2 Spectrophotometry and the Analysis of Riboflavin

Objectives: A) To become familiar with operating the Platereader; B) to learn how to use the Platereader in determining the absorption spectrum of a compound in solution and in constructing a standard curve; C) to use the absorption spectrum to calculate the molar extinction coefficient of riboflavin at its absorbance maximum; D) to use the Beer-Lambert Law to calculate a solution’s concentration from a standard curve.

Introduction: Colorimetry is a technique used widely in biochemistry for qualitative and quantitative investigations. A schematic diagram of the instrumentation (colorimeter or spectrophotometer) employed is indicated below.

When a clear solvent, which does not absorb light, is in the sample well, the following equation relates the percent light transmitted to the intensity of the light.

\[
\% T = \frac{I}{I_0} \times 100 = 100\%
\]

Where \( T \) = transmittance; \( I_0 \) = incident intensity; and \( I \) = transmitted intensity

When a colored substance is dissolved in a clear solvent, the solution absorbs light, i.e., less light is transmitted. Consequently,

\[ I < I_0, \text{ and } \% T < 100 \]

The color of light is related to the wavelength or frequency of the light. Visible light – to which our eyes are most sensitive – falls in the
wavelength range of about 400 to 750nm. This is known as the visible region of the spectrum, and within it are the different colors of the rainbow from violet to red.

Most compounds have a characteristic wavelength of light they absorb. Therefore, the color transmitted depends on the color(s) absorbed. For example, a solution might look green to you because blue and yellow (green) light is transmitted while the red light is absorbed.

Solutions absorb and transmit light as well. There is, however, another relation concerning transmitted light by a solution. The less light transmitted by a solution, the more concentrated is the solution. This is a qualitative statement. The Beer-Lambert (or Lambert-Beer or simply, Beer’s) Law more precisely expresses the relationship between the amount of light absorbed and the concentration of the solution (i.e., it is a quantitative relationship). This law uses the following relationship:

\[ A = \varepsilon c l \]

Where: \( A \) = Absorbance, a.k.a. optical density, O.D.; this number is unit-less.

\[ \varepsilon = \text{Molar extinction coefficient; units - L mole}^{-1}\text{cm}^{-1} \text{ or M}^{-1}\text{cm}^{-1} \]

\[ c = \text{concentration; units - mole L}^{-1} \text{ or M} \]

\[ l = \text{optical path; units - cm} \]

The molar extinction coefficient, \( \varepsilon \), is constant for a given substance in a given solvent at a specific wavelength. The most common \( \varepsilon \) values that appear in the literature are recorded for a path length of 1 cm and a 1M solution. For example, the expression “292.5 nm \( \varepsilon \) mol 7600” indicates that at the wavelength of 292.5 nm, a 1M solution of this compound has an absorbance of 7600 in a cuvette having a diameter of 1 cm. The expression “\( \varepsilon 292.5 = 7600 \)” is another common way to relate this information.

**Standard Curves:**
Practical application of the Beer-Lambert Law comes in determining the concentration of light-absorbing solutions. To determine the concentration of a solution one needs to:

i) Look up $\varepsilon$ for the solute (substance in solution).

ii) Determine the absorbance (A) of the solution in a sample tube of known path length.

iii) Solve the above equation for concentration (c).

$$c = \frac{A}{\varepsilon l}$$

This relationship can be applied to both colored and colorless solutions.

In practice, since $\varepsilon$ depends on a number of variables (solvent, temperature, pH, etc.), the unknown concentration of a substance in solution is obtained by comparing the absorbance of the unknown with the absorbance values of solutions of known concentrations under the exact same conditions. With these data, a standard curve of absorbance (dependent variable; y-axis) at a specific wavelength versus the known concentrations (independent variable; x-axis) of a known solution is plotted. Using the standard curve, the unknown concentration of a solution can be determined:

Riboflavin Standard Curve

$$y = 0.1885x$$

$R^2 = 1$
Because this standard curve is based on the Beer-Lambert Law, there are two important features: 1) the curve is linear and 2) the line passes through the origin. Consequently, all standard curves generated in this course must exhibit both linearity and pass through the origin. A concern when converting experimental data into a linear graph is 'experimental error', i.e., if the data are not very good, the points probably will not fall on a straight line. To generate the best-fit line from experimental data, you must use linear regression analysis. This type of analysis will help you to not only determine the concentration of your unknown from the standard curve (by solving for $X$ in the generated equation) but also let you know how good your data are in generating the best line (from the $R^2$ value). In this course, **all standard curves must be generated via linear regression analysis** of the collected experimental data. *(See instructions on page 12 on how to do linear regression analysis in Microsoft® Excel 2000).*

In addition to being used to determine the unknown concentration of a known solution, the standard curve can be used to determine an extinction coefficient of that substance at the wavelength used to construct the curve. When one rearranges the equation for the Beer-Lambert Law, one can solve for $\varepsilon$ by dividing the slope of the line by the diameter of the cuvette, $l$. However, a more accurate method to determine the $\varepsilon$ of a compound is to construct an absorption spectrum.

**Absorption Spectra:**

An absorption spectrum of a compound is primarily used to ascertain its identity. For this type of analysis, one analyzes the absorbance of a compound at various wavelengths to identify absorption minima and maxima. The characteristic peaks and valleys represent the "fingerprint" of a particular type or class of compounds (*i.e.*, different compounds will have different absorption spectra).

**Absorption Spectra of Two Different Compounds**

![Absorption Spectra of Two Different Compounds](image-url)
An absorption spectrum can also be used to ascertain whether a compound is colored or colorless and the best wavelength to use to construct a standard curve. For example, examination of the above spectra indicate that Compound A is more than likely colorless because all of its absorbance maxima are in the ultraviolet range of the spectrum (< 400nm) whereas Compound B is colored. Because Compound B has at least one significant absorbance maximum in the visible region of the spectrum, a wavelength in the visible range can be used for construction of a standard curve for this compound. The wavelength at which the maximum absorbance (A_max) is observed should be used for quantitation.

Procedures:

We will be using a microplate (or, simply “plate”) reader to analyze multiple samples at multiple wavelengths with minimal sample volume. We will first do an absorption spectrum, using a single riboflavin concentration. This will allow us to determine the wavelength that gives maximal absorbance (the λ_max). Once we have determined the λ_max we will measure the absorbance of varying concentrations of riboflavin at this single wavelength. This will allow us to produce a standard curve and determine the molar extinction coefficient (ε).

NOTE: It is important to handle the plate ONLY by the EDGES! Fingerprints and markings on the bottom of the plate will interfere with the absorbance readings.

A. Prepare the samples. Add the following components to the wells as indicated by the table. Use the micropipette to mix the samples by pulling the liquid into the tip and pushing it out several times. Avoid introducing bubbles as they will interfere with the reading. Once all your samples are added go to the plate reader.

<table>
<thead>
<tr>
<th>Well position</th>
<th>Riboflavin stock 5.31 x 10^{-5} M (µL)</th>
<th>dH₂O (µL)</th>
<th>Riboflavin unknown Concentration (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>200</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A2 and A3</td>
<td>--</td>
<td>200</td>
<td>--</td>
</tr>
<tr>
<td>B1, B2, B3</td>
<td>--</td>
<td>200</td>
<td>--</td>
</tr>
<tr>
<td>C1, C2, C3</td>
<td>40</td>
<td>160</td>
<td>--</td>
</tr>
<tr>
<td>D1, D2, D3</td>
<td>80</td>
<td>120</td>
<td>--</td>
</tr>
<tr>
<td>E1, E2, E3</td>
<td>120</td>
<td>80</td>
<td>--</td>
</tr>
<tr>
<td>F1, F2, F3</td>
<td>160</td>
<td>40</td>
<td>--</td>
</tr>
<tr>
<td>G1, G2, G3</td>
<td>200</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>H1, H2, H3</td>
<td>--</td>
<td>--</td>
<td>200</td>
</tr>
</tbody>
</table>
B. Run the **riboflavinspectrum** protocol
   1. Go to *Protocol* and open the “riboflavinspectrum1” if you are in lab section 1, “riboflavinspectrum2” if you are in section 2, etc. You will know that the Protocol is open when you see “riboflavinspectrum1” in the upper left corner of the screen.

   2. Click the *Read* icon.

   3. Fill in the information: Section, Name, Experiment

   4. Click on the *Start reading* button. A window will appear “Insert plate and press OK”

   5. Place the plate in the plate reader holder with well A1 in the upper left. Click OK.

   The plate reader will automatically take in the plate. It will read the absorbance of the sample in well A1 at wavelengths from 380 through 500 in 5 nm increments. Once it is done, it will open. Leave the plate in the holder.

   6. The computer will ask you to save the data. Save the file by giving it a name. For example, david brown spectrum. It should already be set up to save the data to a folder named “section#”, where the number is your lab section number. Once you save this, the computer will show you the data in a Microsoft Excel format.

   7. Go to *File*

   8. Under *File*, go to *Save Copy As*

   Give the data the same name you did in step 6. It will now be saved as an Excel file that you can e-mail to yourself when you have finished the second part of the experiment (riboextinction).

   9. Close the Excel window

   10. Move the mouse to the little window A! and CLICK

   This will enlarge the data and you can see the $\lambda_{max}$. Record this number in your notebook.

   11. Click on the *Close* button.
12. Click on the New icon.

C. Run the riboextinction protocol
   1. Go to Protocol and open the “riboextinction1” if you are in lab section 1, “riboextinction2” if you are in lab section 2, etc. You should now see “riboextinction1” in the upper left hand corner of the screen.

   2. Repeat steps 2 – 4 above. The plate reader will read wells A1 – H3. First it will read the wells at 977 nm and 900 nm. This is done to measure the amount of water and thus the path-length of each well. ONCE IT HAS READ AT THESE WAVELENGTHS IT WILL OPEN UP. LEAVE THE PLATE IN AND THE MACHINE WILL PULL IT BACK IN AND READ AT 445 nm.

   Note: The purpose of reading at 977 and 900 nm is to measure path-length. Water absorbs at one wavelength but not at the other. The numbers recorded for this experiment will be reported as if the path-length was 1 cm. This is why you will see a difference in the absorbance at 445 nm for well A1 between the two protocols.

3. Once the plate reader is finished reading at 445 nm, it will open up and eject the plate.

4. Save the data as in Steps 6-9 above using a new name, for example “david brown extinction.”

5. E-mail the data (the two Excel files) to yourself and work on them in Excel.

6. Remove the riboflavin solutions from the plate by pipetting them out. Do NOT wash the plates with water. We will use the plates again for two more experiments but we will use different wells.

D. Analyze your experimental data and put them in your laboratory report.
   1. Graph the absorbance spectrum

   2. Calculate the concentration of your riboflavin dilution and report the mean absorbance and standard deviation or standard error. These data should be reported in a table.
3. Perform a linear regression analysis of your standard curve using Excel (chapter 1) and use this information to determine the concentration of your unknown riboflavin solution.

4. Calculate the molar extinction coefficient ($\varepsilon$) for riboflavin at 445 nm and compare your experimental value with one found in the literature. In so doing, you must take note of the experimental conditions. Experimental data about riboflavin can be found at [http://omlc.ogi.edu/spectra/PhotochemCAD/html/riboflavin.html](http://omlc.ogi.edu/spectra/PhotochemCAD/html/riboflavin.html) (once in this site, you can click on “Extinction data” and get a list of extinction coefficients at various wavelengths)

5. Calculate the path-length using data from the spectrum protocol.

Record a copy of the data in your notebook:
1. What does the equation for Beer’s Law look like if the solution has more than one compound that absorbs light?

2. What is plotted in the absorption spectrum of a substance? How can such a spectrum be used?

3. Calculate the molar extinction coefficient of a substance for which a 0.0006 M solution gives an absorbance of 0.750 at 450 nm in a colorimeter tube that is 1.2 cm in diameter. Make sure the molar extinction coefficient has the correct "standard" units.

4. The molar extinction coefficient of flavin adenine dinucleotide at 445 nm is 13,000 M\(^{-1}\)cm\(^{-1}\). What is the molar concentration of a solution of this substance in a 1 cm tube that gives an absorbance of 0.650?

5. What is plotted in the standard curve of a substance like riboflavin? How can this curve be used?

6. If to 5 mL of a 5.31 x 10\(^{-5}\) M riboflavin solution is added 20 mL of H\(_2\)O, what is the molar concentration of the resulting solution?

7. What is the difference between “quantitative” versus “qualitative” statements?

8. Below is given the standard curve for Substance X plotted at an absorption maximum of 550 nm. What is the approximate molar extinction coefficient of X given that the concentration of the stock solution is 5.30 x 10\(^{-5}\)M, and the path length is 0.7cm?