Fluorometric Determination of Riboflavin in Energy Drinks

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1. Overview

This experiment was designed as an at-home lab experiment where students use a portable fluorescence spectrometer to quantitate riboflavin in energy drinks. This experiment can be adapted for a range of educational levels, depending on the background information given. This work is based mainly on work that has been posted by others.[1,2]

This document provides *general* instructions, suggested materials, experiment setup, and analysis. This information can be used to guide students in an at-home or in-lab project, or for faculty to quickly produce an experiment for students.

Active Learning Goals: Students could design studies that…

- Evaluate the linearity of the external calibration
- Evaluate the precision and accuracy of the instrument
- Measure riboflavin concentration in energy drinks
- Perform statistical analysis on their and other student data (see next section)

Topics of discussion and data analysis:

- Discussion of analytical figures of merit (precision, linear range, linearity, sensitivity)
- Data and statistical analysis – comparison of determined concentration of riboflavin
- Discussion of accuracy and precision of solution preparation tools, fluorescence, method development, and statistical analysis
- Discussion on riboflavin in consumer goods

2. Background and Relevance

Riboflavin, also known as vitamin B-2, is a poorly water-soluble vitamin present in dairy products, meat, fish, as well as certain fruits and vegetables, particularly dark-green vegetables. Riboflavin is of biochemical significance and signs of depletion arise within only a few days of dietary deprivation.[3] Exercise stresses metabolic pathways that require several b-vitamins, including riboflavin, therefore the need for these vitamins may be increased in athletes and active individuals.[4] For these reasons, riboflavin is a common additive in energy drinks. Many energy drinks, such as Monster® and Rockstar® contain the recommended daily amount (1.3 mg) or more per serving.

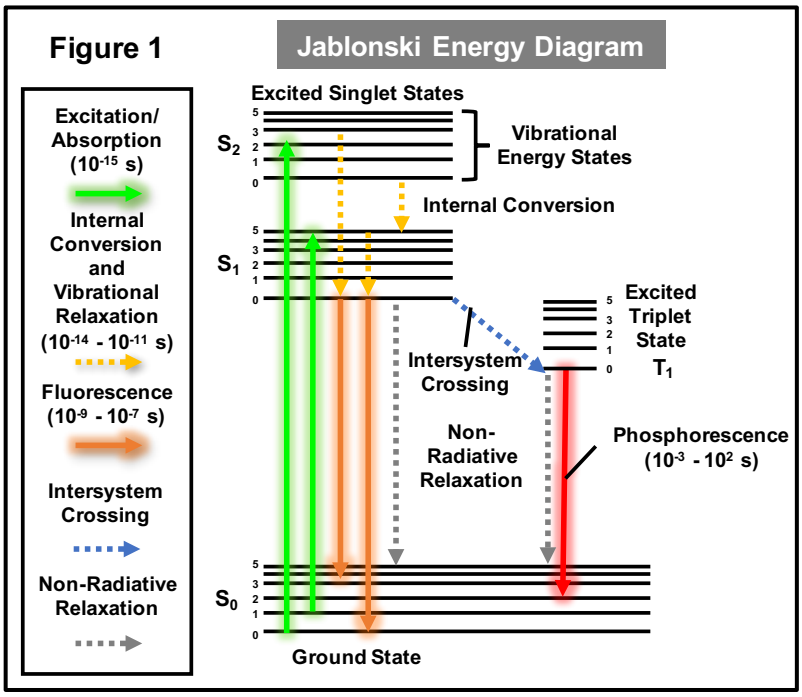
3. About Fluorescence

Luminescence is a general term used to describe the emission of light (a photon) of some wavelength as a material relaxes from an electronic excited state back to the ground state. There are many ways a material can be excited, but in the context of this experiment we are particularly interested in photoluminescence (or PL), where excitation is the result of absorption of a photon of some, usually shorter, wavelength.

Luminescence is a relatively uncommon phenomenon. Most materials relax to the ground state by non-radiative pathways (*i.e.* without the emission of a photon). In photoluminescent molecules, spin-allowed radiative transitions, from a singlet excited state (S_1) to a singlet ground state (S_0) are usually fast (on the order of 10^{-9} – 10^{-8} s) and provide luminescence that we call fluorescence. On the other hand, spin-forbidden radiative transitions from a triplet excited state (T_1) to a singlet ground state (S_0), can lead to luminescence. This pathway, called phosphorescence, exhibits a very slow rate (on the order of 10^{-3} – 10^{2} s for a typical organic molecule). In solution at room temperature, non-radiative relaxation pathways make phosphorescence almost impossible to observe. The majority of molecules are singlet in their

ground state, since singlet-triplet transitions are spin-forbidden. Thus, triplet excited states can be populated only via intersystem crossing (ISC) from the singlet excited states.

PL pathways in molecules are often represented using Jablonski energy diagrams (Fig. 1). In the generic Jablonski diagram shown, a few possible routes of photoexcitation or absorption are shown as glowing, bright green, solid arrows. Once excited, a molecule can undergo internal conversion between excited states and within vibrational levels of an excited state, as shown in gold dashed arrow. Fluorescence, the characteristically fast, emissive, $S_1 \rightarrow S_0$ transition is shown as glowing, bright orange, solid arrows. Spin-forbidden ISC, or $S_1 \rightarrow T_1$ transition is shown by a dashed blue arrow. Finally, phosphorescence, the relatively slow, emissive $T_1 \rightarrow S_0$ transition is given by a glowing, bright red, solid arrow. Other non-radiative paths to S_0 are shown with gray dashed arrows.



Now, why does any of this matter? Well, the usefulness of fluorescence spectroscopy stems from two main factors. First, since the majority of molecules do not exhibit fluorescence the background fluorescence signal is often very small. Therefore, the technique can detect specific molecules with high sensitivity. Second, the spectral properties of the fluorescence emission are usually highly sensitive to local environment (solvent, pH, ionic strength, etc.). Thus, the use of fluorescence is a keystone technology in applications where local environments can vary quite dramatically, for example in the study of macromolecule systems, and biological specimen.

4. Experimental

Materials

For Preparing Stock and Working Standard Solutions

Riboflavin (98 %), Alfa Aesar
Concentrated Sodium Hydroxide (1+ M)
Nanopure Water
Citric Acid-Sodium Citrate Buffer pH ~ 3.5 (I used prepared 1L of ~0.2000 M)
250-mL Volumetric Flask
pH meter (optional)

For Preparing Standard Solutions

Riboflavin Working Standard Solution
Citric Acid-Sodium Citrate Buffer pH ~ 3.5
Disposable transfer pipettes (10+)
Cuvettes (7-10) (and other sample containers, if cuvettes cannot double as sample vials)
Pocket/portable balance

For Analyzing Samples
Vernier GoDirect SpectroVis Plus *Or* Other fluorimeter

Unknowns
Riboflavin-containing energy drinks (in this case, Lo-Carb Monster Energy™ and Sugar-Free (SF) Rockstar®)

Procedure

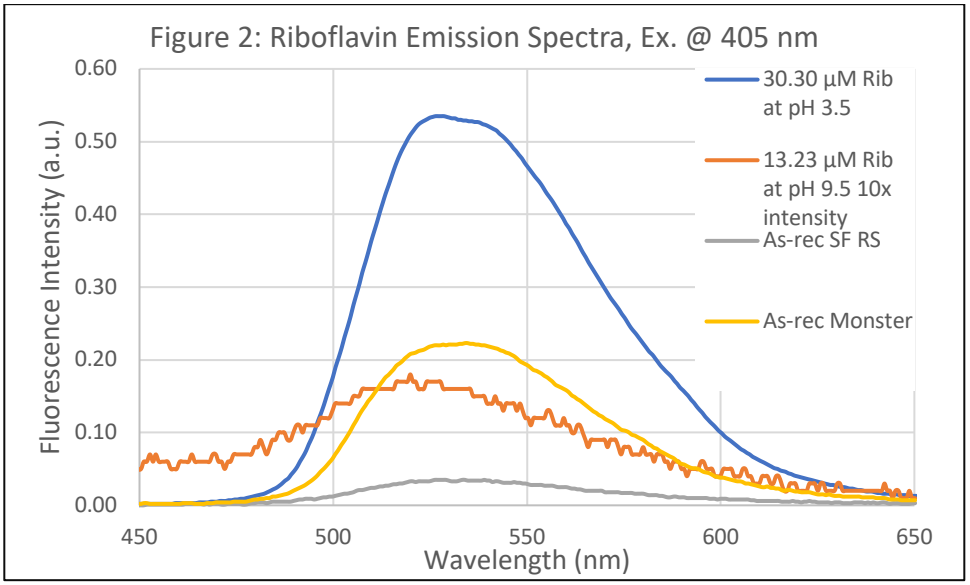
Solution Preparation

Stock Standard Solution Preparation

Makes 250.0 mL of ~ 1 mM riboflavin solution

Add 2-3 drops of concentrated sodium hydroxide to approximately 10 mL of nanopure water. Accurately mass about 0.1000 g of riboflavin, and add it to the basic solution. Mixing the basic solution and riboflavin can be done directly in the 250-mL volumetric flask or in a beaker. Though, it can be hard to dissolve all of the riboflavin in a flask. In either method, if the riboflavin is not dissolving easily, add a few more drops of concentrated sodium hydroxide. If you used an additional beaker, once the riboflavin is completely dissolved, quantitatively transfer the solution to a 250-mL volumetric flask. The dilute to the mark with a pH-3.2-3.5 citrate buffer solution.

Note the pH range. Riboflavin exhibits a pH-dependent fluorescence response, see Figure 2. The citrate buffer pH needs to be just under the pH of the energy drinks to offset the base used to dissolve the riboflavin and keep the pH relatively comparable to that of the carbonated energy drinks. Once mixed the pH of the final solution will be about 0.2-0.3 pH units higher due to the basic riboflavin solution.



Top Standard, or Working Standard Solution Preparation

Makes a ~50-60 µM riboflavin solution

Prepare a 20-fold dilution of the stock standard.

Working Standard Solutions for External Calibration
Use pipettes, cuvettes, and pocket balances, prepare a minimum of 4 standard samples with concentrations spanning 0 to the concentration of the top standard with reasonable spacing. If you are using cuvettes as sample containers, keep in mind that they do not hold much more than 3.5 mL, and keep

in mind that you can make serial dilutions from prior standard dilutions. Use Table 1 to record your standard dilution scheme.

Note that it may be helpful to determine the fluorescence emission of your top standard and as-received energy drinks *before* making your standard solutions. See the next section for measurement instructions.

Table 1: Riboflavin Standard Solution Preparation					
Standard #	Citrate Buffer (g)	[Rib Stock] (μM)	Stock Mass (g)	Total Mass (g)	[Rib] (μM)

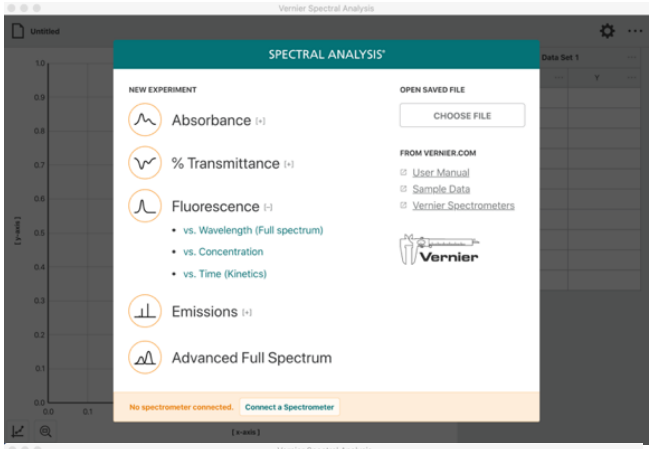
Fluorescence Analysis

Instrument Connection (Instructions will depend on the instrument used)

Setup Vernier GoDirect SpectroVis Plus by plugging the USB into the instrument and your computer. Vernier Spectral Analysis software is required to control the instrument, install it now if needed. The instrument is ready to go when the indicator lights are green next to the power and USB symbols on the bottom righthand corner of the instrument.

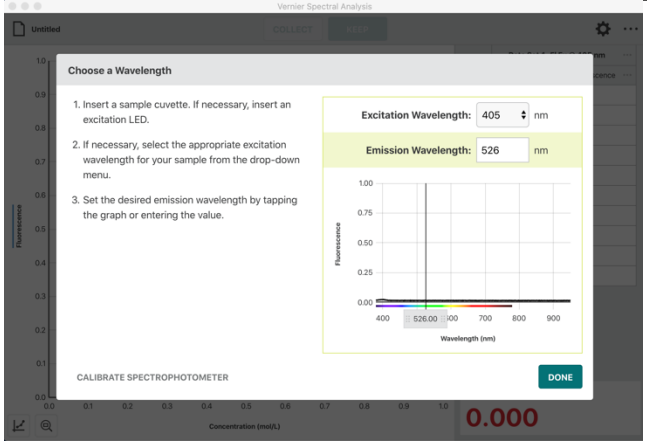
Experiment Setup

Once in Vernier Spectral Analysis, select "Fluorescence" and "vs. Concentration", as shown in the screenshot to the right.



For excitation wavelength, select 405 nm. Note that a 450 nm excitation LED would be preferred because that is where riboflavin has an excitation maximum. However, the stock 405 nm LED will do the job. If a 450 nm LED is available, use that instead. For emission wavelength, select 526 nm.

Author comment: Alternatively, students can acquire absorption and emission spectra to determine ideal excitation and emission wavelengths.



Zeroing the Fluorimeter

Before your begin to make measurements, let the instrument stabilize for a few minutes. Once a few minutes pass, you must calibrate the instrument. To do so, go to the gear on the top righthand side of the window. Place your blank (either buffer or water) in to the cuvette holder, select an integration time (100 ms is suggested), temporal averaging (3-to-5 is suggested), and

confirm excitation wavelength, then press "Calibrate". A new window will appear. With your blank placed in the instrument, select "Finish Calibration". Once complete, your spectrometer should be "zeroed". This is indicated by a reading of approximately 0.000 in red in the bottom corner of the window.

If you find out later down the road that you need to change your settings (e.g., integration time), you will need to re-zero the fluorimeter. *All of your standards and unknowns must be measured with the same acquisition parameters!*

Acquiring Fluorescence Readings
You may use the software to acquire data; however, it is simpler to record the fluorescence intensity (Fl. Int.) for each sample in Table 2 (below). Allow the fluorimeter 10-20 seconds to stabilize after installing a new sample in the instrument. The numbers may fluctuate to some extent; try to estimate a central reading. Do this for each sample a minimum of three times.

Table 2: Riboflavin Fluorescence Results

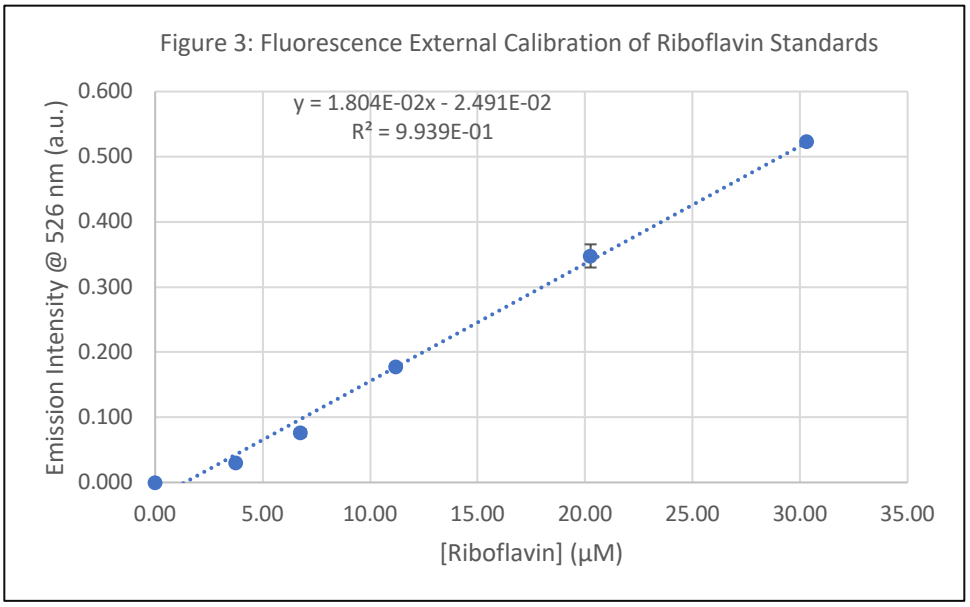
Settings: Ex: _____ nm, Em: _____ nm, Integration Time: _____
Other:

Sample # or [Rib] (μM)	Fl. Int. Rep 1	Fl. Int. Rep 2	Fl. Int. Rep 3	Average Fl. Int.	Stdev
Energy Drink X					
Energy Drink Y					

Data Analysis
Using Excel, plot standard concentration vs average fluorescence intensity. Fit the data with a linear regression and record the equation of the line and coefficient of determination. As with any external calibration method, determine the concentration of your unknowns and report the concentration of riboflavin present in each unknown with the appropriate number of significant figures.

5. Results and Conclusions

A sample external calibration using riboflavin standards is given in Figure 3. The average fluorescence intensities for Lo-Carb Monster Energy™ and Sugar-Free (SF) Rockstar® were determined to be 0.207 and 0.031, respectively. Using the external calibration data shown, these values correspond to concentrations of 12.8 μM for Monster and 3.12 μM for Rockstar. Based on the Nutritional Facts label on the containers, it was expected that Monster would have a concentration of 14.6 μM and Rockstar would be 7.30 μM.



Limitations and Ideas for Improvement

- Students could be assigned to calculate the theoretical concentration of riboflavin in the provided energy drinks, and then statistically compare their calculated value with the expected value. They could also compare their values with the class.
- To account for any issues of ionic strength or pH inconsistencies, etc. a standard addition method could be used as opposed to an external calibration, as shown here.
- The linearity and accuracy of this method is likely limited by using an excitation wavelength far from the excitation maximum. This could be improved by using ~ 450 nm excitation.

In conclusion, results demonstrate that riboflavin in energy drinks can be determined using standard samples prepared by a gravimetric method and a portable fluorimeter.

6. References

1. "Quantification of Riboflavin in Energy Drinks" https://www.stellarnet.us/wp-content/uploads/StellarNet-Exp3_Fluor_Riboflavin.pdf (Accessed July 12, 2020)
2. "Monitoring Vitamin B2 in Energy Drinks" January 31, 2013
3. https://www.vernier.com/vernier-ideas/monitoring-vitamin-b2-in-energy-drinks/ (Accessed July 12, 2020)
4. Powers, H. Riboflavin (vitamin B-2) and health. *American Journal of Clinical Nutrition*. **2003,** *77* (6), 1352-1360.
5. Manroe, M. Effect of physical activity on thiamine, riboflavin, and vitamin B-6 requirements. *American Journal of Clinical Nutrition*. **2000,** *72* (2), 598S-606S.