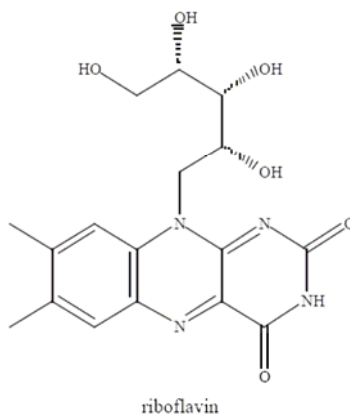


06

FLUOROMETRIC DETERMINATION OF RIBOFLAVIN (VITAMIN B₂)

Principle:

Riboflavin is strongly fluorescent at pH 4-8. Excitation and fluorescence spectra will be obtained in 5 % acetic acid solution to determine the wavelengths of excitation and emission. This information will be then used to analyze standards, create a calibration graph, and determine an unknown concentration.



Materials:

- (1) 1-l volumetric flask
- (1) 100-ml volumetric flask
- (7) 50-ml volumetric flasks
- aluminum foil to wrap the flasks, to protect them from light
- pipette
- fluorometric cuvette (4 walls clear)
- spectrofluorometer and spectrophotometer (Ocean Optics)
- riboflavin, standard and unknown
- acetic acid

Riboflavin standards:

On the day of the experiment a 50.0 ppm solution will be prepared by diluting 50.0 mg riboflavin into a 1-l flask and diluting with 5 % (vol/vol.) acetic acid — Your TA will do this. (CAUTION: Riboflavin is light sensitive and also undergoes rapid oxidation. The flask should be wrapped with aluminum foil to prevent decomposition. Even solutions in dark containers should not be kept for more than several days.) This solution will be subsequently diluted 1:5 in 5 % acetic acid, to make a 10.0 ppm

solution. Further dilute aliquots of this second solution with 5 % acetic acid to prepare standards of 0.00 (blank), 0.300, 0.500, 1.00, 2.00, 3.00 and 5.00 ppm riboflavin.

Procedure:

(Please answer all the questions highlighted in italics in this procedure when you are preparing the results and discussion sections of your laboratory report.)

l) Absorbance Spectrum:

- 1) Turn on the D2000 deuterium light source by pushing the power switch on the back of the D2000 and then pushing the “deuterium” button (blue button) once. Also, make sure the switch on the front of the light source is set to the “Closed” position. Once the LED stops flashing, the light source is ready to be used; it will take 15 minutes for the source to reach thermal equilibrium. You should wait this 15-minute period for the best results. *(Why should you wait the stated 15 minutes?)*
- 2) Open the “OOIBase32” software located on the desktop of the computer. If the USB device is not recognized, please get the TA for assistance.
- 3) Align the fiber optic cables with the sample holder in a way that is appropriate for an absorbance experiment. Use the narrow bore optical fiber pair of cables for the transmission experiment. With the wider bore there is too much light passing through and the detector will be overloaded. Have your TA check your experiment setup before continuing. *(Why are the fiber optic cables aligned in this manner?)*
- 4) Make sure the software is in “Scope Mode” by clicking on the “Spectrum” menu and selecting “Scope Mode.” Make sure the integration time is 25 ms and the boxcar is set to a value of 5. Ask the TA to check the software setup before continuing.
- 5) Right click the black background of the real-time spectrum display and select the “Auto-Scale” option. You should see a signal that is typical for noise. This is the dark signal of the detector — the noise with no light — but this noise will be present in fluorometric and absorbance measurements also. *(What type of noise is this? What can be done to decrease the magnitude of this noise?)* Once you have observed the dark noise, click on the “View” menu and select “Spectrum Scale” → “Unscale.”
- 6) Click on the “Spectrum” menu and select “Store Dark.” Next, click the “File” menu and then choose “Save” → “Dark.” Save the dark current as “dark absorbance 01” in a new folder labeled with the last name of you and your partner in the “My Documents” folder.
- 7) Click on the “Spectrum” menu and select “Scope Minus Dark.” You have just subtracted the dark current from all proceeding spectra.
- 8) Put a 5% acetic acid “blank” or “reference” into a clean cuvette. Be sure to rinse the cuvette with the solution going into it three times and then wipe the exterior of the cell. *(What is the purpose of rinsing the cell and wiping the exterior clean?)* Place the cuvette into the sample holder—Ask your TA to

show you how the first time—and be sure you put it in the same way every time. *(What is the purpose of putting the cuvette into the same holder the same way each time?)* Change the switch on the front of the D2000 light source from the “closed” to the “open” position. Light is now incident on the reference cell.

- 9) Once light is passing through the reference solution, the signal should increase above zero. Once the signal has stabilized, after several 25 ms periods, click “store reference,” then save the reference by clicking “File”, then “save” then “reference” and save the file as something like “reference absorbance”. Then it is necessary to save the spectrum, first click on the “Edit” menu and select “Copy Spectral Data” → “All Spectrometer Channels.” Now open up a blank Microsoft Excel worksheet and paste the data into the first two columns by clicking on cell “A1” once and then clicking on the “Edit” menu and selecting “Paste” (or use control-V shortcut). Save this file in the subfolder named after you and your partner in the “My Documents” folder. Be sure to name the file something like “absorbance reference 01.”
- 10) Fill a clean cuvette with the 10 ppm riboflavin solution and place it in the sample holder. Now select “Absorbance Mode” from the “Spectrum” menu. Copy and paste the data into a blank excel workbook as explained in step 9. Name the file something like “absorbance spectrum 01” and save it in the same folder as in step 9. Close the software, close all open windows, and change the switch on the front of the D2000 light source from the “closed” to the “open” position.
- 11) From the absorbance spectrum, determine what wavelength of light used to excite the analyte and induce fluorescence. *(Of the available light sources, choose which one will produce the best fluorescence signal and explain why in your report. You may ask the TA for advice on choosing the appropriate light source.)*

II) Fluorescence Spectra

- 1) Open the “OOIBase32” software located on the desktop. If the USB device is not recognized please get the TA for assistance.
- 2) Switch to the appropriate fiber optic cables (Use wider bore cables. The cables from the transmission experiment do not pass enough light through.) and the appropriate light source for fluorescence. Ask TA for assistance if necessary. Then align the fiber optic cables with the sample holder in a way that is appropriate for a fluorescence experiment. Have your TA check your experiment setup before continuing. *(Why are the fiber optic cables aligned in this manner?)*
- 3) Make sure the software is in “Scope Mode” by clicking on the “Spectrum” menu and selecting “Scope Mode.” Make sure the integration time is 12000ms and the boxcar is set to a value of 5. Ask the TA to check the software setup before continuing.
- 4) Click on the “Spectrum” menu and select “Store Dark.” Next, click the “File” menu and then choose “Save” → “Dark.” Save the dark current in your folder.

- Click on the "Spectrum" menu and select "Scope Minus Dark." You have just subtracted the dark current from all proceeding spectra.
- 5) Put a 5% Acetic acid "blank" or "reference" into a clean cuvette. Be sure to rinse the cuvette with the solution going into it three times and then wiping the exterior of the cell to remove dust and fingerprints. Place the cuvette into the sample holder. Change the switch on the back of the light source from the "off" to the "continuous" position. Light is now incident on the reference cell.
 - 6) Once light is passing through the reference solution the signal should increase above zero. (*What is this signal (i.e., is it the analytical signal, the dark signal, etc.)?*) Once the signal has stabilized, after several 12000 ms (12 s) periods, click on the "Edit" menu and select "Copy Spectral Data" → "All Spectrometer Channels." Now open up a blank Microsoft Excel worksheet and paste the data into the first two columns by clicking on cell "A1" once and then clicking on the "Edit" menu and selecting "Paste." Save this file in a new folder labeled with the last name of you and your partner in the "My Documents" folder. Be sure to name the file something like "fluorescence reference 01." Repeat this procedure a total of three times for the reference solution, the calibration standards, and the unknown—Be sure to clean the cuvette as described in step 5 after every sample. This will give you a total of 24 spectra. Name the calibration standard files something like "3.0 ppm riboflavin 01" for the first measurement of the 3.00 ppm riboflavin solution and similarly for others.
 - 7) Deselect "Scope Mode Minus Reference" by clicking on the "Spectrum" menu and clicking on "Scope Mode Minus Reference." Now Change the integration time to 3000 ms collect and save a new dark current as instructed in step 4. Now, measure the fluorescence intensity of the reference, the 0.50 ppm riboflavin, and the 1.0 ppm riboflavin solutions again as you did previously. Collect the data for these spectra as described in step 6 and save the files for these spectra in the same folder as before. Name the files as suggested before but with the integration time attached to the end of the individual file names. *In your laboratory report, compare the S/N ratios for the 1ppm and 0.5 ppm riboflavin standards at the 12000 ms and 3000 ms integration times. Why are the S/N ratios different and by what factor have they changed. How does this relate to the question proposed in section 1 step 5.*
 - 8) Using observations from this lab and sources such as your text, answer the following question: *Why are fluorescence spectrometry detection limits inherently better than with spectrophotometry techniques?*
 - 9) Retrieve the data from the computer and save them on a thumb drive and take it home with you for processing.

Results:

Include the following items in your report:

- 1) Absorbance spectrum: Label wavelength of maximum absorbance
- 2) Fluorescence spectrum for 3.00-ppm riboflavin solution: Label wavelength of maximum emission.
- 3) Calibration curve for riboflavin: Include equation of line and R^2

value. If a point or several points exhibit rollover, do not include them in your calibration curve and give justification in your report for excluding this point.

- 4) Table with the following values for the 3 runs of each solution (including blank--0.00 ppm riboflavin):
 - a. Raw signal
 - b. Background signal
 - c. Analytical signal
 - d. Standard deviation of background
 - e. S/N
 - f. S/B
- 5) Table comparing S/N values for 0.50 and 1.00 ppm solution at the 12000 ms and 3000 ms integration times.
- 6) Detection limit, limit of quantitation, and limit of linearity for the 12000 ms integration time experiment. These can be determined from the calibration curve.
- 7) Concentration of unknown determined from each run including an average value and the standard deviation of your unknown determination.

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