Lab #4

SPECTROPHOTOMETRY

Adapted from: C. Greene MJC

INTRODUCTION:

In this exercise, you will learn the basic principles 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.

Objectives:

- State the basic mechanics of the spectrophotometer
- Describe the basic principles of spectrophotometry, including transmittance and absorbance.
- Determine the **A***max* for a compound.
- Generate and use a standard curve to analyze data
- Perform serial dilutions

Background Information:

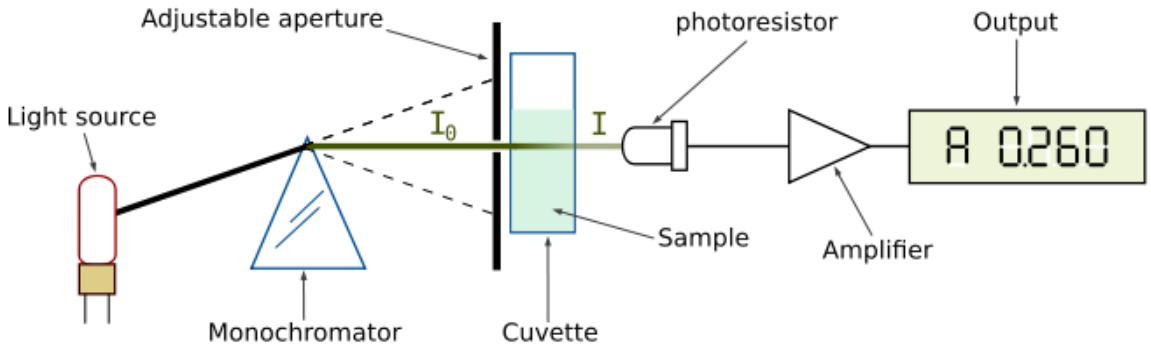
An absorbance spectrophotometer is an instrument that measures the fraction of the incident light transmitted through a solution. In other words, it is used to measure the amount of light that passes through a sample material and, by comparison to the initial intensity of light reaching the sample, they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

Spectrophotometers are designed to transmit light of narrow wavelength ranges. 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.

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. **Note**: 100% Absorbance = 0% Transmittance



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 ate trying to analyze or measure. For instance, in today's lab exercise you will be measuring the absorbance of a dye, bromophenol 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 to the energy transmitted through the reference blank. Since the compound being tested is not present in the reference blank, the transmittance of the reference blank is defined as 100%T. (0% Absorbance)

For most biological applications however, we measure absorbance (**A**, also referred to as **Optical Density** or OD, where **λ** is the wavelength used for the measurements), the amount of light absorbed by a solution. 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 = 0)

Visible light (see your text) 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 (Amax} for measuring absorbance is that wavelength that is most absorbed by the compound in question. This provides maximum sensitivity for your measurements.

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. 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.

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.

Beer's Law: **A = εlc**

A = Absorbance

ε = Extinction Coefficient of the substance: Units are $M^{-1}*cm^{-1}$ (unique to each substance)

l = Sample path length measured in cm (width of cuvette) usually 1 cm

c = Molar concentration of the solution

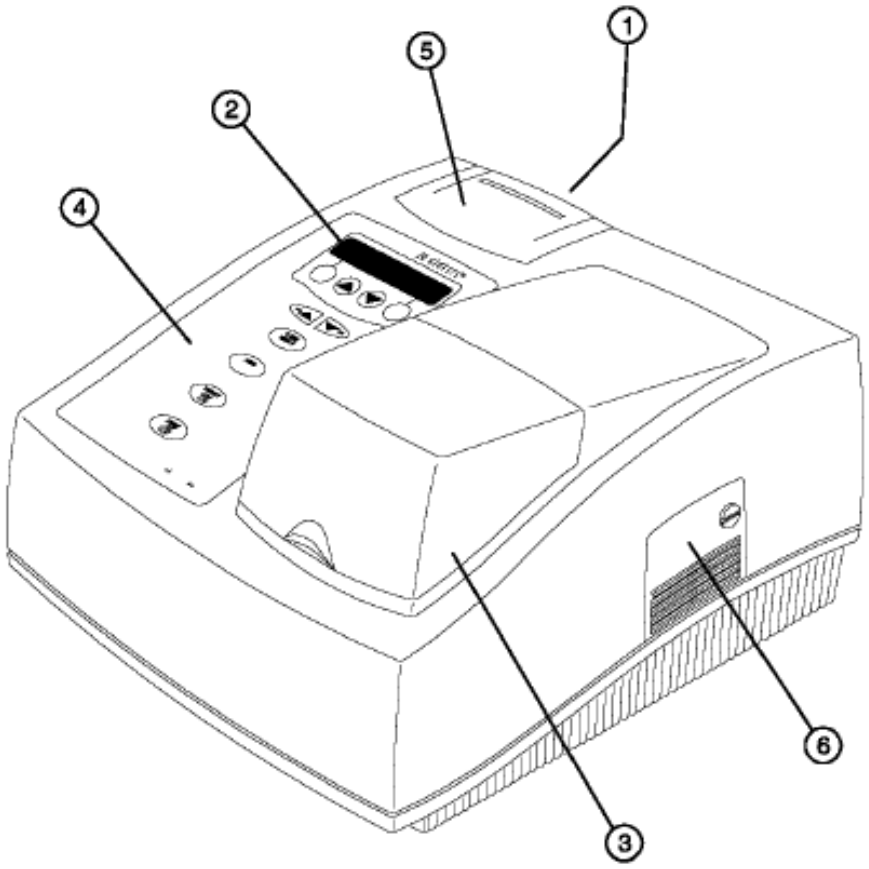
It is because of this relationship that biologists measure absorption rather than transmission. The Lambert-Beer law can be used to calculate the concentration of a solution if its extinction coefficient is known. To determine the extinction coefficient, you measure the absorbance of a known concentration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

Algebraic solution for ε: ε = A /lc

SPECTROPHOTOMETER OPERATION:

1. Press in the power button (located on the back of the instrument} to the ON position.
2. Let the machine warm up for 10 min before you use it
3. Enter the desired wavelength
4. Load sample (glass tubes 3ml min)
5. Wipe off your cuvette containing the blank solution with a Kim Wipe and place it in the single cuvette holder.

   *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!*
6. Close the sample compartment door.
7. Press the **AUTO ZERO** button on the keypad. The display shows "ZEROING ... "
8. 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.
9. Read the absorbance displayed and record it



SPECTRONIC 20

1. Power
2. LCD Display
3. Sample Door
4. Keyboard
5. Optional Printer Connect
6. Lamp Compartment Door

1. DETERMINATION OF THE A*max* OF BROMOPHENOL BLUE:

MATERIALS:
Beaker of dH20
Tube of Bromophenol blue (BPB) (18.6µM}
Cuvettes
P-1 000 Micropipettor and blue tips
Spectrophotometer

PROCEDURE: 

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

DETERMINATION OF THE A*max* of BROMOPHENOL BLUE

1. Look at the bromophenol blue (BPB) dye. What color light is being transmitted? What color light is being absorbed? Using your knowledge of color and wavelengths, estimate what the wavelength might be the **A**max. *Record this on your worksheet*
2. To determine the Amax 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 write your data on the board and compile and graph using the data for the whole class to determine the **A**max for BPB.
3. Follow the Spec 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 bromophenol blue into the spec. Read the absorbance at the first wavelength in your range and record it in a table in your lab book.
6. **Repeat steps 6 - 8** to read the absorbance of the bromophenol 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 dH20 and place upside down on a Kim Wipe to drain, you will use them again in Part B.

- Record your numbers in the results section on your worksheet.

- Once all the data is obtained and present on the board, draw a quick graph in your lab book and determine the Amax for bromophenol blue. This is the wavelength you will use in Part 2.

2. THE EFFECT OF CONCENTRATION ON ABSORBANCE: (*use of serial dilutions*)

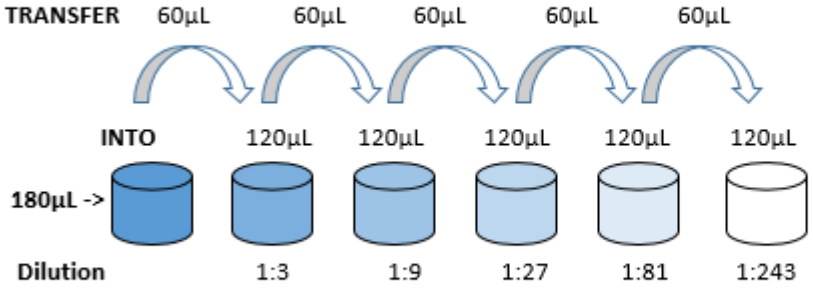
In Part B of this exercise, you will make serial dilutions of bromophenol blue and measure their absorbance to see the relationship between concentration of a compound and its absorbance.

- You will then generate a standard curve and use it to determine the concentration of an unknown sample of BPB.

Serial Dilutions (Background)
A dilution series is a succession of step dilutions, each with the same dilution factor, where the diluted material of the previous step is used to make the subsequent dilution.

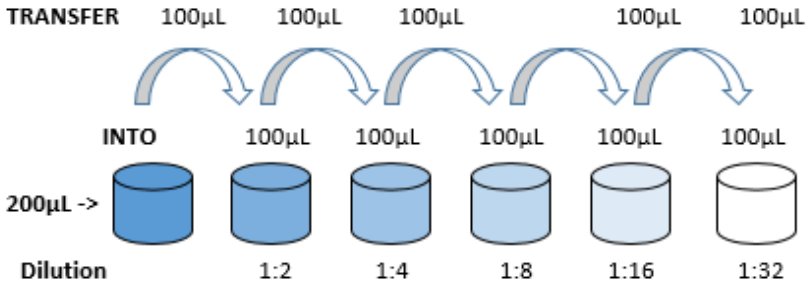
EXAMPLES

33.3% OR Serial Dilution Series – Dilution Factor of 3



Starting with 180 µl, 60 µl from each tube will be transferred into 120 µl of diluent (water). This will ensure that 60 µl (1/3 of the initial volume) *is transferred each time.*

50.0% OR Serial Dilution Series – Dilution Factor of 2



Starting with 200 µl, 100 µl from each tube will be transferred into 100 µl of diluent (water). This will ensure that 100 µl (1/2 of the initial volume) *is transferred each time.*

Single Dilutions (Background)

Stock solutions are constantly diluted in biological lab settings. It is critical that you master both serial and simple (single) dilutions. The following formula can easily be used to carry out a simple dilution.

EXAMPLES

Calculation of Concentration Using C1V1 = C2V2

To make a fixed amount of a dilute solution from a stock solution, you can use the formula: C1V1 = C2V2 where:

- V1 = Volume of stock solution needed to make the new solution
- C1 = Concentration of stock solution
- V2 = Final volume of new solution
- C2 = Final concentration of new solution

Example: Make 5 mL of a 0.25 M solution from a 1 M solution.

- Formula: C1V1 = C2V2
- Plug values in: (V1)(1 M) = (0.25 M) (5 ml)
- **Solve for V1**: V1 = [(0.25 M) (5 ml)] **/** (1 M)
- V1 = 1.25 ml
- Answer: Place 1.25 mL of the 1 M solution into V2-V1 (5ml – 1.25 ml) = 3.75 ml of diluent

Practice this technique – if you are going to be a successful biology student you will need to be very comfortable carrying out this type of activity.

2A: THE EFFECT OF CONCENTRATION ON ABSORBANCE: (*use of serial dilutions*)

MATERIALS:
Bromophenol blue (BPB) (18.6µM}
Cuvettes
P-1000 Micropipettor and blue tips
Spectrophotometer
Test tubes
Unknown solution of bromophenol blue

PROCEDURE: 

1. Obtain 4 test tubes. Number them 1 to 4.
2. Use a P1000 to pipet 2000 µl of dH_20 into each tube.
3. Add 2000 µl of 18.6 µM bromophenol blue to tube 1. This tube represents a 50% (or a **1:2 dilution**). The concentration of bromophenol blue in this tube is 9.3 µM (18.6 µM * 0.5).
   To mix the contents of tube 1, use a vortex mixer if available. Place your tube in the black rubber cup of the vortex and press down to mix your sample.
4. Determine the Absorbance of your solution in tube 1 and record it on your worksheet
5. Pipet 2000 µl of the Tube 1 contents into Tube 2. Mix using the vortex. Tube 2 is a 25% (or **1:4 dilution**). (You made a 50% dilution of a 50% dilution. 0.5 * 0.5 = 0.25) What is the concentration of bromophenol blue in this tube?
6. Determine the Absorbance of your solution in tube 2 and record it on your worksheet
7. Transfer 2000 µl of Tube 2 into Tube 3. What dilution is this? You made a 50% dilution of a 1:4 dilution, making it a **1:8 dilution**. What is the concentration of bromophenol blue in this tube?
8. Determine the Absorbance of your solution in tube 3 and record it on your worksheet
9. Transfer 2000 µl of Tube 3 into Tube 4. (**1:16 dilution**)
10. Set the spec. to the A*max* wavelength that you determined in Part 1 of this exercise.
11. Zero the spec using your blank cuvette.
12. Determine the Absorbance of your solution in tube 4 and record it on your worksheet

2B: DETERMINATION OF CONCENTRATION OF AN UNKNOWN:

PROCEDURE: 

1. Obtain a solution of Bromophenol Blue from your instructor. The concentration of this solution is not known.
2. Take the Absorbance of the unknown solution and record it on your worksheet
3. Determine the concentration of the unknown using your data from section 2A
4. Is the absorbance in the usable range of your graph from part 2B? If not you will either need to adjust the axis parameters of you graph, or – to save massive erasing – try making a dilution of your unknown that will fall into the usable range!
5. Try a 1:2 dilution of the unknown and take the absorbance. Does it fall into the usable range of your concentration plot? If so, you will now use the plot to estimate the concentration for the unknown. DON'T FORGET TO MULTIPLY THE RESULTING CONCENTRATION BY A FACTOR OF 2!
6. Your 1:2 dilution may not be enough, so also prepare a 1:3 dilution. Just don't forget that a 1:3 dilution would need to be multiplied by 3 to determine the final concentration.

NO CONTENT