

Using the Spectrophotometer

Introduction

In this exercise, you will learn the basic principals of spectrophotometry and serial dilution and their practical applications. You will need these skills to complete other exercises throughout the semester. A spectrophotometer is a very powerful tool used in both the biological and chemical sciences yet operates by simply shining a beam of light, filtered to a specific wavelength (or very narrow range of wavelengths), through a sample and onto a light meter. Some basic properties of the sample can be determined by the wavelengths and amount of light absorbed by the sample. The instrument we will be using in class is the Spectronic 401.

Learning Objectives (in no particular order)

Conceptual

You should understand:

- the basic mechanics of the spectrophotometer
- the basic principles of spectrophotometry including *transmittance* and *absorbance*.
- the A_{\max} for a compound and how it is determined.
- the application of Beer's law to determine *concentrations* and *extinction coefficients*.
- the use of spectrophotometry to identify compounds
- the use of standard curves in analyzing data

Practical

- basic operation of the Spectronic 401.
- making serial dilutions.

Underlying Science

Basic principles of spectrophotometry

An *absorbance spectrophotometer* is an instrument that measures the fraction of the incident light transmitted through a solution. In other words, it is used to measure the amount of light that passes through a sample material and, by comparison to the initial intensity of light reaching the sample, they indirectly measure the amount of light absorbed by that sample.

Spectrophotometers are designed to transmit light of narrow wavelength ranges (see Figure 1 the electromagnetic spectrum). A given compound will not absorb all wavelengths equally—that's why things are different colors (some compounds absorb only wavelengths outside of the visible light spectrum, and that's why there are colorless solutions like water). Because different compounds absorb light at different wavelengths, a spectrophotometer can be used to distinguish compounds by analyzing the pattern of wavelengths absorbed by a given sample. Additionally, the amount of light absorbed is directly proportional to the concentration of absorbing compounds in that sample, so a spectrophotometer can also be used to determine concentrations of compounds in solution. Finally, because particles in suspension will scatter light (thus preventing it from reaching the light detector), spectrophotometers may also be used to estimate the number of cells in suspension.

We will be using a spectrophotometer several times this semester to quantify the concentration of chemicals present in a solution.

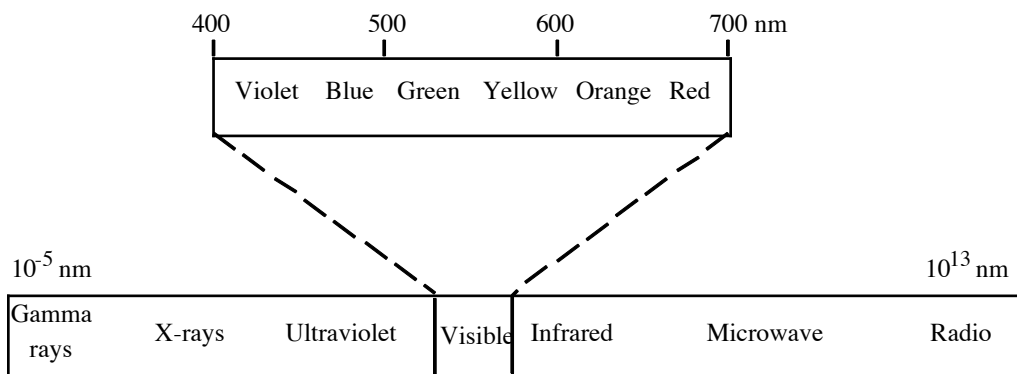


Figure 1. The electromagnetic spectrum. Visible light (400-700 nm) constitutes only a small portion of the spectrum that ranges from gamma rays (less than 1 pm long) to radio waves that are thousands of meters long

When studying a compound in solution by spectrophotometry, you put it in a sample holder called a *cuvette* and place it in the spectrophotometer. Light of a particular wavelength passes through the solution inside the cuvette and the amount of light transmitted (passed through the solution—*Transmittance*) or absorbed (*Absorbance*) by the solution is measured by a light meter. While a spectrophotometer can display measurements as either transmittance or absorbance, in biological applications we are usually interested in the absorbance of a given sample.

Because other compounds in a solution (or the solvent itself) may absorb the same wavelengths as the compound being analyzed, we compare the absorbance of our test solution to a *reference blank*. Ideally, the reference blank should contain everything found in the sample solution *except* the substance you are trying to analyze or measure. For instance, in today's lab exercise you will be measuring the absorbance of a dye, bromphenol blue that was dissolved in water. The reference blank in this case would be water alone.

The amount of light transmitted through a solution is referred to as *transmittance* (T). The transmittance is defined as the ratio of the light energy transmitted through the sample (I) to the energy transmitted through the reference blank (I_0). Since the compound being tested is not present in the reference blank, *the transmittance of the reference blank is defined as 100%T*.

$$T = I/I_0$$

This number is multiplied by 100 to determine the *percent transmittance* (%T), the percentage of light transmitted by the substance relative to the reference blank.

$$\%T = I/I_0 * 100$$

A certain portion of the light will be absorbed by the compound in the test cuvette, therefore its %T will be lower than that of the blank (by definition, 100%).

For most biological applications however, we measure *absorbance* (A_{λ} , also referred to as Optical Density or OD_{λ} , where λ is the wavelength used for the measurements), the amount of light absorbed by a solution. Absorbance is related logarithmically to transmission thusly.

$$A = -\log T$$

Again, a reference blank is used. In this case, to ‘zero out’ any light absorbed by anything in the solution other than the compound of interest. *By definition, the absorbance of the reference blank is set at zero ($A_{\lambda} = 0$)*

Visible light (see Figure 2) is composed of wavelengths from 400 to 700 nm (nanometers). When visible light passes through a colored solution, some wavelengths are transmitted and others are absorbed. You see the color of the transmitted wavelengths. For instance, a red color results when a solution absorbs short wavelengths (green and blue) and transmits longer wavelengths (red). An *absorbance spectrum* (a plot of absorbance as a function of wavelength) is determined to select the optimal wavelength for analyzing a given compound. The optimal wavelength (A_{\max}) for measuring absorbance is that wavelength that is most absorbed by the compound in question. This provides maximum sensitivity for your measurements. A hypothetical absorbance spectrum is shown in Figure 2.

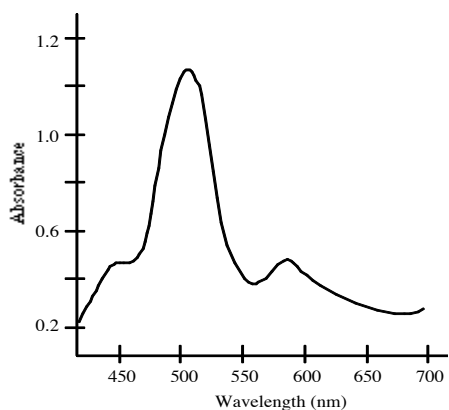


Figure 2. Absorption spectrum. A graph of absorbance vs. wavelength for a hypothetical compound. The A_{\max} for this compound is about 500 nm.

The light from the spectrophotometer’s light source (in the case of measurements in the visible range, a simple incandescent bulb) does not consist of a single wavelength, but a continuous portion of the electromagnetic spectrum. This light is separated into specific portions of the spectrum through the use of prisms or a diffraction grating. A small portion of the separated spectrum then passes through a narrow slit. When you adjust the wavelength on a spectrophotometer, you are changing the position of the prism or diffraction grating so that different wavelengths of light are directed at the slit. The smaller the slit width, the better the ability of the instrument to resolve various compounds. The slit width in the Spectronic 401 is < 8 nm. Very high quality spectrophotometers have slit widths of < 2 nm. This small band of light then passes through the cuvette containing the sample. Light that passes through the sample is detected by a photocell and measured to yield the transmittance or absorbance value (optical density) for the sample. See Figure 3 for a schematic of a spectrophotometer.

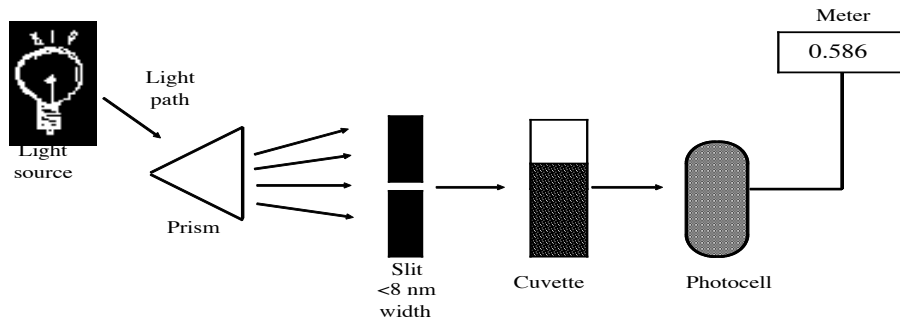


Figure 3. Components of a spectrophotometer.

There is a relationship between concentration and absorbance. This relationship is expressed by the Lambert-Beer law, which is more commonly known as Beer's law. This law states that the absorbance of a light absorbing material is proportional to its concentration in solution.

$$A = \epsilon lc$$

ϵ = the *extinction coefficient* of the substance, has units of $M^{-1} * cm^{-1}$ (unique for each substance)

l = the sample path length measured in centimeters (i.e. the width of the cuvette—almost always 1 cm)

c = the **molar** concentration of the solution (you must express concentration in terms of molarity)

It is because of this relationship that biologists measure absorption rather than transmission. The Lambert-Beer law can be used to calculate the concentration of a solution if its extinction coefficient is known. To determine the extinction coefficient, you measure the absorbance of a known concentration of solution and then rearrange the equation to solve for ϵ

$$\epsilon = A/lc$$

The Exercise:

Controls and Variables

Controls:

Positive Controls:

Part B: standard curve with know BPB concentrations

Negative Controls:

Parts A & B: Water blank,

Variables:

Independent variables: (the variables that are intentionally changed)

Part A: Wavelengths to measure A_{max}

Part B: Concentration of bromphenol blue

Dependent variable: (or results or thing you are measuring)

Parts A & B: Absorption

Controlled variables: All parameters that are kept constant between sample readings (e.g. same spectrophotometer for all readings, constant wavelength for measuring concentration)

Part A. Determination of the A_{max} of bromphenol blue

In Part A of this exercise you will determine the A_{max} (the optimal wavelength for measuring absorbance) of bromphenol blue.

Materials

Beaker of dH₂O

Tube of Bromophenol blue (BPB) (18.6 μ M)

Cuvettes

P-1000 Micropipettor and blue tips

Spectrophotometer

Method

Watch the demonstration on how to use the Spec 401 in class and follow the instructions below.

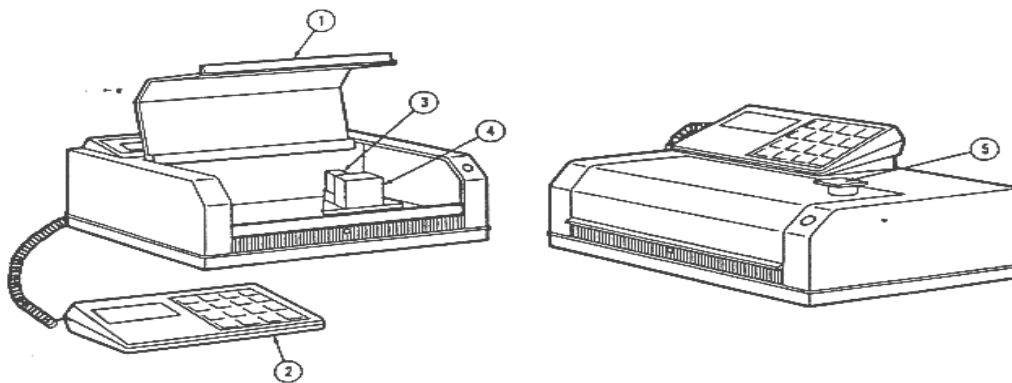


Figure 4. The Spectronic 401 spectrophotometer. 1) Sample compartment door 2) Keyboard/ display unit 3) Cuvette holder 4) Detector 5) Test tube access door.

Use of the Spectronic 401 spectrophotometer

- Make sure there are no samples in the cuvette holder and that the sample compartment and test tube access doors are closed (see Figure 5).
- Press in the power button (located on the back of the instrument) to the ON position.
- “SPECTRONIC 401” will show up on the display unit followed by something like “E 546NM -0.123A”
- Let the machine warm up for 10 min before you use it.
- Enter the desired wavelength (ie, 530) then press the [GO TO \square] key.

- The display will read, “GOING TO 530 NM” and then will read “E 530NM -0.123A”
 - Wipe off your cuvette containing the blank solution with a KimWipe and place it in the single cuvette holder or in position 1 of the multiple cuvette holder in the sample compartment. *Make sure that the cuvette is aligned with the light source.* Be sure to have the **clear** faces of the cuvette facing towards the **front** of the machine!
 - Close the sample compartment door.
 - Press the AUTO ZERO button on the keypad. The display shows “ZEROING...” and then will show “E 530NM 0.000A”
 - Remove the blank. Wipe off the cuvette containing the sample with a Kim Wipe, insert it into the sample compartment and close the sample compartment door.
 - Read the absorbance displayed (ie, for the display, “E 580NM 0.586A,” the absorbance is 0.586) and record it in a table.
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1. Look at the bromphenol blue (BPB) dye. What color light is being transmitted? What color light is being absorbed? IN your lab notebook, speculate on what wavelength be the the A_{\max} .
2. To determine the A_{\max} of the compound, each team will be assigned a range of 100 nm within the visible range of the spectrum (400nm to 700 nm). Read the absorbance of the sample every 10 nm within your team’s assigned range. You will then give your data to the TA who will compile and graph the data for the whole class to determine the A_{\max} for BPB.
3. Follow the Spectronic 401 instructions above to set the wavelength of the spectrophotometer to the lowest wavelength in your range.
4. Place the reference blank cuvette (usually referred to as a “blank”) into the spectrophotometer (“spec”) and follow the above directions to zero the absorbance.
5. Remove your blank and place the cuvette containing the bromphenol blue into the spec. Read the absorbance at the first wavelength in your range and record it in the table provided in the results section.
6. Repeat steps 6 - 8 to read the absorbance of the bromphenol blue every 10 nm for the rest of your range.
7. **You must "re-zero" the spec at each wavelength using the blank.** Don’t worry if the display of the spec starts flashing “UNFL.” This will go away when you hit the AUTO ZERO button.
8. Rinse out your two cuvettes with dH₂O and place upside down on a KimWipe to drain, you will use them again in Part B.

Record your numbers in the results section in your lab notebook and share your data with the TA. The TA will graph all the data for your section (WE get to use computers to make graphs! ha ha) and provide you with a printout of the graph. From the graph, determine the A_{\max} for bromphenol blue. This is the wavelength you will use in Part B.

Part B. The effect of concentration on absorbance

In Part B of this exercise, you will make serial dilutions of bromphenol blue and measure their absorbance to see the relationship between concentration of a compound and its absorbance. You will then use the Lambert-Beer law to determine the extinction coefficient for bromphenol blue and determine the concentration of BPB in an unknown solution.

Materials

Bromophenol blue (18.6 μM)
Cuvettes
P-1000 Micropipettor and blue tips
Spectrophotometer
Test tubes
Unknown solution of bromphenol blue

Method

1. Obtain 4 test tubes. Number them 1 to 4.
2. Use a P1000 to pipet 2000 μl of dH_2O into each tube.
3. Add 2000 μl of 18.6 μM bromphenol blue to tube 1. This tube represents a 1/2 (or a 1:1) dilution. The concentration of bromphenol blue in this tube is 9.3 μM (18.6 μM * 1/2).
4. To mix the contents of tube 1, use a vortex mixer.
5. Set the vortex speed on 4. Make sure the vortex switch is on TOUCH.
6. Place your tube in the black rubber cup of the vortex and press down to mix your sample.
7. Pipet 2000 μl of the Tube 1 contents into Tube 2. Mix using the vortexer. Tube 2 is a 1/4 (or 1:3) dilution. (You made a 1/2 dilution of a 1/2 dilution. $1/2 * 1/2 = 1/4$) What is the concentration of bromphenol blue in this tube?
8. Transfer 2000 μl of Tube 2 into Tube 3. What dilution is this? You made a 1/2 dilution of a 1:4 dilution. What is the concentration of bromphenol blue in this tube?
9. Transfer 2000 μl of Tube 3 into Tube 4.
10. Set the spec. to the A_{max} wavelength that you determined in Part A.
11. Zero the spec using your blank cuvette.
12. Read the absorbance of tube 4. Record it in your results section.
13. Pour the contents of tube 4 down the sink and read the absorbance of tubes 3, 2, and 1. Record them in the results section.
14. Remeasure the absorbance of your undiluted BPB.
15. Have T.A. check your data.
16. **Make a graph of absorbance vs. concentration of bromphenol blue** (you can do this outside of lab.)
17. Obtain a bromphenol blue sample of unknown concentration.
18. Read the absorbance at the A_{max} and record it in the results section of your notebook.
19. Based on your data in Table 2, calculate the extinction coefficient for bromphenol blue and determine the concentration of bromphenol blue in your unknown sample (also outside lab).
20. Return your BPB stock tubes to your instructors
21. When you are finished, pour the dye dilutions into the sink, rinse out the cuvettes and testubes with tap water followed by distilled water and place them upside down to dry.

Results

Make and complete the following items in your lab notebook exercise:

TABLE 1. Record the absorbance values for your assigned wavelengths for bromphenol blue.

Wavelength (nm)	Absorbance

When making graphs, remember the independent variable (the thing you change, wavelength in this case) goes on the X-axis and the dependent variable (the thing you measure, absorbance in this case) goes on the Y-axis. You can do your graphs by hand on graph, but be sure to use the whole page. Large graphs are more accurate because they are easier to read. Be sure to label your axes and give your graph a title.

TABLE 2. Determination of the effects of dilution on absorbance of bromphenol blue.

Collect your data by filling out the values in the table

Tube number	Dilution	Concentration of bromphenol blue	Abs _____nm
Undiluted	0	18.6 μM	
1	1/2	9.3 μM	
2	1/4		
3			
4			

Graph of the data from Table 2. Absorbance vs. concentration of bromphenol blue. Be sure to label the axes and give your graph a title.

What was the concentration of bromphenol blue in your unknown solution?

Assignment

Study questions to be tuned in sparately from your notebooks

1. Briefly outline how would you determine the A_{\max} of an unknown compound?
2. What is the relationship between absorbance and concentration?
3. Use your data in Table 2 and the Lambert-Beer Law to determine the extinction coefficient for bromphenol blue. Assume that l (the path length) = 1 cm (because IT IS!). Be sure to convert your concentration values into molar from micromolar first! Calculate the value *for each concentration:absorbance pair in your table*. Show your work. How consistant are your values? Which one is best? How would you manipulate those values to report your “*best estimate*” of the real value in a scientific paper?
4. What was the concentration of bromphenol blue in your unknown solution?

Calculate your answer by using Beer’s law and using the graph of your data in Table 2. List your Unknown number in your notebook and give the results from your graph and from the Beer’s calculation. Be sure you include the appropriate units in your answer. Are your results different when using the two methods? Briefly discuss your thoughts on this.