

Spectrophotometers and the Standard Curve

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Introduction

Spectrophotometers are a standard research tool used in biology and chemistry labs world wide. Among the many uses they can follow the progress of an enzymatic reaction (as you will do later in this course), estimate the number of bacteria in sample and detect contaminating protein in a DNA sample. Knowing the principles behind the spectrophotometer and how to use one is a valuable laboratory skill and you will learn the basics of spectrophotometry in this exercise.

Learning Objectives

Upon completion of this lab you should be able to:

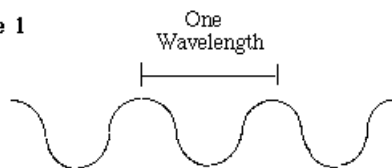
1. Define
blank light wavelength Absorbance
% Transmittance serial dilution absorption spectrum
absorbance maximum standard curve
2. Describe how a spectrophotometer works.
3. Use a micropipet and measuring skills to set up an experiment.
4. How to set up a Spec 20 and collect scientific data.
5. Determine the absorbance maximum for a chemical in solution.
6. How to set up a serial dilution series.
7. Construct a line (“connect the dots”) graph of the scientific data using Cricketgraph™.
8. How to make and use a standard curve.
9. How to use the equation $C_1V_1=C_2V_2$ to calculate concentration.

Introduction to Spectrophotometers

The spectrophotometer is in principle a fairly simple instrument. But before I describe it I need to say a couple things about light. Light is a type of electromagnetic energy and this type of energy travels as a wave. Just like waves at the beach, electromagnetic waves have peaks and troughs. One useful way to describe light is to measure the distance between peaks. This distance is known as the **wavelength** (see Figure 1). For visible light, these wavelengths range from about

380 nm (nanometers) to about 750nm. You can see that we are talking about *very* small waves!

Figure 1

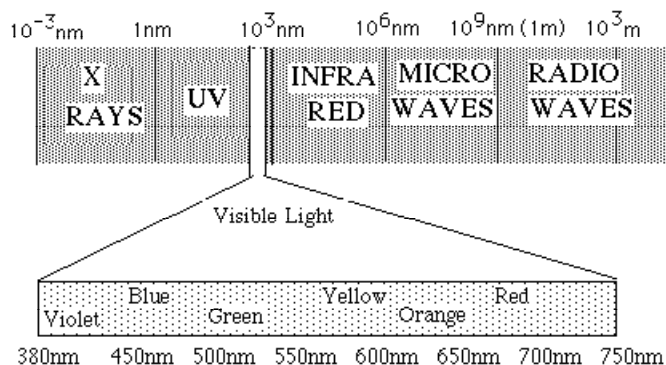


Light Traveling as a Wave

What's more, various wavelengths of light are seen by you as the colors of the spectrum. Most of the **electromagnetic spectrum** is shown in Figure 2. As you can see, light is only one part of the electromagnetic spectrum.

As you may already know, white light is made up of light of many wavelengths. Essentially a white light source generates all visible wavelengths of light and that is a problem when using a spectrophotometer. To get clear results, you can only measure the absorbance of one wavelength of light at a time. Therefore, in spectrophotometer, a white light is shown on a prism and the prism brakes up the white light into its individual wavelengths. A resulting single wavelength of light is then selected by the operator of the spectrophotometer and shown on a sample.

Figure 2



An experimental sample for use in a spectrophotometer usually contains a chemical in solution or particles in a suspension which will absorb light. The amount of light the chemical or particles absorb is measured by the spectrophotometer. That is very useful because the amount of light absorbed can be correlated to the amount of that substance present.

Once the light is shown on a sample and some of the light is absorbed, the rest of the light passes through the sample. The light that passes through is collected and measured by a phototube and the results are displayed on a scale.

The scale on a spectrophotometer is something else that deserves mention. Look at a spectrophotometer. You will notice that there are actually two scales: **Absorbance** and **% Transmittance**. No, these weren't placed here specifically to confuse you. It turns out that some experiments or procedures work better with one scale or the other.

But let's bypass that and move on to what the scales actually measure.

% Transmittance is a scale that measures the amount of light that shines through a solution. For example, if 75% of the light shining through a sample makes it all the way through, that would be 75% Transmittance. If a sample completely blocked the light shining on it, what would be the % Transmittance? If you answered 0%

Transmittance, you have the idea.

How about Absorbance? Absorbance measures the amount of light absorbed by a sample (i.e. blocked from passing through). That sounds like the opposite of the definition of % Transmittance and it is! To further confirm this, look at the scales. They run in the opposite directions.

The Absorbance scale as you can see is not linear like the % Transmittance scale but exponential, starting at zero and ending at infinity. This allows for great sensitivity at the low end of the scale but poor sensitivity at the high end. For this reason Absorbance readings above about 1.0 are usually not used. Also note that the Absorbance scale is only a relative scale and therefore has no units.

Before you go on with this exercise, there are some things about the absorbing molecule (or particle) and the amount of light absorbed by that molecule (or particle) that you should know. The amount of light absorbed depends on several factors.

1. The higher the concentration of the absorbing molecule, the more light is absorbed.
2. The longer the distance that the light travels through the sample, the greater the absorbance. This factor is held constant because all of the samples are measured in the same sized test tube.
3. A substance that absorbs a light of one specific wavelength very strongly may absorb light of another wavelength only weakly. For this reason, different wavelengths of light are used to measure the concentration of various substances. How do you know what wavelength to use? That is what you will discover in Exercise A!

One more thing before you tackle the experiments. When an object or a solution is illuminated, some wavelengths of light are absorbed and some are reflected. The color of an object or solution that you see is composed of the wavelengths of light that are being reflected. The colors that you don't see are the wavelengths of light that are being absorbed.

The Experiments

Exercise A: The absorption spectrum of Biuret reagent

1. Introduction

a. What is Biuret reagent? This blue colored reagent is commonly used to stain proteins. Proteins by themselves are usually colorless and therefore it is difficult to measure the presence of protein without a special kind of spectrophotometer (which happens to be very expensive). An easy (and inexpensive!) way to remedy this problem is to stain the protein with Biuret reagent and measure the amount of absorption of the Biuret reagent.

However you don't know what wavelength of light is best absorbed by the Biuret reagent. In Exercise B you are going to use the reagent to figure out the concentration of a protein solution. But without the knowledge of which wavelength of light to use you won't be able to do that experiment. Exercise A will determine which wavelength of light to use in Exercise B.

b. You will be using **Blanks** in these experiments. Blanks are useful when there are other substances in the experimental tube besides the substance you are trying to measure. Since those other substances are not the chemical that you are trying to measure, they often interfere with the absorbance reading of the chemical of interest. Remember: you are interested only in the Absorbance due to the substance you want to measure.

How do you deal with this problem? One way would be to remove the offending substances, but this is usually not practical and often the substances are necessary in the experiment. A much more suitable way to deal with this problem is to exclude these other substances from our spectrophotometer reading without removing them from the experimental tube. The way to do that is to use a blank. A blank contains all the substances (or substance) in the experimental tube except the substance that is being measured. Then, before reading your experimental tube, you place the blank tube in the spectrophotometer, set the spec. to "0" Absorbance (or 100% Transmittance), then read the experimental tube. What you have done, is to set the spec. to read only the absorbance that is due to the substance that you are interested in!

c. Here are some tips to help make your experiments with the spectrophotometer more successful.

- 1) Let the spec. warm up for 15 minutes.
- 2) Tubes need to be at least half full.
- 3) Make sure that the spec. tubes are clean and free from scratches.
- 4) Make sure that all of your readings are with the same side of the tube forward (most spec. tubes have a vertical line on the front to help with this)
- 5) Make sure the solution is free from bubbles.
- 6) Recheck the "0" Absorbance reading with the blank often to make sure your Spec is free from drift.

2. Experimental Procedures

a. Read all of Exercise A. You should do this before starting any experiment. It allows you to anticipate what is coming up and lets you avoid nasty surprises.

b. Turn on the Spectrophotometer 20 (left front knob) and set the wavelength (right top knob) to about 460nm. Let the machine warm up for 15 min.

c. While the Spec 20 is warming up, collect 2 spectrophotometer tubes and make sure that they are clean and free of scratches. Now fill 1 tube with Biuret reagent and the other with DI H₂O. The tube with DI H₂O will be your blank. Since the Biuret reagent is made with water, you need a blank for the water in the Biuret reagent.

d. When the Spec 20 is warmed up, set the range so that your sample will read on the scale. Do this by

- 1) With nothing in the sample holder and with the cover closed, use the zero control knob (same as the on/off knob), left front, to set the meter to 0% Transmittance.

- 2) Insert your clean, fingerprint free blank in the sample holder. Using the transmittance control knob (right front) , set the meter to 100% Transmittance.

3) You can now read your sample by inserting your fingerprint free sample into the sample holder and closing the cover. You will get a full scale reading in either Absorbance or % Transmittance.

e. Now you are ready to start taking experimental readings. With the wavelength set to 460nm, insert the sample into the sample holder and record the reading on the Absorbance scale. Record your results in Table 1.

f. Reset the wavelength to 480nm, reset the scale by the procedure described in (d) above and take another Absorbance reading.

g. Repeat taking readings every 20nm until you reach 600nm. Remember to reset the scale with the water blank each time you change wavelengths.

Table 1: Absorbance maximum of Biuret reagent

Wavelength Absorbance

460nm | _____

480 | _____

500 | _____

520 | _____

540 | _____

560 | _____

580 | _____

600 | _____

h. Clean up when finished. Then plot Absorbance (on the “Y” axis) vs. wavelength (on the “X” axis) as a line graph (“connect the dots”). Do this by hand on graph paper or use Cricketgraph™ or other graphing software if available. This graph will be turned in with the exercise.

i. Answer these questions on a separate piece of paper

1) What is the absorption maximum for Biuret reagent and what is the wavelength of light you will use in Exercise B? Why would it be best to use this wavelength?

2) Why is a blank necessary in this experiment?

3) Why must you use the blank each time you change wavelengths?

4) If the concentration of Biuret reagent were changed in this experiment, would the wavelength absorbance maximum change? Why or Why not?

5) What color light do plants reflect and which are absorbed and used to drive photosynthesis?

6) How much light is being absorbed by a sample when a spectrophotometer reads infinity?

7) Why must a spectrophotometer use a single wavelength of light in measuring Absorbance?

8) Some spectrophotometers use wavelengths of light in the ultraviolet range. What are the wavelengths of light that are in the ultraviolet range? Can you think of a situation in which a "UV spectrophotometer" would be more useful than a visible light spectrophotometer?

Exercise B: Making and Using a Standard Curve

1. Introduction

- a. What is a **serial dilution**? A serial dilution is a set of dilutions in which the important reagent is present in a regularly decreasing concentration. You will be making this type of dilution in this experiment.
- b. What is a **standard curve**? As the concentration of a light absorbing chemical in a solution decreases, the absorbance of the solution decreases. If you make a serial dilution of the chemical in a solution and then take absorbance readings, you can construct a graph which shows the relationship between concentration and absorbance. That is a standard curve and the standard curve has many applications in the biology and chemistry lab when the concentration of a solute in a sample must be determined.

2. Experimental Procedure

- a. Read all of Exercise B.
- b. Set the Spectrophotometer to _____ nm (You fill in the blank).
- c. Collect a test tube rack and 6 spectrophotometer tubes, making sure that they are clean and free of scratches.
- d. You will also need
~15ml of DI H₂O
4ml of 1% Bovine Serum Albumin (a blood protein also known as BSA)
~40ml of Biuret Reagent
1000µl and 100µl micropipetors and tips (or 1ml serological pipets)
Two 5ml serological pipets
- e. Before you make up your experimental tubes and the blank, lets do a little exercise that will help you to do this step.
- 1) Read step f.
 - 2) Notice that you will be making a serial dilution. Remember that a serial dilution is a set of dilutions in which the important reagent is present in a regularly decreasing concentration.

3) In Table 2, fill in the column for “Amount of BSA in ml” for each tube. (Hint: remember that an amount of BSA is removed from each tube)

4) Make the conversion from ml to μl . In Table 2, fill in the column for “Amount of BSA in μl ” for each tube.

5) Leave the column “Final concentration of BSA” empty for now. The reason is that you will be adding other reagents to the tubes.

f. Make your sample tubes. Since none of the reagents are sterile, you may use the same micropipet tip for a single reagent, as long as you do not dip the tip when you deliver the reagent. Remember to change micropipet tips when you change to another reagent. When you are finished, all of the tubes should have the same amount of solution in them. If they don't, you have made a mistake in measurement and should correct it by making that tube(s) over again.

1) Label your spec tubes 1 through 5. Using the 5ml pipet, remove 4ml from the stock BSA solution and place in tube 1.

2) Using the 1000 μl micropipetor, remove 2ml from tube 1 and place in tube 2.

3) Using the same tip, remove 1ml from tube 2 and place in tube 3.

4) Using the same tip, remove 0.5ml from tube 3 and place in tube 4.

5) Using the same tip, remove 0.25ml from tube 4 and place in tube 5.

6) Using the same tip (or for better accuracy, using a 100 μl pipet), remove 0.125ml from tube 5 and discard it.

g. Using the 1000 μl micropipetor and a new tip, place the indicated amount of DI H₂O in the correct tube.

Tube 1: no DI H₂O

Tube 2: 1ml DI H₂O

Tube 3: 1.5ml DI H₂O

Tube 4: 1.75ml DI H₂O

Tube 5: 1.875ml DI H₂O

- h. Using a clean 5ml pipet, place 5ml of Biuret reagent in each of the 5 experimental tubes and mix well. Remember that when you are finished, all of the tubes should have the same amount of solution in them.
- i. Make a blank tube with 2ml of DI H₂O and 5ml of Biuret reagent. This is the blank that you will use to set the 100% Transmittance on the spec in this experiment.
- j. Zero and set the range on your spectrophotometer with your blank (see Exercise A, p.6 if you need to refresh your memory).
- k. Now you are ready to start taking experimental readings. With the spec wavelength set to the Absorbance maximum for Biuret reagent (discovered in Exercise A), insert tube 1 into the sample holder and record the Absorbance reading. Place this reading in Table 2.
- l. Repeat until all five tubes and the solution which contains an unknown concentration of protein have been read and recorded.
- m. Now it is time to figure out the final concentration of BSA in each of the experimental tubes. The simple equation that follows will allow you to do this.

$$C_1V_1=C_2V_2$$

C_1 is the concentration of the original solution

C_2 is the concentration of the final solution

V_1 is the volume of the amount transferred

V_2 is the volume of the final solution

For each of the five experimental tubes you already know C_1 , V_1 and V_2 . All you need to do is plug in the numbers and solve for C_2 . Put these results in the “Final concentration of BSA” column of Table 2.

Table 2: Exercise B experimental tubes

<u>Tube #</u>	<u>Amount of BSA in ml</u>	<u>Amount of BSA in μl</u>	<u>Final concentration of BSA in mg/ml</u>	<u>Abs.</u>
1				
2				
3				
4				
5				
<u>Unk.</u>				

n. Clean up. When finished, graph a “best fit” line graph of your results by hand on graph paper or using Cricketgraph™ if available. The Y” axis should be the Absorbance scale and the “X” axis will be the Concentration scale. This graph of a standard curve will be turned in with the exercise.

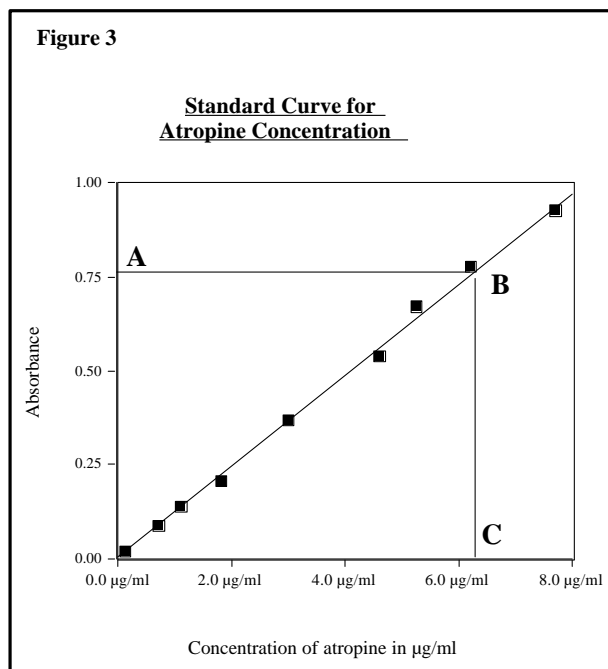
o. Determination of the concentration of an unknown sample is simple once you have a graph of a standard curve for that solute. Refer to Figure 3, which is a hypothetical a standard curve for Atropine, a medicinal compound derived from plants, as you read the following explanation.

1) Locate the absorption value of the unknown on the Absorbance scale(“X” axis) (see **A** in Figure 3).

2) Draw a horizontal line at this value across the graph until it intersects with the standard curve (see **B** in Figure 3).

3) At this point, draw a line vertically down until it intersects the Concentration scale (“Y” axis) (see **C** in Figure 3).

4) The concentration value at this point will be the solute concentration of the unknown solution.



p. Answer these questions on a separate piece of paper

- 1) Why is a blank necessary in this experiment?
- 2) What is the purpose of a standard curve?
- 3) Once a standard curve has been made for a compound, could it be used to determine the concentration of another compound? Why or why not?

4) Once a standard curve has been made for a compound, could it be used to determine the concentration of the same compound in a solution different from the solution used to make the standard curve? Why or why not?

5) Can you think of a laboratory procedure that might utilize a standard curve?

6) Apply your knowledge in making a serial dilution: You needed to make 10 fold (or 1 in 10) serial dilution of a 0.5 M solution of arginine (an amino acid). Describe how you would make this serial dilution if the most dilute tube is to contain 5 mM arginine. If you need help, review p. 10.

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