FAU Florida Atlantic University

Lab Manual 8: Spectrophotometry

General Chemistry for Health Sciences

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Introduction

Absorption of light is very important to living organisms. The simplest example of the absorption light in nature is photosynthesis. During this process, carbon dioxide from the air is combined with water from the ground and energy from the sun to form simple sugars. These simple sugars are then either put together to form more complex carbohydrates or they are broken down through the process of respiration to release the energy from the simple sugars to do cellular work. Our body can also absorb UV (ultraviolet) light from the sun and use this energy to transform cholesterol into vitamin D.

In chemistry, absorption of light is measured by a spectrophotometer. Spectrophotometry is a method to measure how much a chemical substance absorbs light. When a colored solution is excited, it will absorb light at certain wavelength because the electrons in molecules can jump up to different energy levels. Different chemicals absorb different amounts of light of different colors. When light is not absorbed, the light is transmitted through the solution. The more light a solution can absorb, the less light can be transmitted through the solution. Different chemical compounds absorb different wavelengths of light depending upon their chemical structure; for that reason, appropriate wavelength has to be chosen for absorbance measurements. The amount of light that can be absorbed at this wavelength will vary with the concentration. This relationship is described in Beer's Law and is very important in the fields of chemistry and physics and relates color intensity and concentration of solution.

Goal of Lab 8: Spectrophotometry

The purpose of this lab is to become familiar with common laboratory techniques to prepare stock solution, be able to do serial dilutions, perform concentration calculations, understand the relationship between absorbance and concentration, and find concentration of unknown solution from a standard (calibration) curve.

Theory and Background

Properties of Light

Light is a type of energy that travels as a wave-particle. The **wavelength** of light is the distances between peaks in the waves as light travels. Wavelengths are measured in nanometers (nm) and different wavelengths of light represent differing colors. White light is a mixture of the visible light **spectrum**. Light of long wavelengths (infra-red) and very short wavelengths (ultraviolet) are invisible to humans but can be observed by other organisms. As the wavelength decreases, the energy of the light is increased (Figure 8.1).

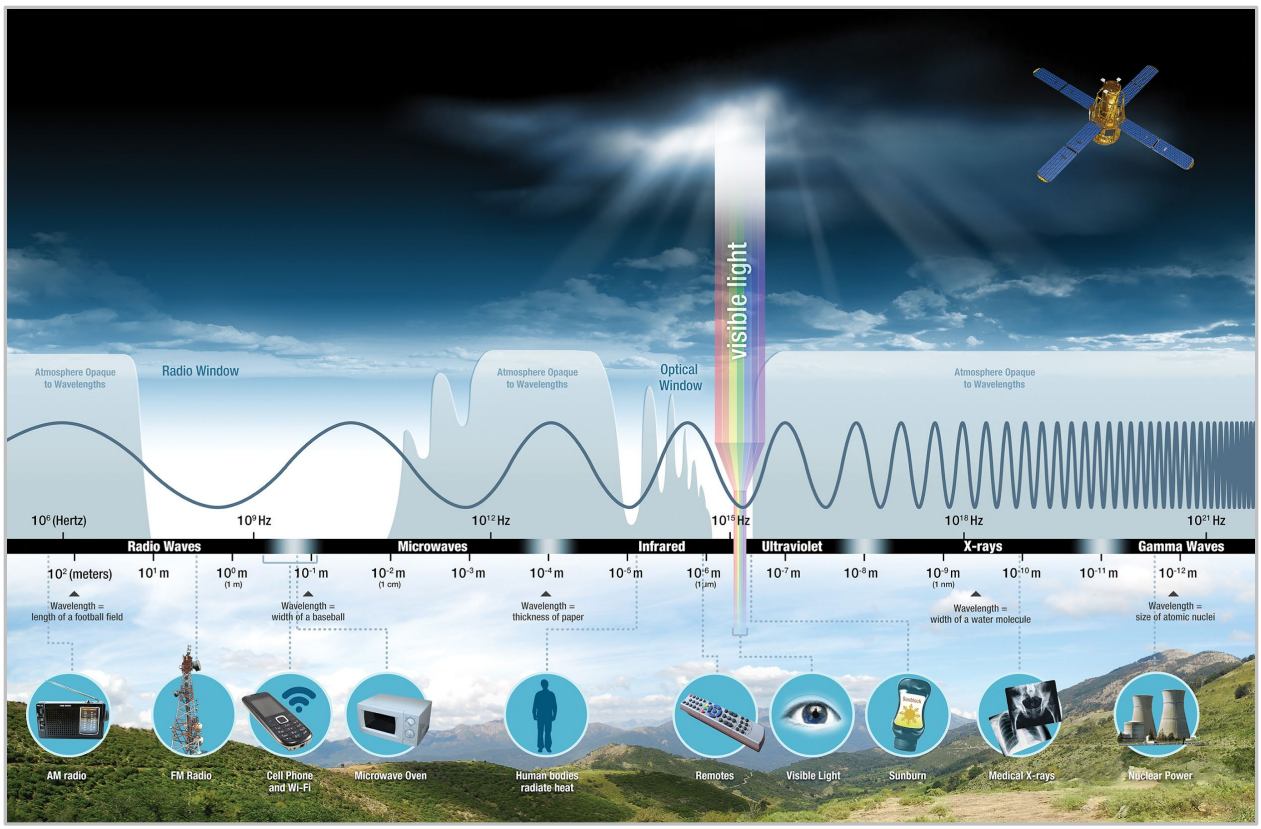


Figure 8.1 Electromagnetic spectrum and associated peak wavelength emitted. [credit: Diagram of the Electromagnetic Spectrum. NASA (2010). Public domain.]

For a deeper introduction into the electromagnetic spectrum, watch this NASA video What is Electromagnetic energy? Illustrated in Figure 8.2, diffraction of light through a prism exposes the component wavelengths of light.

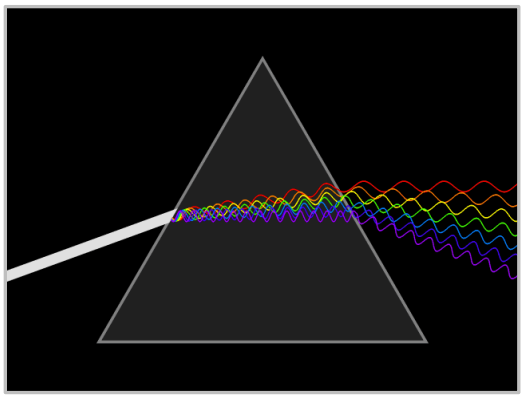


Figure 8.2 Diffraction of light through a prism. [credit: LibreTexts *Biology*, Introduction. CC BY-NC-SA.]

Spectrophotometry

Spectrophotometers (***spectro***-image/color; ***photo-****light****; meter***-measure) are used for chemical analysis of solutions based on properties of absorption or transmission. Figure 8.3 shows a schematic of a spectrophotometer.

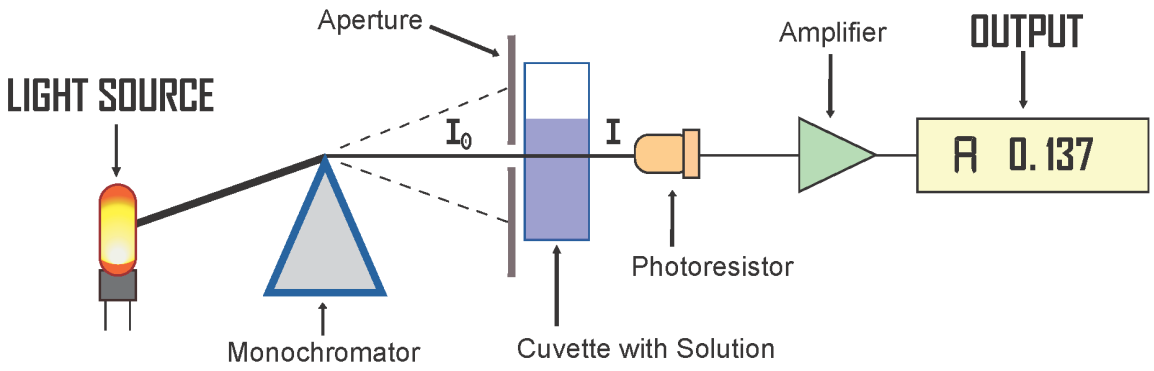


Figure 8.3 A spectrophotometer splits light through a monochromatic prism, reducing the light from the light bulb to a single wavelength. A small portion of the single wavelength light passes through the aperture, which is a hole that captures the single wavelength light. The aperture focuses the light to go through the solution contained in the cuvette. [credit: Center for Online and Continuing Education in partnership with Ozlem Yavuz-Petrowski and the College of Science, Florida Atlantic University. (2020). Spectrophotometer Schematic. CC BY.]

For a practical exploration in using a spectrophotometer as an instrument, you may optionally explore this Spectrophotometer Simulation.

Beer's Law

Beer's Law is a relationship between the concentration or amount of a dissolved substance in a solution that is reducing the amount of transmitted light due to the absorption of the radiant energy. **Lambert's Law** states that the reduction of transmittance was related to the length of the path of light. As the light path increases through a substance, there is a reduction in transmittance. Collectively, these ideas are referred to as **Beer-Lambert Law**, but most observers will control the path length and simply refer to it as Beer's Law.

The Beer-Lambert law relates the attenuation of light to the properties of the material through which the light is traveling. This page takes a brief look at the Beer-Lambert Law and explains the use of the terms absorbance and molar absorptivity relating to UV-visible absorption spectrometry.

The Absorbance of a Solution

For each wavelength of light passing through the spectrometer, the intensity of the light passing through the reference cell is measured. This is usually referred to as I_o - that's I for Intensity. **Transmittance** refers to the amount of light that passes through the solution.

$$T = \frac{I}{I_o}$$

The transmittance of a light source through a cuvette. The intensity of light, **I0**, decreases as it passes through the solution. The light detected by the sensor, **I**, reflects the transmittance of the solution (see Figure 8.4).

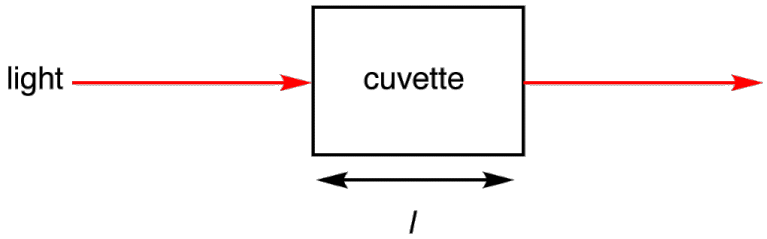


Figure 8.4 Light absorbed by sample in a cuvette. [credit: LibreText *Biology*, The Beer-Lambert Law Figure 1. CC BY-NC-SA.]

The intensity of the light passing through the sample cell is also measured for that wavelength - given the symbol, I. If I is less than I_o, then the sample has absorbed some of the light (neglecting reflection of light off the cuvette surface). A simple bit of math is then done in the computer to convert this into something called the absorbance of the sample - given the symbol, A. If the light is being absorbed by chemicals in the solution, this results in a lower transmission. **Absorbance** is therefore inversely related to transmittance as expressed by the equation:

$$A = -log_{10}T$$

The absorbance of a transition depends on two external assumptions:

1. The absorbance is directly proportional to the concentration (c) of the solution of the sample used in the experiment.
2. The absorbance is directly proportional to the length of the light path (l), which is equal to the width of the cuvette.

Assumption one relates the absorbance to concentration and can be expressed as,

$A \propto c$

The absorbance (A) is defined via the incident intensity $I_o$and transmitted intensity I by

$A = log_{10}\left(\frac{I_o}{I}\right)$

Assumption two can be expressed as,

$A \propto l$

Combining equations from assumption one and two gives,

$A \propto cl$

This proportionality can be converted into an equality by including a proportionality constant (ϵ).

$A = \epsilon cl$

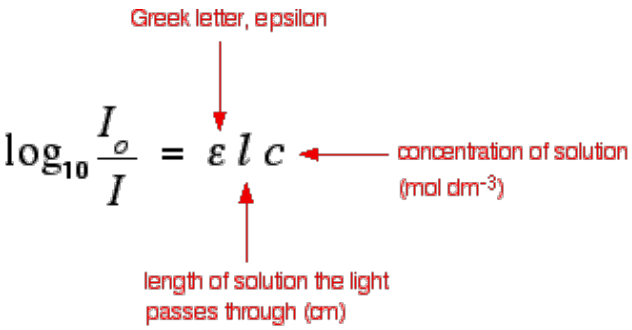
This formula is the common form of the *Beer-Lambert Law*, although it can be also written in terms of intensities:

$A = log_{10}\left(\frac{I_o}{I}\right) = \epsilon lc$

The constant ϵ is called **molar absorptivity** or **molar extinction coefficient** and is a measure of the probability of the electronic transition. On most of the diagrams you will come across, the absorbance ranges from 0 to 1, but it can go higher than that. An absorbance of 0 at some wavelength means that no light of that particular wavelength has been absorbed. The intensities of the sample and reference beam are both the same, so the ratio $\frac{I_o}{I}$ is 1 and the $log_{10}$of 1 is zero.

The Beer-Lambert Law

You will find that various different symbols are given for some of the terms in the equation, particularly for the concentration and the solution length.



The Greek letter epsilon, ϵ, in these equations is called the **molar absorptivity** - or sometimes the molar absorption coefficient. The larger the molar absorptivity, the more probable the electronic transition. In UV spectroscopy, the concentration of the sample solution is measured in mol L^{-1} and the length of the light path in cm. Thus, given that absorbance is unitless, the units of molar absorptivity are L mol^{-1} cm^{-1}. However, since the units of molar absorptivity is always the above, it is customarily reported without units.

The Importance of Concentration

The proportion of the light absorbed will depend on how many molecules it interacts with. Suppose you have got a strongly colored organic dye. If it is in a reasonably concentrated solution, it will have a very high absorbance because there are lots of molecules to interact with the light. However, in an incredibly dilute solution, it may be very difficult to see that it is colored at all. The absorbance is going to be very low. Suppose then that you wanted to compare this dye with a different compound. Unless you took care to make allowance for the concentration, you could not make any sensible comparisons about which one absorbed the most light. Figure 8.5 shows $CuSO_4$ solutions that are prepared in different concentrations from stock solution. Very diluted solutions do not have color and do not have many $CuSO_4$ particles.

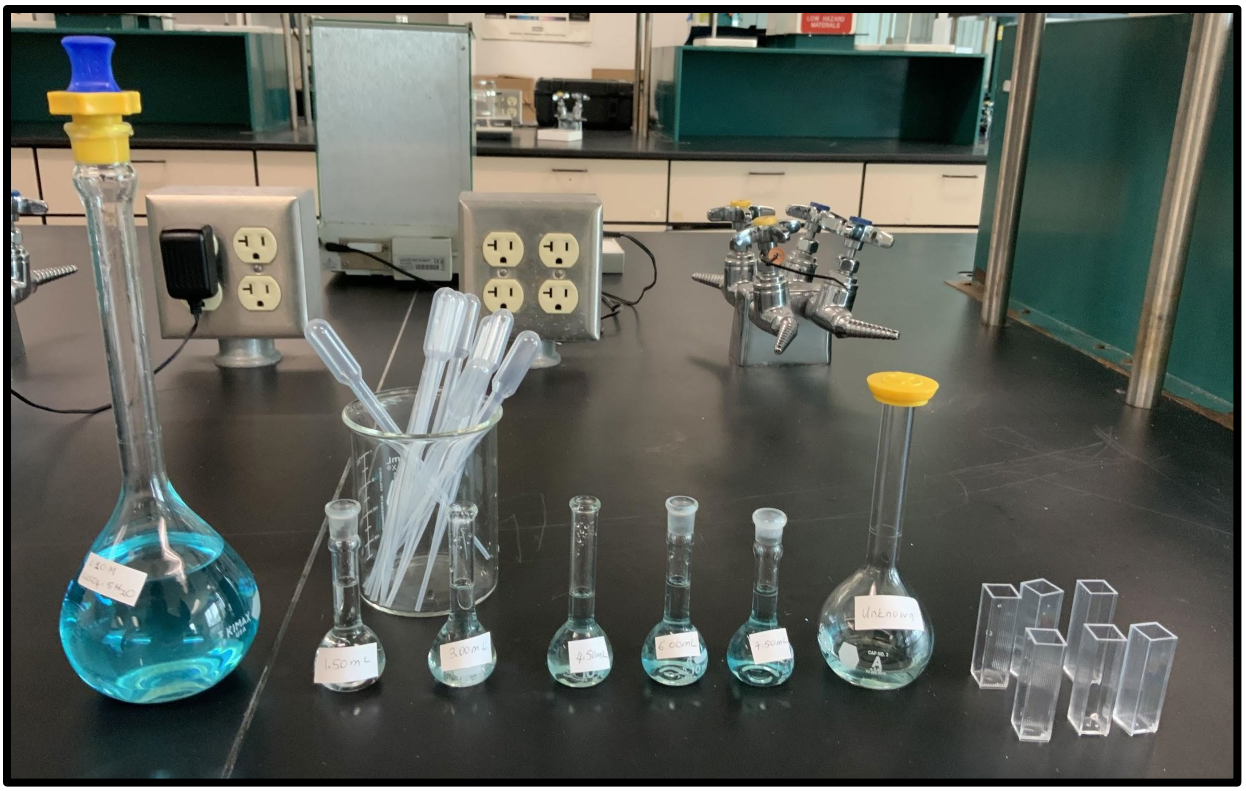


Figure 6.5 Solutions for absorption measurements to make a calibration curve. [credit: Yavuz-Petrowski, O., & the College of Science, Florida Atlantic University. (2020). Absorption Measurement Solutions.]

The Importance of the Container Shape

Suppose this time that you had a very dilute solution of the dye in a cube-shaped container so that the light traveled 1 cm through it. The absorbance is not likely to be very high. On the other hand, suppose you passed the light through a tube 100 cm long containing the same solution. More light would be absorbed because it interacts with more molecules. Again, if you want to draw sensible comparisons between solutions, you have to allow for the length of the solution the light is passing through. Both concentration and solution length are allowed for in the Beer-Lambert Law.

Molar Absorptivity

The Beer-Lambert law can be rearranged to obtain an expression for ϵ (the molar absorptivity):

$$\epsilon = \frac{A}{lc}$$

Remember that the absorbance of a solution will vary as the concentration or the size of the container varies. Molar absorptivity compensates for this by dividing by both the concentration and the length of the solution that the light passes through. Essentially, it works out a value for what the absorbance would be under a standard set of conditions - the light traveling 1 cm through a solution of 1 mol dm^{-3}. That means that you can then make comparisons between one compound and another without having to worry about the concentration of solution length.

Values for molar absorptivity can vary hugely. For example, ethanal has two absorption peaks in its UV-visible spectrum - both in the ultra-violet. There are no units given for absorptivity. That's quite common since the length is often in cm and the concentration is mol dm^{-3}, the units are mol^{-1} dm^3 cm^{-1}.

What Does an Absorption Spectrum Look Like?

The diagram in Figure 8.6 shows a simple UV-visible absorption spectrum for buta-1,3-diene - a molecule. Absorbance (on the vertical axis) is just a measure of the amount of light absorbed. The higher the value, the more of a particular wavelength is being absorbed.

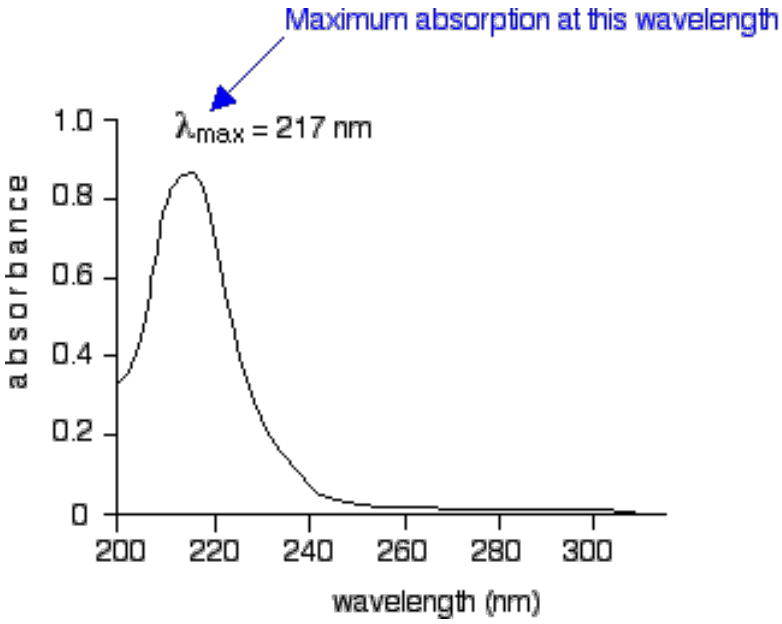


Figure 8.6 Diagram of UV-visible absorption spectrum. [credit: LibreText *Chemistry*, What causes molecules to absorb UV and visible light. CC BY-NC-SA.]

You will see that absorption peaks at a value of 217 nm. This is in the ultra-violet and so there would be no visible sign of any light being absorbed - buta-1,3-diene is colorless. You read the symbol on the graph as "lambda-max".

What Happens When Light Is Absorbed by Molecules?

Absorption is the process in which light of other electromagnetic radiation gives up its energy to an atom or molecule (see Figure 8.7). A photon is the smallest unit of light/electromagnetic energy. When a photon is absorbed by a molecule, the molecule gains the photon's energy and moves to an excited state, higher energy state. Once the electron is being excited to a higher energy level, it becomes more unstable and quickly falls back to the lower energy level and emits a photon. Difference in energy between the lower and higher energy levels is the same amount of energy absorbed by photons (NASA & GSFC, 2013).

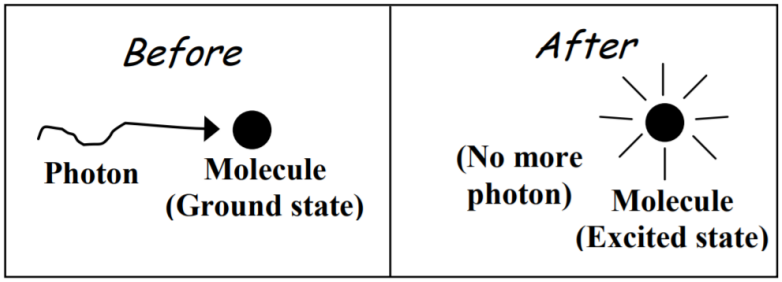


Figure 8.7 Before and after snapshots of photon exciting a molecule. [credit: MITOpenCourseware Spectroscopy in frequency and time domains, Figure I. CC BY-NC-SA.]

Beer's Law Using Graphical Analysis

Beer Lambert Law states that there is a direct and linear relationship between the absorbance and concentration of the sample. The amount of light absorbed will vary with the concentration of the solution.

Beer's law only be applied if there is a linear relationship. If the concentration of a solution is unknown, the concentration can be measured by determining the amount of light absorbed at a particular wavelength. Absorbance and concentration, c, are directly related in the Beer's Law.

$$A = \epsilon c l$$

ϵ is the "molar absorptivity" and it is a constant and depends on the compound at the particular wavelength) and "l" (lowercase, not capital "L") is the path length, or distance the light travels through the solution, because a standard spectrometer uses a cuvette that is 1 cm in width, "l" is always equal to 1 cm and "c" is the concentration of the compound in solution expressed in mol/L.

To prepare a Beer's Law plot (standard curve), a set of solutions of known concentration must be prepared by diluting a stock solution. Amounts chosen of diluted solutions need to be in the range of concentration expected to be found in the unknown concentration. The absorbance for each solution is then measured at the wavelength of maximum absorbance, and the data is used to prepare the plot (see Figure 8.8).

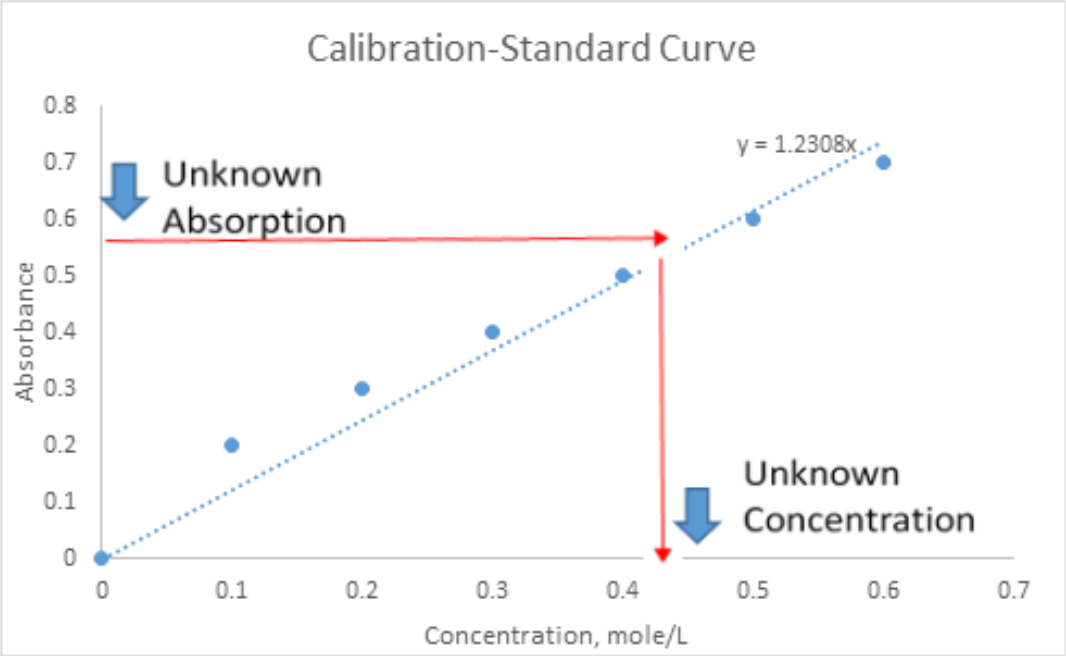


Figure 8.8: Example of Standard Curve for Beer's Law.

When a graph of absorbance vs. concentration is plotted for the diluted solutions, a direct relationship should result, as shown in Figure 8.8. This is called a calibration plot since all concentrations are known. The linearity of this plot arises from Beer's law. The equation for Beer's law is a straight line with the general form of $y = mx + b$. When the y-intercept is zero then b=0 because a solution with a zero concentration would result in zero absorbance. The line of equation can be rewritten in terms of Beer's Law ($A = \epsilon cl$), and result:

$$y = mx$$

where the slope, m, is equal to ϵl, and is a constant. In addition, y is absorbance and x is concentration. The absorbance is measured for an unknown solution, then the slope of the best fit line obtained from the graph is used to determine the unknown concentration of the solution.

Another way to determine the unknown concentration of substance in the solution, intersect across the absorbance measurement for unknown on y axis with standard concentration and down to x, and this is the concentration of substance in unknown sample (Figure 8.8).

Lab Examples

Example 8.1: Virtual Exploration of Beer's Law

In this section, you may explore the Beer's Law Lab Simulation to alter the properties involved in spectrophotometry. Figure 8.9 shows the color star to help you in the simulation. Increase and decrease the concentration slider in the simulation:

1. What happens to the contents in the cuvette?
2. How does this change the Transmittance and Absorbance readings?
3. Click "variable" and use the slider. What happens to the readings when the Wavelength of the laser is a similar color as the solution in the cuvette?
4. Consult the color star below and find the color wavelength that is opposite of the color of the solution. Set the laser to this color using "variable" and the slider.

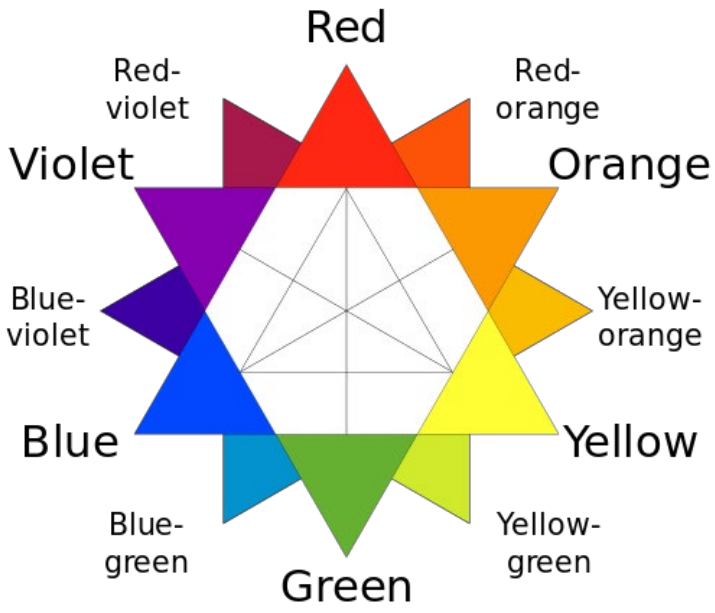


Figure 8.9 Color star to find the color wavelength opposite of the color of the solution. [credit: LibreTexts Biology, Exploring Beer's Law (Virtual). CC BY-NC-SA.]

Continue to play around with the simulation. Think about what your answers might be to these two additional questions:

5. What is the effect on Transmittance and Absorbance with this color?
6. Using the previous observations (using variable wavelength slider), how would you use the relationship on Transmittance/Absorbance to best measure the concentration of a solution?

EXAMPLE 8.2: RELATIVE LIGHT ABSORPTION

In a sample with an absorbance of 1 at a specific wavelength, what is the relative amount of light that was absorbed by the sample?

SOLUTION

This question does not need Beer-Lambert Law to solve, but only the definition of absorbance,

$$A = log_{10}\left(\frac{I_o}{I}\right)$$

The relative loss of intensity is,

$$\frac{I - I_o}{I_o} = 1 - \frac{I}{I_o}$$

The equation can be rearranged using the properties of logarithms to solve for the relative loss of intensity:

$$10^A = \frac{I_o}{I}$$

$$10^{-A} = \frac{I}{I_o}$$

$$1 - 10^{-A} = 1 - \frac{I}{I_o}$$

Substituting in $A = 1$,

$$1 - \frac{I}{I_o} = 1 - 10^{-1}$$

$$= 1 - \frac{1}{10}$$

$$= 0.9$$

Hence 90% of the light at that wavelength has been absorbed and that the transmitted intensity is 10% of the incident intensity. To confirm, substituting these values into the equation to get the absorbance back:

$$\frac{I_o}{I} = \frac{100}{10} = 10$$

And

$$log_{10}10 = 1$$

EXAMPLE 8.3: GUANOSINE

Guanosine has a maximum absorbance of 275 nm. ϵ_{275} = 8400 M^{-1} cm^{-1} and the path length is 1 cm. Using a spectrophotometer, you find that A_{275} = 0.70. What is the concentration of guanosine?

SOLUTION

To solve this problem, you must use Beer's Law.

$$A = \epsilon lc$$

$$0.70 = (8400\ M^{-1}cm^{-1})(1\ cm)(c)$$

Next, divide both sides by $[(8400\ M^{-1}cm^{-1})(1\ cm)]$, yielding,

$c = 8.33 \times 10^{-5}\ mol/L$

EXAMPLE 8.4: EXTINCTION COEFFICIENT

There is a substance in a solution (4 g/L). The length of cuvette is 2 cm and only 50% of the certain light beam is transmitted. What is the extinction coefficient?

SOLUTION

Using Beer-Lambert Law, we can compute the absorption coefficient. Thus,

$$-log\left(\frac{I_t}{I_o}\right) = -log\left(\frac{0.5}{1.0}\right) = A = 8\epsilon$$

Then we obtain,

$\epsilon = 0.0376$

EXAMPLE 8.5: MOLAR ABSORPTION COEFFICIENT

In Example 8.4 above, what is the molar absorption coefficient if the molecular weight is 100?

SOLUTION

It can be obtained by multiplying the absorption coefficient by the molecular weight. Thus,

$\epsilon = 0.0376 \times 100 = 3.76\ L \cdot mol^{-1} \cdot cm^{-1}$

EXAMPLE 8.6: THE IMPORTANCE OF CONCENTRATION

In Example 8.4 above, how much is the beam of light is transmitted when 8 g/L?

SOLUTION

Since we know ϵ, we can calculate the transmission using Beer-Lambert Law.

Thus,

$$log(1) - log(I_t) = 0 - log(I_t) = 0.0376 \times 8 \times 2 = 0.6016$$

$$log(I_t) = -0.6016$$

Therefore,

$$I_t = 0.2503 = 25\%$$

EXAMPLE 8.7: CONCENTRATION WITH TRANSMISSION PERCENTAGE

The absorption coefficient of a glycogen-iodine complex is 0.20 at light of 450 nm. What is the concentration when the transmission is 40% in a cuvette of 2 cm?

SOLUTION

It can also be solved using Beer-Lambert Law. Therefore,

$$-log(I_t) = -log_{10}(0.4) = 0.20 \times c \times 2$$

Thus,

$$c = 0.9948$$

Relations to Health Sciences

Spectroscopy finds widespread application in our daily life. It is used in biological, chemical, and environmental science and medicine. By measuring the intensity of light detected, this method can be used to determine the concentration of solute in the sample. In addition, the wavelength of light absorbed tells the nature of the compound. Figure 8.10 shows an apparatus set-up for using a spectrophotometer in the laboratory. Figure 8.11 shows a zoomed-in view of the spectrophotometer used in the laboratory.

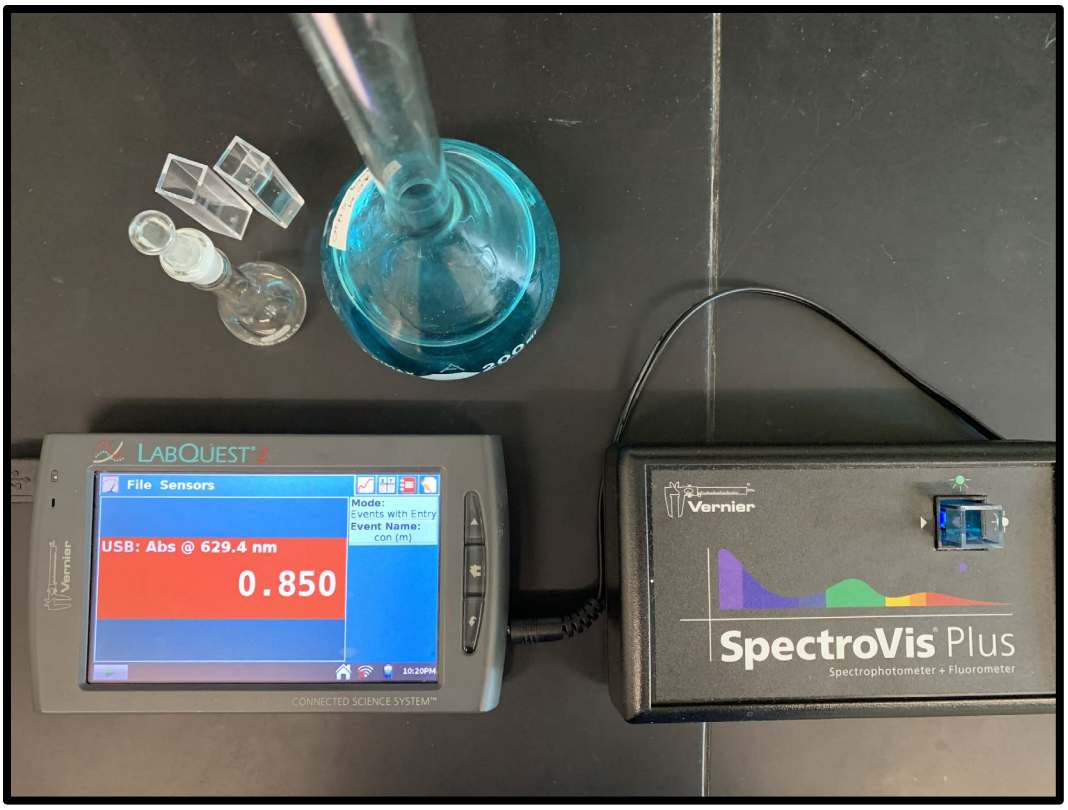


Figure 8.10 Laboratory apparatus set-up for spectrophotometer usage. [credit: Yavuz-Petrowski, O., & the College of Science, Florida Atlantic University. (2020). Spectrophotometry Laboratory Equipment.]

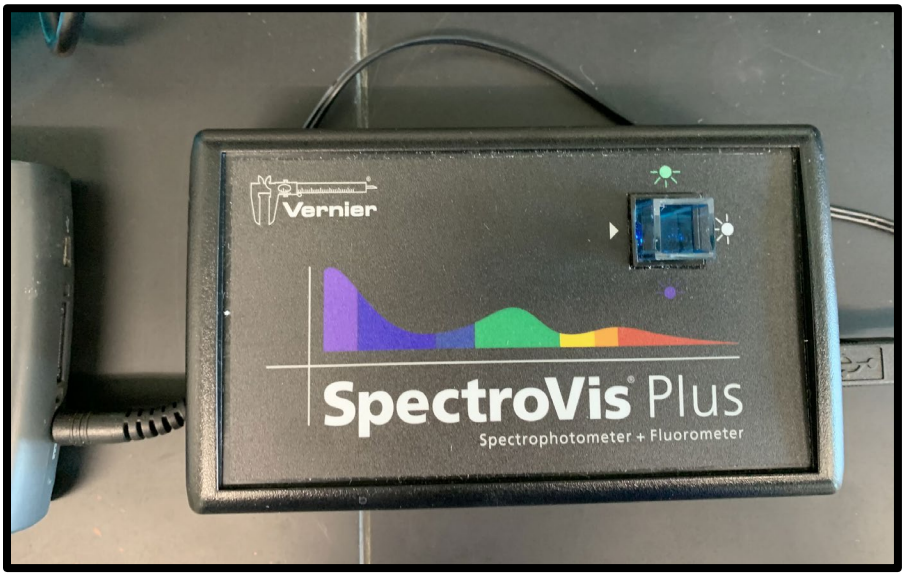


Figure 8.11 Zoomed-in view of spectrophotometer used in the laboratory at Florida Atlantic University. [credit: Yavuz-Petrowski, O., & the College of Science, Florida Atlantic University. (2020). Spectrophotometer in Lab.]

Spectrometers can be also used to measure kinetic or enzymatic reaction by the disappearance of reaction intermediate over time. Different types of spectroscopic techniques can be used in biomedical and environmental research, such as:

- UV-spectroscopy
- Infra-red spectroscopy
- NMR spectroscopy
- Atomic absorption spectroscopy

These techniques can be classified according to the energy range and interaction with samples. Environmental scientists have used emission spectroscopy in the UV-Vis region to analyze metals in water for years. Spectrophotometers are used in pharmaceutical laboratories to detect different compounds and are used in digital printing for color selection and confirmation. UV-Vis spectrophotometer is also used in agriculture in industry to detect phosphate ion in solutions.

Attributions

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National Aeronautics and Space Administration (NASA) & Goddard Space Flight Center (GSFC). (2013). Imagine the Universe! Dictionary. Webpage. Public domain. https://imagine.gsfc.nasa.gov/resources/dictionary.html