Absorbance and Fluorescence Characterization of Vitamin B2

Vitamin B2, also known as riboflavin, was first isolated in 1933. It is an essential human nutrient that is a precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FAD plays a key role in several important enzymatic reactions in metabolism, including the Krebs cycle. Its molecular formula is C17H20N4O6 and its molecular weight is 376.37 g/mol.

Riboflavin is also a relatively stable, strongly fluorescing compound that makes it ideal for investigation using a fluorescence spectrophotometer. In this experiment, you will measure the absorbance spectrum of riboflavin to determine the best wavelength for fluorescence excitation. You will then measure the fluorescence spectrum of riboflavin and determine its Stokes shift.

Materials

Chromebook or computer

Vernier Spectral Analysis app

Go Direct SpectroVis Plus or Vernier Fluorescence/UV-VIS Spectrophotometer

cuvette(s)

riboflavin stock solution (20.0 mg/L)

Procedure

1. Obtain and wear goggles and gloves.

2. Launch Spectral Analysis. Connect the spectrometer to your Chromebook, computer, or mobile device. Click or tap Advanced Full Spectrum.

3. Calibrate the spectrophotometer in Absorbance mode.

1. Absorbance mode is selected by default. To calibrate the spectrophotometer, select Calibrate from the Collection Settings area.
2. Prepare a blank by filling the empty cuvette 3/4 full with distilled water.
3. Place the cuvette in the spectrophotometer.
4. Select Finish Calibration.

4. Collect absorbance *vs.* wavelength data.

1. Remove the blank cuvette from the spectrophotometer. Empty the cuvette.
2. Fill a cuvette with ~3 mL of the 20 mg/L riboflavin sample.
3. Place the sample in the spectrophotometer.
4. Click Collect to start data collection. Once the absorbance spectrum is displayed, click Stop to end data collection. The data are automatically stored.

5. Examine the plot of absorbance *vs.* wavelength. Make sure the absorbance values are between 0.1 and 1.0 absorbance units. Any values outside this range may introduce an error. If your sample is outside this range, dilute or concentrate it and repeat Step 4.

6. Highlight the first absorbance peak and, using the Statistics feature, record the wavelength of maximum absorbance. To access Statistics, click on Graph Tools, , and choose View Statistics. Record both the maximum absorbance and the wavelength of its occurrence. Repeat this process for each absorbance peak present.

7. Switch to Fluorescence mode and set the LED.

1. To switch to fluorescence mode, click the spectrometer settings and choose Fluorescence.
2. Set the Integration Time to 50 ms, if necessary. Values are saved as you type.
3. Set the Wavelength Smoothing to 1 nm, if necessary.
4. Set the Temporal Averaging to 6, if necessary.
5. If using the Go Direct SpectroVis Plus: Set the Excitation Wavelength to 405 nm.
   1. If using the Vernier Fluorescence/UV-VIS Spectrophotometer, obtain the 450 nm LED and insert it into the LED slot on the spectrometer, then Set the Excitation Wavelength to 450 nm.
6. If using the Vernier Fluorescence/UV-VIS Spectrophotometer, set the LED Intensity to 50%.

8. Calibrate the spectrophotometer for Fluorescence mode.

1. While still in the spectrometer settings dialog box, click or tap Calibrate.
2. Prepare a blank by filling the empty cuvette 3/4 full with distilled water.
3. Place the cuvette in the spectrophotometer.
4. Select Finish Calibration.

9. Collect fluorescence *vs.* wavelength data.

1. Remove the blank cuvette from the spectrophotometer. Empty the cuvette.
2. Fill a cuvette with ~3 mL of the 20 mg/L riboflavin sample.
3. Place the sample in the spectrophotometer.
4. Click Collect to start data collection. Once the spectrum is displayed, click Stop to end data collection. **Note**: the fluorescence spectrum is automatically graphed on the right   
   y-axis. The data are automatically stored.
5. Examine the plot of fluorescence vs. wavelength. Make sure the fluorescence values are between 0.1 and 1.0 relative units. Any values outside this range may introduce an error. If your sample is outside this range, adjust the integration time or LED intensity as in   
   Step 7. **Note**: If you adjust these values too much, you may need to recalibrate with the new settings as you did in Step 8.

10. Using the Statistics feature, record the maximum fluorescence and the wavelength at which it occurred.

Discussion

* Compare the wavelength where the maximum absorbance peak occurred, and the maximum fluorescence peak occurred. The difference between these two values is the Stokes shift. What is the Stokes shift for riboflavin?
* Compare the absorbance analysis of the riboflavin sample with the fluorescence analysis. Which technique is more sensitive?

EXTENSIONS

* Measure the fluorescence emission of riboflavin with different excitation wavelengths. Collect fluorescence spectra of riboflavin with other excitation LEDs available. Discuss the dependence of fluorescence measurements on excitation wavelength.
* Measure the halide quenching of riboflavin fluorescence. Prepare five solutions of   
  20 mg/L riboflavin with the following concentrations of KBr: 0 mM, 2.5 mM, 5.0 mM, 7.5 mM, and 10.0 mM. Measure the fluorescence of the five solutions. Discuss the changes in fluorescence emission of riboflavin as a function of halide concentration.
* If you have the Vernier Fluorescence/UV-Vis Spectrophotometer, measure the excitation intensity dependence of riboflavin. Choose one sample and measure its fluorescence as a function of LED intensity. Discuss the changes in fluorescence emission of riboflavin when the excitation intensity is adjusted.