.

Procedure

Whenever the procedure requires you to 'dilute', ensure that you are diluting with 0.02M acetic acid.

A. Solution Preparation

1. Prepare 1.0 L of 0.02 M acetic acid from 4.0 M acetic acid and nanopure water. The concentration does not need to be exact, a 1 L beaker or other suitable (clean) containers with approximate volume markers can be used.

   Your TA will assign you to perform either step 2 or step 3. You will share the stock solution you make with another group:

2. Prepare 500 mL of a 50.0 ppm stock solution of riboflavin in 0.02 M acetic acid. Riboflavin takes about an hour to dissolve. Weigh the required amount of riboflavin to 0.1 mg precision, place in an **Erlenmeyer flask** (volumetric glassware cannot be heated!) with ~450 mL of 0.02 M acetic acid, add a stir bar, and place on a stir plate. Gentle heating of the solution (to 50 – 70 °C) may accelerate dissolution. Riboflavin is dissolved when no suspended particles remain in the solution. Once riboflavin is fully dissolved, cool the solution to room temperature, transfer to a 500 mL volumetric flask, and fill/dilute to the mark.

3. Weigh out about 50 mg of the powdered multivitamin unknown to 0.1 mg precision and dissolve it in 250 mL of 0.02 M acetic acid. The powder also dissolves slowly, so use the same strategy as stated in step 2: first dissolve the powder in about 200 mL with stirring, than transfer into a 250 mL volumetric flask, and dilute to volume.

   (While waiting for the riboflavin and multivitamin to dissolve, find and read the article "Linearizing the Calibration Curve in Determination of Sulfate by the Methylthymol Blue Method" (Parry, et. al., Analytical Chemistry, 48(12), 1693, 1976) in your handouts. This topic is related to the seawater lab at the end of the quarter.)

4. Using the 50.0 ppm riboflavin stock solution, make 7 – 10 standard solutions using 25 mL volumetric flasks. Solution concentrations should range from 1.0 to 20.0 ppm. Possible concentrations are:

Target Concentration	**Take x mL of 50 ppm Stock**	**Dilute to mark in volumetric flask**
0 ppm	–	Solvent only for blank
2 ppm	1 mL	25 mL
4 ppm	*Calculate*	25 mL
6 ppm	*Calculate*	25 mL
8 ppm	*Calculate*	25 mL
10 ppm	*Calculate*	25 mL
12 ppm	*Calculate*	25 mL
14 ppm	*Calculate*	25 mL
20 ppm	*Calculate*	25 mL

5. You need one set of standard solutions per pair. Compare the color of the unknown solution with the standard solutions. Estimate the concentration of the unknown solution based on appearance alone.

B. Absorbance Spectrum

1. Open Logger Pro. If the program doesn't automatically recognize the spectrometer, unplug it and plug it back in. Go to **Experiment** and choose **Calibrate**. Let the lamp warm up for at least 90 seconds. When the spectrometer is ready, fill a clean cuvette with 0.02 M acetic to use as a blank.

2. Prerinse your cuvette with your least concentrated calibration solution that is not your 0 ppm blank. Then, fill your cuvette with this solution and place in the spectrometer. Press **Collect**. Once the spectrum is acquired, press **Stop**. Prerinse and fill the cuvette with your most concentrated calibration solution. Make sure you keep consistency between runs by using the same cuvette and having the same side facing the light source and detector throughout the experiment. There is a marker you can use to label the correct side of cuvette. Press **Collect** and choose **Store Latest Run**. Save the spectrum. Copy your data in the Vernier software table into Excel.

3. By examining the spectrum, chose the best excitation wavelength for the fluorescence of riboflavin. The best wavelength is where the absorption is reasonably strong but the emission is weak. With only two excitation wavelengths at your disposal (405 nm and 500 nm), the choice should be quite obvious.

4. Before moving on to take fluorescence measurements of your calibration solutions, take an absorbance spectrum of your unknown solution (it is good practice as a chemist to characterize your analyte as much as possible).

C. Fluorescence Measurements

Important: The fluorometer you are using has a very small dynamic range. If the concentration is too low (< 1 ppm or so), no signal will be seen. If it is too high (> 10 ppm or so) the measured fluorescence intensity will be saturated. Therefore, the dilution factors chosen below will determine if your fluorescence intensity falls in range.

1. Go to **Experiment** and choose **Change Units**. Change the units to fluorescence, selecting the appropriate fluorescence wavelength. Fill a cuvette with the most concentrated calibration solution (20 ppm). Press **Start**. Wait a few seconds then record the wavelength of maximum fluorescence and fluorescence intensity. Save the spectrum. Remove the cuvette, rinse well, fill with the next solution, and repeat until you have spectra of all the standard solutions, the unknown, and the solvent (the blank). Save all spectra as you go, the data is needed for your report.

2. Plot the fluorescence intensity vs. the concentration. Based on this plot, determine the concentration range over which the signal is proportional to the concentration.
   a. Are there enough calibration points in this range? If not make additional calibration solutions, and record their fluorescence as well.
   b. Does the unknown signal fall within the calibration range? If not dilute the unknown by an appropriate factor (e.g., by 2), and collect another spectrum. Once the fluorescence is in range, take 2-3 measurements (with different aliquots of unknown solution).

D. Method of Standard Additions

1. Make an unknown solution that produces a fluorescence signal falling within the calibration range. Add 5.0 mL of this unknown solution into five 25.0 mL volumetric flasks. Add one of the following amounts of the 10.0 ppm stock solution to make 5 standard addition solutions: 0 mL, 1.0 mL, 2.0 mL, 3.0 mL, and 4.0 mL. Dilute to the calibration mark with 0.02 M acetic acid. *If the fluorescence intensity is too high (the signal is saturated), dilute the initial unknown or/and use a lower concentration stock for the standard additions.*

2. Record the fluorescence spectra of the 5 standard addition solutions. Save the spectra. You will use them to build a plot of the peak intensity as a function of the added standard concentration.

E. Shutting Down

The solutions produced during this lab can be safely poured down the drain. Please turn off the program and disconnect the spectrometer from the computer (following proper steps for disconnecting USB powered hardware). Return kits and clean 1L glassware to the stockroom.