Laboratory: Spectrophotometry
Skills= 13 points

Objectives:

At the end of this unit, the student will be able to:

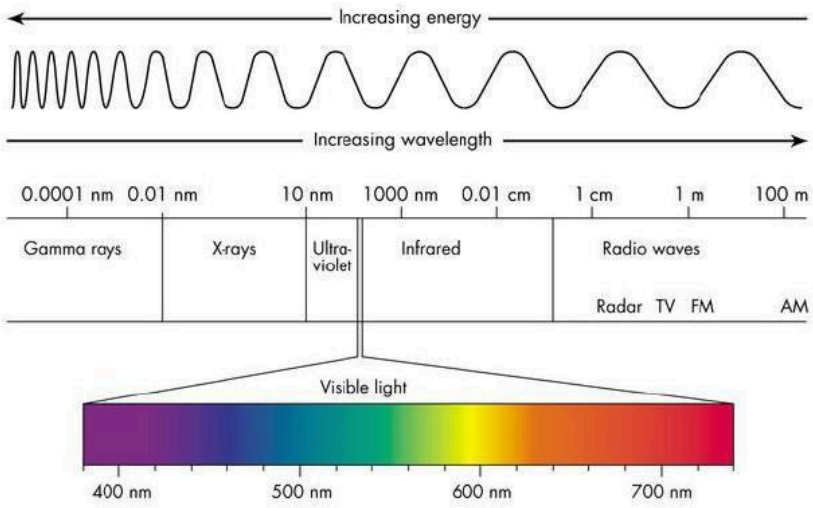
1. Identify the regions of the electromagnetic spectrum that are used in laboratory analysis.
2. Given a color of a solution, determine what wavelength range of the spectrum would be most appropriate to use for spectrophotometric analysis for that substance.
3. Define percent transmittance and absorbance.
4. Identify the parts of a spectrophotometer.
5. Calculate concentration of an unknown when given the concentration of the standard and the absorbances of the standard and the unknown.
6. State the units for reporting wavelength.

Materials:

Biomodel Website: http://biomodel.uah.es/en/lab/abs/espectro.htm

Discussion:

Light is electromagnetic radiation that travels in waves. The wavelength (**λ**) of light is measured in nanometers which is 10^{-9} meters. The wavelength is the distance between adjacent peaks or troughs in a continuous wave. Visible light falls between 400-700nm, Ultraviolet light falls between 200-400nm, and infrared light falls between 700-800nm. These three regions are the most commonly used regions in clinical laboratory analysis.



When a beam of white light is shone through a substance that absorbs yellow

light, the complementary color of blue will be transmitted through the substance. The blue color is observed because the substance absorbs the yellow color. The table below shows the wavelengths of light when a color is absorbed and what complementary color is observed. Laboratory application will be discussed below.

Wavelength in nm	**Approx. Maximum absorbance Wavelength in nm**	**Color Absorbed**	**Color Observed**
380-450	420	Violet	Yellow-green
450-495	470	Blue	Yellow
495-570	530	Green	Purple
570-590	580	Yellow	Blue
590-620	620	Orange	Greenish blue
620-750	700	Red	Bluish green

Spectrophotometric measurements are based on detection and quantification of energy that is transmitted after passing a beam of light through the solution being analyzed.

Transmitted energy is expressed in terms of percent transmittance (% T) by the following equation:

$$\%T = P/P_0 \text{ X } 100$$

P=radiant energy (light) transmitted
P_0=intensity of incident light (original)

If all of the incident light is absorbed, then no light is transmitted and the %T=0. If none of the incident light is absorbed, all of the light is transmitted and the %T is 100%.

Percent transmittance is often expressed as absorbance (A) for ease of use in practice because %T is not a linear relationship. Absorbance is directly proportional to the concentration of the absorbing substance. The following Beer's Law equation is used:

$$A = 2 - \log \%T$$

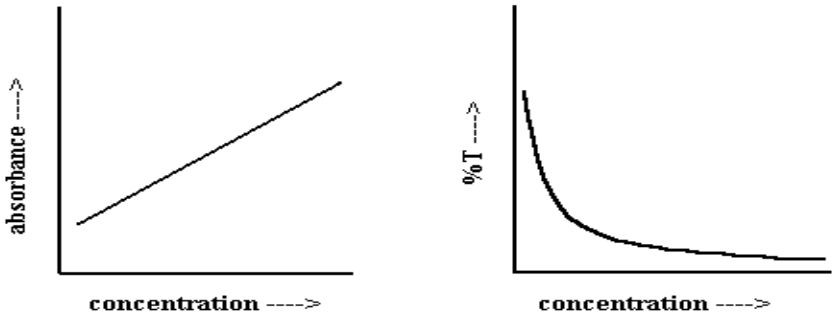
Beer- Lambert's law states that absorbance is directly proportional to the length of the light path through the sample, which is 1.0 cm in most spectrophotometers.

The Beer-Lambert Law is as follows:

$$A=abc$$

A=absorbance
a=absorptivity constant in moles/cm^2
b=path length in cm (ie. 1.0 cm)
c=concentration

Because (a) and (b) are constant, absorption has a directly proportional linear relationship with concentration.



When Beer's Law is followed, the concentration of an unknown can be determined by analyzing the absorbance of a standard of known concentration. If we measure the absorbance of an unknown solution and the absorbance of a standard of known concentration, we can determine the concentration of the unknown by using the following equation:

$$\text{Concentration}_{unk} = (A_{unk}/A_{std}) * \text{Concentration}_{std}$$

If you are given a color of solution, you can select the appropriate wavelength to measure the substance's absorbance by selecting the wavelength that corresponds to maximum absorbance of its **complementary** color. Recall from the table above, that if you "see" a color, then its complementary color is being absorbed by the substance.

It is best to select a wavelength for measurement in which the absorbance for that substance is the greatest, so that changes in concentration are easily differentiated. For instance, bilirubin absorbs more violet and blue light in the 430-400 nm wavelength range than other colors; therefore, bilirubin measurements are typically analyzed on amniotic fluid at 450 nm. The wavelength/color chart above helps explain why icteric plasma has a yellow-green tint. The figure below shows the best wavelength that range for this substance is between 520-550 nm because the absorbance is greater and the differences between samples is greatest within that range. This correlates with the information in the chart above as well.

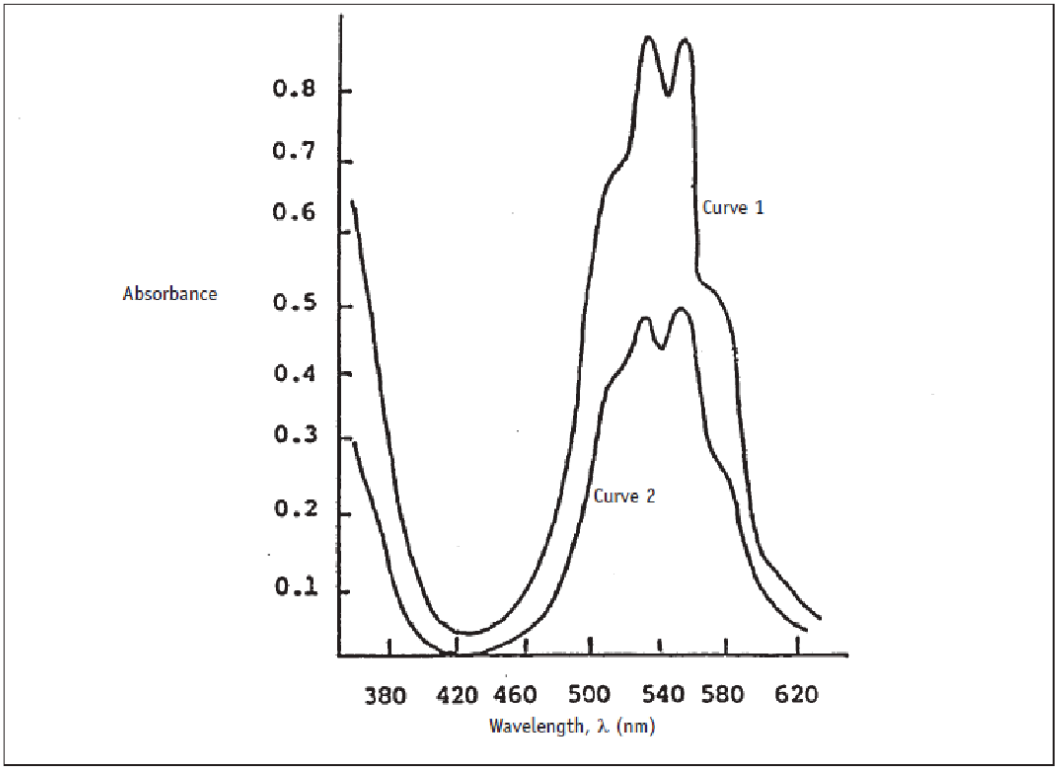


FIGURE The absorption spectrum of solutions of potassium permanganate ($KMnO_4$) at two different concentrations. The solution for curve 1 has a *higher* concentration than that for curve 2.

Assignment:

In this assignment, you will use a virtual spectrophotometer to first establish a standard curve using three concentrations of hemoglobin. Once the curve is established, you can "run patient samples" and determine the concentration of your unknowns based on the absorbance patterns of your standards.

Procedure:

1. Click on the link to open the Biomodel virtual spectrophotometry lab
    a. http://biomodel.uah.es/en/lab/abs/espectro.htm
    b. Review the information under the instructions tab, then follow the directions below to complete the lab. Please note the image icons on the instructions tab to assist you with this lab.
    c. DO NOT click on any other tabs in the virtual UV-VIS spectrophotometer.

Creating a Standard Curve

2. Power on the spectrophotometer by clicking the power button
3. Click on the "Record Spectrum" button
4. Set wavelength to 400 nm by clicking on the dropdown arrow next to the lamba (λ) sign.
5. Blank instrument
    a. Click the set absorbance to zero button (A=0)
6. Prepare hemoglobin standards
    a. Using the solutions in the table, prepare the following concentrations:

Solutions	First Cuvette	Second Cuvette	Third Cuvette
Hemoglobin	1 mL	2 mL	3 mL
Water	2 mL	1 mL	0 mL

    b. Preparing each standard:
        i. For the first cuvette:
            1. Click on the H_2O bottle to fill pipette.
            2. Click on the first radio button under the cuvette.
            3. Click on the pipette to dispense 1 mL of liquid into cuvette.
            4. Click on the haemoglobin bottle to fill the pipette with 1 mL.
            5. Click on the pipette to dispense 1 mL of hemoglobin into cuvette.
        ii. For the second cuvette:
            1. Click on the H_2O bottle to fill pipette.
            2. Click on the second radio button under the cuvette 2.
            3. Click on the pipette to dispense 1 mL of liquid into cuvette 2.
            4. Click on the haemoglobin bottle to fill the pipette with 1 mL.
            5. Click on the pipette to dispense 1 mL of hemoglobin into cuvette 2.
            6. Repeat steps 1-3 to dispense another 1 ml of hemoglobin into cuvette 2.
        iii. For the third cuvette:
            1. Click on the haemoglobin bottle to fill the pipette with 1 mL.
            2. Click on the pipette to dispense 1 mL of hemoglobin into cuvette 3.
            3. Repeat steps 1-2 to dispense another 1 ml of hemoglobin into cuvette 3.

***Careful not to overfill the cuvette**
***Click on the 'Chemical Waste' to start over.**

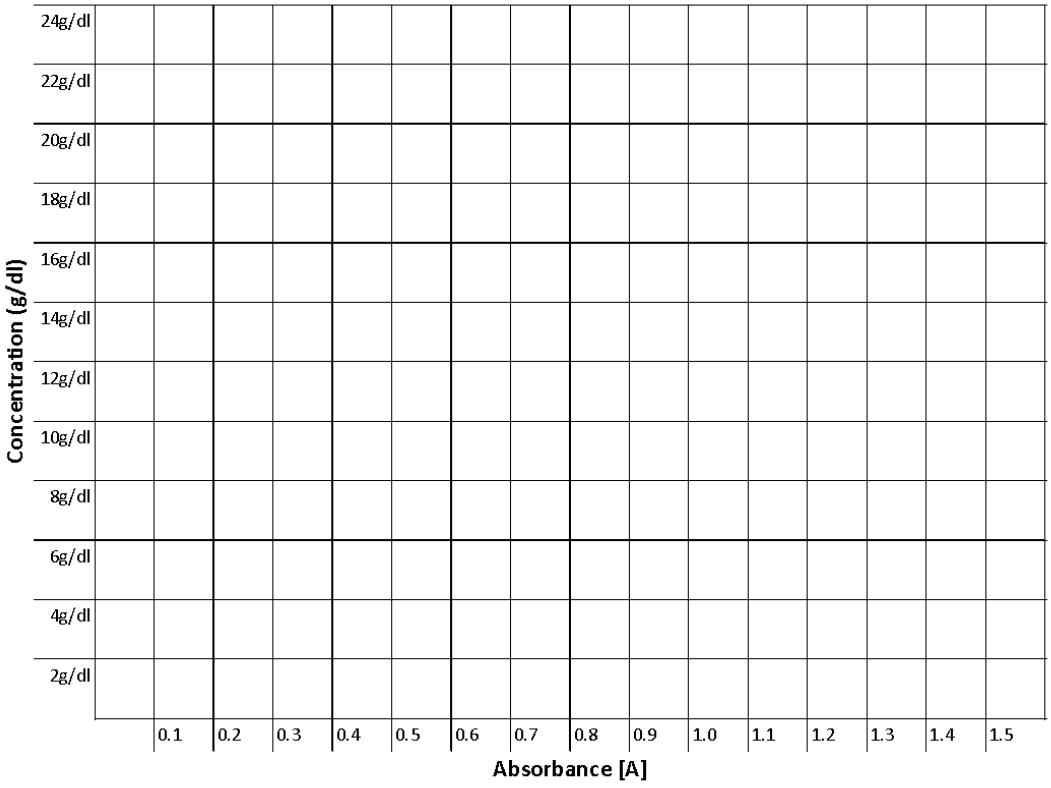
7. Test the absorbance of each sample
    a. Click on the sample
    b. Open the spectrophotometer by clicking the arrow button at the foot of the door.
    c. Click the green arrow button to place the sample
    d. Close the door by again clicking the arrow button at the foot of the door.
    e. Once door is closed, absorbance should be visible
    f. Record absorbance for cuvette 1 on report form.
    g. Remove cuvette by opening the door, then clicking on the red arrow
    h. Repeat with cuvettes 2 and 3.
8. Record absorbance readings for cuvette 2 and 3 in the chart provided
9. Using the chart below
    a. Graph the absorbance readings from the standards (cuvette 1, 2, and 3) using the provided chart
    b. Draw a "line of best fit" through your graph to represent your standard curve
10. Using your standard curve, determine the concentration of your "patient samples" using the absorbance readings provided.

NAME _____ **DATE** _____

Laboratory: Spectrophotometry
Lab Report Form
13 pts.

Grading Rubric (1 point each: absorbance value, patient concentration, patient concentration units, each plotted data point, best fit line)

Standards	Concentration (g/dL)	Absorbance
First Cuvette	6 g/dL	
Second Cuvette	12 g/dL	
Third Cuvette	18 g/dL	
Dirt, Joe 1/10/85		0.555
Wing, Chicken 8/24/07		0.355



Name:_____ **Date:**_____

Laboratory: Spectrophotometry
Study Questions
20 pts

Using your textbook, lecture notes, or Internet, answer the following questions.

1. Electromagnetic radiation is described as: (1 pt)

2. Using the table on page 2 of the lab, give the following maximum absorbance wavelengths for each of the following colors that are absorbed. (4pts)

   Yellow:
   Red:
   Green:
   Blue:

3. White light is defined as: (1pt)

4. When concentration increases light transmittance (increases/decreases). Circle one (1pt)

5. What component of the spectrophotometer is the wavelength selector that promotes spectral isolation and increases sensitivity and specificity? (1 pt)

6. Name 2 types of light sources used frequently to detect absorbance of wavelengths within the **ultraviolet spectrum**. (2 pts)

7. What is the definition of bandpass with respect to the monochromator? (1 pt.)

8. _____ light is any wavelength of light reaching the detector outside the range of wavelengths being transmitted by the monochromator. (1 pt)

9. Visible light is typically what range of wavelengths? (1pt)

10. Fill in the blank in the Beer-Lambert law equation for absorbance. (1pt)

A= 2-log ____

11. State the equation used to determine an unknown concentration using spectrophotometric absorbance and Beer's Laws. (1pts)

12. What is the path length of a cell in most spectrophotometers? (1pt)

13. Given the following information, what is the concentration of the unknown substance? (Show your work) (4pts)

a. A_{std}: 0.042
A_{unk}: 0.137
$Conc_{std}$: 1.6 mg/dL

b. A_{std}: 0.341
A_{unk}: 0.984
$Conc_{std}$: 4.6 mg/dL