SPECTROPHOTOMETRY

Photometry means "measurement of light." A camera light meter is an example of a photometer that quantifies the total light intensity striking the photocell. Many determinations made in the clinical laboratory are based on the measurements of light (also known as radiant energy) absorbed or transmitted under controlled conditions. The instrument used to measure this absorbed or transmitted light energy is the **spectrophotometer**. A review of some of the basic characteristics of light may make this section more understandable.

Light is a type of electromagnetic radiation that travels in the form of a wave. There are two ways of describing a light wave. A light wave can be described by its wavelength or by its frequency. The **wavelength** (λ) is the distance between the peaks of a light wave. The wavelength is a function of the energy of the light wave. The shorter the wavelength, the greater the energy associated with the wave of light. The **frequency** (ν) is the number of waves that pass an observation point in a unit of time. The wavelength is inversely related to frequency and energy, that is, the shorter the wavelength, the higher the frequency and energy, and, vice versa. The relationship between energy and frequency of light is given by the equation:

$$E = h\nu$$

where E refers to the energy (in ergs), h is Planck's constant (6.62×10^{-27} erg · second), and ν is the frequency (Hertz). The frequency of light is related to the wavelength by:

$$\nu = c/\lambda$$

where c is the speed of light in a vacuum (3×10^{10} cm/sec) and λ is the wavelength (cm). If one substitutes the expression of ν from this equation into the previous equation, the following is obtained:

$$E = hc/\lambda$$

This equation shows that energy is inversely proportional to the wavelength of light. Of the two ways of describing light (wavelength and frequency), wavelength is most commonly used in clinical laboratory applications. The table below shows the relationship between the types of electromagnetic radiation and wavelength.

Electromagnetic Radiation Spectrum	
Radiant Energy	**Wavelength (nm)***
Gamma Rays	0.1
X-rays	1
Ultraviolet (UV)	180
Visible Light	380
Infrared Light	780
Microwave	400,000

* The wavelength where the lowest type of respective radiant energy occurs.

In the clinical laboratory, the wavelengths of primary interest in spectrophotometric measurement fall between 180 and 2500 nm. This corresponds to the UV, the visible, and the near infrared (IR) regions. The visible region can be further subdivided into various color regions as illustrated below.

Colors and Complementary Colors of the Visible Spectrum		
Wavelength (nm)	**Color Absorbed**	**Complementary Color**
350 - 430	Violet	Yellow-green
430 - 475	Blue	Yellow
475 - 495	Green-blue	Orange
495 - 505	Blue-green	Red
505 - 555	Green	Purple
555 - 575	Yellow-green	Violet
575 - 600	Yellow	Blue
600 - 650	Orange	Green-blue
650 - 700	Red	Blue-green

The wavelengths are approximate. The colors of the light absorbed and reflected change gradually from one color to the next with no clear line of demarcation. The sum of the colors of the reflected light forms the apparent color of the object to the viewer.

Light from the sun or from incandescent light bulbs contains the entire visible spectrum. This continuum of light typically appears white at the source and colorless as the continuum moves away from the source. Objects that appear colored, absorb light from the continuum at particular

wavelengths and reflect the other parts of the continuum. For example, a substance that absorbs violet light at 400 nm reflects all other light and appears yellow-green. Also, a substance that absorbs yellow light at 590 nm is seen as blue, which is the sum of the reflected light. These absorbed and reflected colors are shown in the table above.

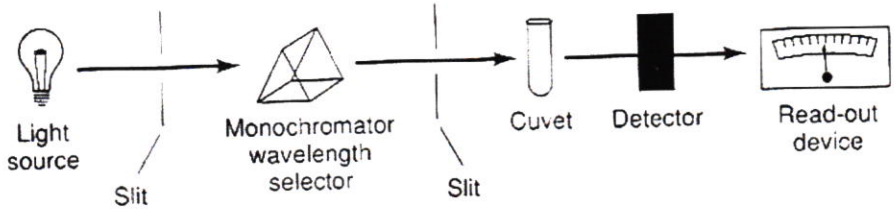
NOTE: please do not memorize information in the two tables above. This information is given for illustrative purposes only.

Spectrophotometry takes advantage of the property where a colored solution results from absorption of a specific wavelength of light. To measure the concentration of a blue solution, light is passed through the solution at 590 nm. The amount of yellow light absorbed at 590 nm varies in direct proportion to the concentration of the blue substances in the solution.

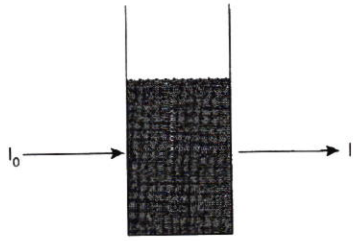
Note that when we move outside of the visible range, humans cannot see color. Therefore, any laboratory analysis of a solution using a wavelength outside of the visible range will appear colorless.

Beer's Law

As we begin to consider the quantitative aspects of spectrophotometry, defined by Beer's Law, a brief review of the schematic of a spectrophotometer may be helpful. The components illustrated in this schematic will be discussed in detail in a later section.



Suppose we intend to use this spectrophotometer to measure the absorbance of a blue solution (i.e., the compound we are interested in measuring absorbs yellow light). The monochromator in the instrument will isolate the 590 nm wavelength of light from the continuum. This 590 nm light will next pass to the cuvet that hold the sample of interest as illustrated below.

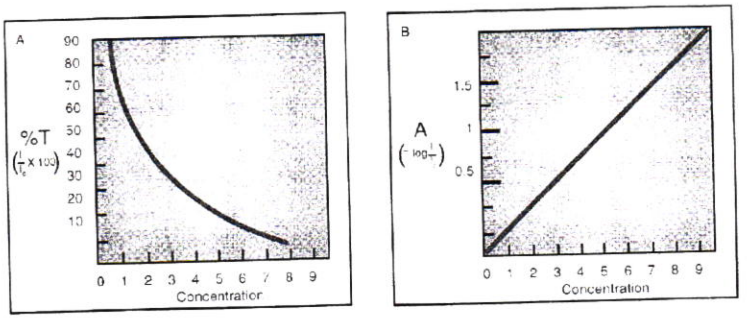


When light of an appropriate wavelength strikes a cuvet that contains a solution of an absorbing material (e.g., glucose, cholesterol, etc.), some of the light is absorbed by the material of interest. The remainder of the light is transmitted through the solution and passes to the detector. The proportion of the light that reaches the detector is known as transmittance (T). Percent transmittance (%T), transmittance multiplied by 100, can be represented by the following equation.

$$\frac{I}{I_o} \text{ X } 100 = \%T$$

In this equation I_o is the intensity of light striking the sample and I is the intensity of light transmitted through the sample. In actual practice, the light transmitted by a **blank** (perhaps abbreviated as I_B instead of I_o) is measured first. The blank may be water or the entire reagent mixture except for the sample to be measured. Use of a blank tells the system the maximum amount of light that can be transmitted through the solution when no absorbing compound is present.

Once the blank is set, the blank cuvet is removed from the spectrophotometer and a cuvet containing the sample of interest is placed in the light path. As the concentration of the absorbing material is increased, the I will decrease along with the %T. As can be seen in part A of the diagram below, as concentration increases, %T decreases. This relationship between concentration and %T is not linear; it is curvilinear, i.e., a curve. If, however, the **logarithm** of %T is plotted against concentration, a straight line will be obtained. If we were to go one step further and consider the **negative** logarithm of %T, not only would we obtain a straight line, the slope of the straight line would change producing a direct relationship between negative logarithm of %T and concentration. The negative logarithm of %T is known as **absorbance**. This type of direct relationship between absorbance (A) and concentration where absorbance increases as concentration increases is desirable because of ease of interpretation. This is illustrated in part B of the diagram below.



Based on the above discussion, absorbance and transmittance can be related as follows:

$$A = -\log T = \log\left(\frac{1}{T}\right) = \log\left(\frac{100}{\%T}\right) = 2 - \log \%T$$

Absorbance is not a directly measurable quantity; only transmittance can be directly measured by spectrophotometers. Absorbance can only be obtained by mathematical calculation from transmittance data. It is desirable to work with absorbance, however, because of the ease of the relationship between absorbance and concentration, as illustrated in the graphs above. All modern spectrophotometers carry out this mathematical calculation and display absorbance information automatically.

Beer's Law states that the concentration of a material is directly proportional to the amount of light absorbed or inversely proportional to the logarithm of transmitted light. This law can be expressed by the following equation

$$A = abc = \log\left(\frac{100}{\%T}\right)$$

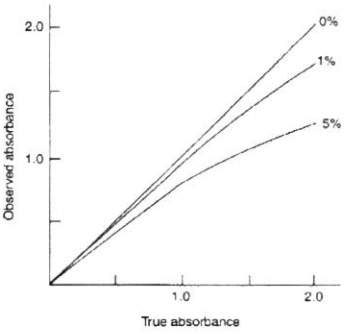
where:

A = absorbance
a = absorptivity of the compound (a constant as long as the same compound is being measured)
b = light path of the solution (a constant as long as the same spectrophotometer is being used)
c = concentration of the compound
$\%T$ = percent transmittance

In its most common application, Beer's Law is expressed as: $\boldsymbol{A = abc}$. When using this equation, a and b are constant in a given spectrophotometric procedure. Thus, absorbance ($\boldsymbol{A}$) is directly proportional to concentration ($\boldsymbol{c}$), as illustrated in graph B above.

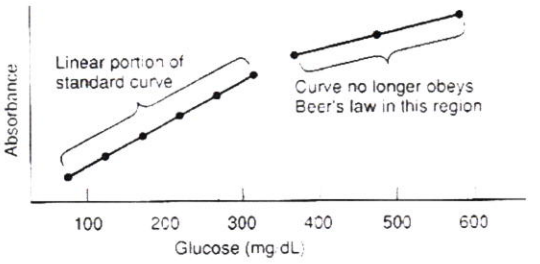
This law is an ideal mathematical relationship that has some limitations in practice. Essentially, this law will be followed only if (1) the light striking the sample ($\boldsymbol{I_o}$) is monochromatic (i.e., a single wavelength of light), (2) the solvent absorbance is insignificant compared with the solute absorbance (blanks are important here), (3) the solute concentration is within linearity limits, (4) no **stray light** is present in the system, and (5) no chemical reaction occurs between the compound of interest and other solute or solvent molecules.

Two of these limitations require further discussion. First, **stray light** (also known as stray radiation) is radiant energy that reaches the detector at wavelengths other than those isolated by the monochromator. All radiant energy that reaches the detector, with or without having passed through the sample in the cuvet, will be detected by the detector. The graph below shows the effects of stray light on Beer's Law. As the amount of stray light increases (or monochromicity decreases - i.e., no longer a single wavelength of light), deviation from Beer's Law increases (i.e., the range where there is a linear relationship between concentration and absorbance decreases).



The second limitation requiring further discussion is **the solute concentration must be within linearity limits**. For each assay that uses spectrophotometry as its method of quantitation, the solute concentration range over which there will be a linear relationship between concentration and absorbance **must** be proven. One reason for this is, with all chemical reactions, we will eventually reach a point where we run out of reagent. A second reason for this is we may reach a point where we exceed the spectrophotometers limits for measuring absorbance. Whichever the problem, we need to know what solute concentration causes us to lose the linear relationship between concentration and absorbance, i.e., deviate from Beer's Law.

In order to determine this linear range, we analyze a series of standards of known concentration by the same exact method used to analyze unknown samples. Once the absorbance readings are taken for the known standards, a Beer's Law plot is prepared on linear graph paper where concentration of the standards is plotted on the x-axis and measured absorbance is plotted on the y-axis. The concentration range over which we have a straight line is the linear range for that assay using that spectrophotometer. This is illustrated in the diagram below.



Notice in the above diagram the phrase "standard curve" is used instead of the more correct phrase "standard plot." Based on the above discussion, you know that we desire to work with a straight and linear Beer's Law plot. Yet, you will frequently hear the word "curve" when reading about or discussing this linear relationship. The reason for this is many years back, spectrophotometers were not capable of mathematically converting transmittance data into absorbance readings. Therefore, during this time, transmittance (or percent transmittance) had to be related to concentration. Recalling the appearance of graph A above, the relationship between transmittance and concentration is a curve. When spectrophotmeters made the transition to reading in terms of absorbance allowing the development of linear Beer's Law plots, for some

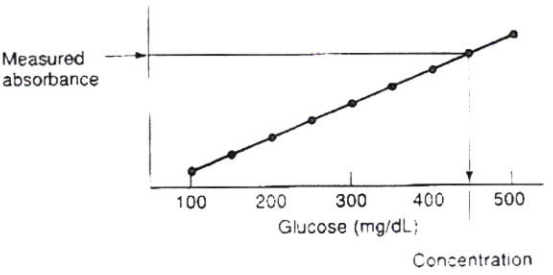
reason, terminology did not make the appropriate transition. Therefore, today, even though we are working almost exclusively with linear relationships, you will very frequently hear and see the word "curve" to describe the straight and linear relationship between concentration and absorbance.

As illustrated by the graph above, deviations from linearity at the upper end of the standard plot are easily detected. By running standards of higher concentrations, a point is reached where the relationship between absorbance and concentration is no longer linear. In most situations, the absorbance does not rise as rapidly as the concentration increases, so the actual level is under-estimated. The graph above illustrates a situation in which the standard plot is not linear above approximately 300 mg/dL (Note: on a properly developed Beer's Law plot using graph paper, you can get much more exact than with the graph illustrated above.) The situation illustrated here may occur when the absorbance of the solution is too strong for the instrument to measure accurately. In other cases, there may be more analyte present than detecting reagent. Not all the analyte reacts with the assay system, and the concentration appears to be less than it actually is. The upper limit of detection must be established so that high levels of an analyte are not under reported.

Two approaches may be taken to deal with a patient's sample that produces an absorbance that exceeds the linear range. The report may be sent out as "greater than" whatever the upper limit of the proven linear range might be. This option is taken in cases where the value is obviously so elevated that no further useful clinical information would be obtained by further re-analysis of the sample. This approach may also be useful in emergency situations when the medical staff needs to know simply that an analyte is very elevated so that appropriate treatment can be initiated. Another option is dilution of the original sample followed by re-assay. The value obtained on the diluted sample is multiplied by the inverse of the dilution for the final result. For this second option, you should not simply dilute the final assay mixture because the analyte may be present in excess of the assay reagent.

When preparing standard plots, our natural tendency is to extend the line through the origin, i.e., through zero. This is true even though we did not analyze a zero concentration standard. When we do this, our unconscious assumption often is that we can then detect any concentration on the line all the way down to a zero level. In practice, this assumption is never valid. For maximum reliability, the lower end of the Beer's Law plot should stop at the lowest standard concentration. Thus, as illustrated in the graphs above, the linear range proven by a set a standards never exceeds the range of standards used. If, in assaying patient samples, we obtain an absorbance that falls below that of our lowest standard, we should simply report "less than" whatever the lower limit for the standard plot might be.

Once we have established our linear range, the unknown samples can be analyzed and the resulting absorbance readings that fall within the linear range can be used to determine concentration by **extrapolation** using this graph. This is illustrated below.



Thus, extrapolation from a standard Beer's Law plot is one method of determining concentration following a chemical reaction. This method is most frequently used when the standard plot will be stable over an extended period of time. In this situation, a new standard plot will need to be prepared at some predetermined interval (e.g., once a month, every time a new lot of reagents is used, etc.).

As an alternative to extrapolation, **once the linear range of the assay is known**, we can perform an assay using one standard within the linear range to compare with our unknown patient sample. This approach is typically referred to as a "ratio of standard to unknown." Here we determine the absorbance values for a known concentration of the compound of interest (standard) and the compound of interest in the patient sample (unknown). If we let A_s and A_u indicate the absorbance values of the standard and unknown respectively, and c_s and c_u indicate the concentrations of the standard and unknown, we can write the following equations:

$$A_s = abc_s$$

and

$$A_u = abc_u$$

These two equations can be stated as a ratio.

$$\frac{A_s}{A_u} = \frac{abc_s}{abc_u}$$

Since we are measuring the same material (e.g., glucose) in both the standard and unkown, the absorptivity term (a) will be the same for both. Therefore, a will divide out. Also, since the standard and the unknown are measured in the same spectrophotometer, the light path (b) will be the same and divide out as well. This will leave the following ratio:

$$\frac{A_s}{A_u} = \frac{c_s}{c_u}$$

This equation can be rearranged to determine the concentration of the unknown sample.

$$C_u = \frac{A_u C_s}{A_s}$$

The following example illustrates the principle. We wish to determine the bilirubin concentration in a patient sample. The absorbance of our patient sample (unknown) is 0.428 and the absorbance of the 5.0 mg/dL standard is 0.372. If we put these numbers into the equation above, we get the following:

$$C_u = \frac{0.428 \text{ x } 5.0 \text{ mg / dL}}{0.372}$$

$$C_u = 5.75 \text{ mg / dL bilirubin}$$

Another way to determine concentration from absorbance measurements involves the use of **molar absorptivity**. The use of molar absorptivity requires a specific knowledge for the absorptivity term ***a*** which is specific for a given compound under defined conditions, e.g., wavelength. When we know the specific absorptivity in terms of molar units, the absorptivity term ***a*** is then known as the molar absorptivity (ϵ). In the clinical laboratory, the main material commonly measured utilizing the molar absorptivity is the reduced form of nicotinamide adenine dinucleotide (NADH). This is a coenzyme that is associated with many enzymatic reactions.

The reason NADH is so useful in clinical laboratory measurements is that the oxidized form of nicotinamide adenine dinucleotide (NAD) does not absorb light at 340 nm while the reduced form, NADH, absorbs light strongly at 340 nm. Many of the chemical reactions that are run in the chemistry section of the laboratory are oxidation-reduction reactions. Every oxidation reaction must be coupled with a reduction reaction. Hence, as the material of interest is oxidized, NAD is reduced to NADH. Thus, as the concentration of material of interest increases, there is increased oxidation of that material. This is coupled with an increased reduction of NAD to NADH, resulting in an increased absorbance at 340 nm. In other words, as the concentration of material of interest increases, the measured absorbance increases.

Returning to Beer's Law, a direct calculation of concentration can be made using absorbance data for NADH. This is because the molar absorptivity for NADH is known to be 6.22×10^3 L/mol-cm. The units for molar absorptivity may seem quite unusual, but they serve the purpose of canceling out the other units on the right hand side of the Beer's Law equation (i.e., light path ***b*** which is in **cm** and concentration ***c*** which is in **mol/L**). This is necessary because absorbance (A) is a unitless term.

$$A(unitless) = \frac{L}{molxcm} \text{ x } cm \text{ x } \frac{mol}{L}$$

This concept is illustrated with the assay that is used to measure ethanol in body fluids by an enzymatic reaction. The reaction used to quantitate ethanol in blood involves the enzymatic oxidation of ethanol to acetaldehyde with the simultaneous reduction of NAD to NADH.

$$\text{ethanol} + \text{NAD} \xrightarrow{\text{alcohol dehydrogenase}} \text{acetaldehyde} + \text{NADH}$$

For every molecule of ethanol oxidized, one molecule of NAD is reduced to form NADH. If we measure the molar concentration of NADH, we can then determine the molar concentration of ethanol originally present since this reaction occurs in a one-to-one ratio. Recall that NADH absorbs light strongly at 340 nm while NAD does not absorb light at this wavelength.

Our assay produces the following data:

Blank absorbance: 0.000
Absorbance (***A***) at the end of the reaction: 0.421
Path length (***b***): 1 cm
Absorptivity (***a***)/Molar absorptivity (ϵ): 6.22×10^3 L/mol×cm
Concentration of ethanol (***c***): ?

Using Beer's Law:

$$A = abc$$

$$c = \frac{A}{ab}$$

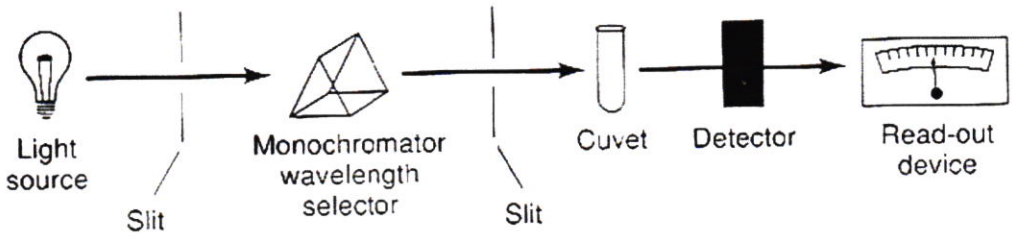
$$c = \frac{0.421}{(6.22^2\ 10^3\ L/mol\,x\,cm)(1\,cm)}$$

$$c = 6.77\times10^{-5}\ \text{mol/L}$$

From this point you could use the molecular weight of ethanol and your vast knowledge of the metric system to convert to mg/dL.

Components of a Spectrophotometer

Basic components of a spectrophotometer consist of the light source, the entrance slit, the dispersion element, the exit slit, the analytical cell or cuvet, and the detector. The entrance slit, dispersion element and exit slit make up the monochromator. A schematic of a basic spectrophotometer is illustrated on the next page. The light source provides electromagnetic radiation as ultraviolet (UV), visible, or infrared light that will pass through the monochromator to be separated into discrete wavelengths. Light of a selected wavelength is made incident on the cuvet containing the solution of which the absorption is to be measured.

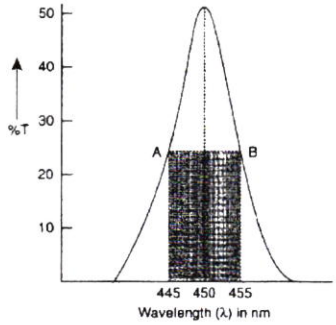


The first component is the **light source**. The light source must be a stable lamp that provides the desired wavelength and provides enough light to penetrate the solution being measured. No single type of light source can provide all the wavelengths that might be needed for all applications. For spectrophotometric work in the visible and near-infrared ranges, tungsten and halogen quartz lamps are good sources of radiant energy. Several types of vapor lamps are available for ultraviolet range. The deuterium lamp is more widely used than the hydrogen lamp because the deuterium lamp emits a greater amount of ultraviolet light. A mercury lamp is less desirable owing to its uneven emission spectrum. A xenon lamp gives a brilliant light that is ideal for applications requiring a narrow slit, but it is not suited for routine application owing to problems resulting from stray light. Collimating lenses are often inserted between the exciter lamp and the entrance slit of the monochromator to focus the light into a beam of parallel light rays.

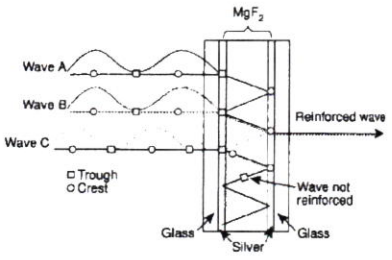
The next component is the **monochromator**. The monochromator consists of three components: the entrance slit, the dispersion element, and the exit slit. The goal of the monochromator is to isolate a single wavelength of light from the continuum provided by the light source.

The first component of the monochromator is the entrance slit. The function of the entrance slit is to reduce stray light and prevent scattered light from entering the monochromator. If stray light were allowed to pass through the analytical cell, this would cause a deviation from Beer's Law. The result would cause a significant error in the measurement, as discussed above.

The dispersion element is a device that produces light of specific wavelengths from a light source. Types of dispersion elements include interference filters, prisms, and diffraction gratings. As we consider these different types of dispersion elements, the term **bandpass** needs to be introduced. With the exception of laser optical devices, the light isolated by a dispersion element in a spectrophotometer is not truly monochromatic (i.e., it is not of a single wavelength). Instead, a narrow range of wavelengths will be isolated. The actual range of wavelengths isolated by the dispersion element is called the bandpass. The bandpass is defined as the range of wavelengths between points at which the light transmitted is equal to one-half (or 50%) the peak light intensity, or greater. Considering the diagram below, the wavelength at which the maximum light is transmitted (known as the nominal wavelength) is 450 nm. The wavelength range at which we find 50% or more of the peak light intensity extends from 445 nm to 455 nm, a 10 nm range. Thus we say that this particular spectrophotometer has a 10 nm bandpass. The more narrow the bandpass, the more specific the measurement. This will be considered in more detail below, following the discussion of double beam spectrophotometers.



The first type of dispersion element is the interference filter. Interference filters are made by placing semitransparent silver films on both sides of a dielectric such as magnesium fluoride (illustrated below). When light perpendicular to the silvered surface enters the filter, is passes through the dielectric and is reflected from the second silvered surface back to the first surface. This process repeats itself until the light is finally transmitted through the filter and through the exit slit. Constructive and destructive interferences occur as the light is reflected between the silver films. Constructive interference refers to a process where light waves of the same amplitude combine and reinforce each other, developing enough energy to exit the filter. Destructive interference refers to light wave that are out of phase and are thus destroyed. Interference filters allow transmission of 40% to 60% of the incident light, with a bandpass between 10 and 20 nm. The dielectric thickness may be varied to produce filters of different bandpasses.



Passage of light waves through an interference filter. Wave A reinforced by wave B breaks through. Wave C is not reinforced by any wave and does not break through.

The second dispersion element is the prism. Prisms are wedge-shaped pieces of glass, quartz, or fused silica. Polychromatic light that enters a prism is refracted at the air-prism interface. The shorter, more energetic wavelengths are refracted more than the longer wavelengths. This results in light being dispersed into a continuous spectrum. Glass prisms are suitable for work in the visible range, but quartz or fused silica is essential for the UV range because glass does not transmit light efficiently at wavelengths shorter than 320 nm. The main problem with the use of prisms as dispersion elements is that dispersion is not linear. Above 550 nm, linearity suffers significantly requiring the use of variable exit slits (referred to below).

The third dispersion element is the grating. Diffraction gratings are made by cutting tiny grooves or slits into an aluminized surface of a flat piece of crown glass. These grooves are cut at a precise angle and at an equal distance from each other. There are usually 1,000 to 50,000 grooves per inch. Each of these grooves produces an individual spectrum. As the spectrum moves away from the grating surface, it interacts with other spectra. Some light waves undergo

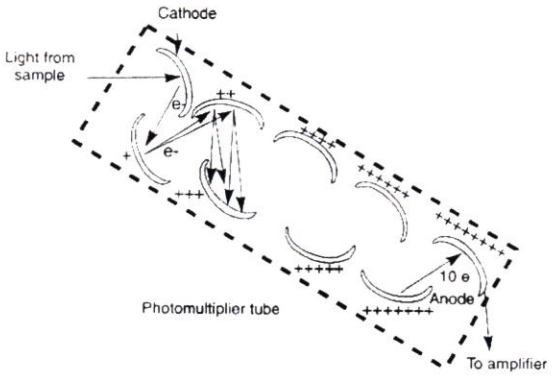
constructive interference while others undergo destructive inference. The final result is a single spectrum where each wavelength is well resolved. Usually, gratings are capable of better resolution than prisms or filters. Gratings also have the additional advantage of covering all essential wavelengths, in contrast to the glass prisms, which cannot be used in the ultraviolet region. Because high-quality gratings can now be produced economically, most spectrophotometers incorporate diffraction gratings as their dispersion elements.

The final component of the monochromator is the exit slit. The exit slit determines the bandwidth of the light that will be selected from the dispersed spectrum. By increasing the width of the exit slit, the band width of the emerging light is broadened, with a resultant increase in energy intensity but a decrease in spectral purity. In diffraction grating monochromators, the exit slit may be a fixed width, resulting in a constant bandpass. In contrast, prism monochromators have variable exit slits. The purpose of both entrance and exit slits in filter photometers is to make the light parallel and reduce stray light.

The **cuvet** holds the solution for which the absorbance is to be measured. Glass cuvets are satisfactory for use above 320 nm. For measurements below 320 nm, quartz or fused silica cuvets are required. As noted with glass prisms, glass does not transmit light efficiently at wavelengths shorter than 320 nm. Both round and rectangular cuvets are available. A rectangular cuvet, which presents a flat surface to the incident light, has less radiant energy loss from reflection than does a round cuvet. For routine work, this loss is usually not significant, accounting for about 4% of the incident energy for most round cuvets. Rectangular and round cuvets typically have a standard 1 cm inside diameter (i.e., path length or ***b*** in Beer's Law calculations). Finally, room light entering the cuvet will cause measurement errors. A light shield should be placed over the cuvet well when a spectrophotometric reading is being made.

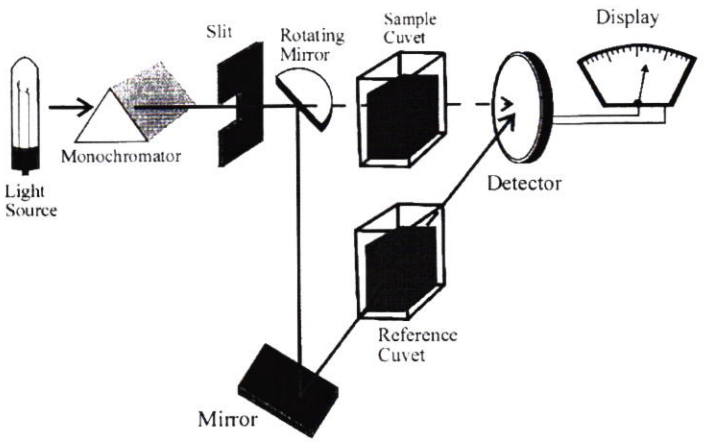
The final component of the basic spectrophotometer is the **detector**. Types of detectors include barrier-layer cell, phototube, photomultiplier tube, and a variety of semiconductor detectors. All detectors use photosensitive materials in their cathodes that release electrons when they are emitted from the cathode. When a closed electrical circuit is provided, the free electrons produce a current. Thus, the purpose of the detector is to convert light energy into electrical energy.

Perhaps the most common type of detector is the photomultiplier tube. An example of a photomultiplier tube is illustrated below. Photomultiplier tubes consist of a photoemissive cathode, an anode, and an internal electron-multiplying series of dynodes. Many photomultiplier tubes have 9 to 16 photosensitive dynodes. A dynode is an electrode used to generate secondary emissions of electrons. All of these components are encased in a glass evacuated tube. When radiant energy strikes the cathode, the emitted electrons are attracted to the first adjacent dynode. On striking the dynode, each electron causes the emission of several other electrons. The electrons emitted from the first dynode are subsequently attracted to the second dynode, where the same emission cycle is repeated. This process continues through the entire series of dynodes, resulting in a multiplication of the number of electrons, until the anode is reached. The amplification factor achieved by a photomultiplier tube may be as high as 10. Because of their excellent sensitivity and rapid response, all stray light and room light must be carefully shielded from the photomultiplier tube to prevent burn out.



Double-Beam Spectrophotometers

Up to this point, we have primarily considered what is known as a single-beam spectrophotometer. In double-beam spectrophotometers, monochromatic light is split into two segments. One segment of light is directed through a reference cuvet and one segment of light is directed through the sample cuvet. The intensity of these two light segments is then measured alternately by one detector. The sample beam intensity is compared with the reference beam intensity as a ratio. An illustration of a double-beam schematic is shown below.



In this system, the light beam is split with a rotating chopper that alternately presents a mirror and an opening. During the time the light passes through the opening, the segment of light continues through the sample cuvet. During the time the light is reflected off of the mirrored surface, the segment of light is directed through the reference cuvet. After each segment, consisting of a pulse of electromagnetic radiation separated in time by a dark interval, passes through the appropriate cuvet, it is directed onto the detector. The detector measures all of the light that hits it and the electronics of the spectrophotometer distinguishes between the segments that passed through the sample cuvet and the reference cuvet. This is possible because the amplifier of the instrument is tuned with the rotating chopper. Therefore, the amplifier knows which segment of light is being processed at any given point in time.

This concept is illustrated with the assay that is used to measure ethanol in body fluids by an enzymatic reaction. The reaction used to quantitate ethanol in blood involves the enzymatic oxidation of ethanol to acetaldehyde with the simultaneous reduction of NAD to NADH.

$$\text{ethanol} + \text{NAD} \xrightarrow{\text{alcohol dehydrogenase}} \text{acetaldehyde} + \text{NADH}$$

For every molecule of ethanol oxidized, one molecule of NAD is reduced to form NADH. If we measure the molar concentration of NADH, we can then determine the molar concentration of ethanol originally present since this reaction occurs in a one-to-one ratio. Recall that NADH absorbs light strongly at 340 nm while NAD does not absorb light at this wavelength.

Our assay produces the following data:

Blank absorbance: 0.000
Absorbance (***A***) at the end of the reaction: 0.421
Path length (***b***): 1 cm
Absorptivity (***a***)/Molar absorptivity (ϵ): 6.22×10^3 L/mol×cm
Concentration of ethanol (***c***): ?

Using Beer's Law:

$$A = abc$$

$$c = \frac{A}{ab}$$

$$c = \frac{0.421}{(6.22^2\ 10^3\ L/mol x cm)(1\ cm)}$$

$$c = 6.77 \times 10^{-5}\ \text{mol/L}$$

From this point you could use the molecular weight of ethanol and your vast knowledge of the metric system to convert to mg/dL.

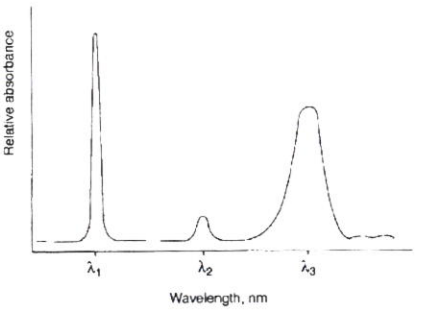
Components of a Spectrophotometer

Basic components of a spectrophotometer consist of the light source, the entrance slit, the dispersion element, the exit slit, the analytical cell or cuvet, and the detector. The entrance slit, dispersion element and exit slit make up the monochromator. A schematic of a basic spectrophotometer is illustrated on the next page. The light source provides electromagnetic radiation as ultraviolet (UV), visible, or infrared light that will pass through the monochromator to be separated into discrete wavelengths. Light of a selected wavelength is made incident on the cuvet containing the solution of which the absorption is to be measured.

The first step in the use of a double-beam spectrophotometer involves placing the blank solution in both the sample cuvet and reference cuvet. The absorbance reading for the spectrophotometer is then adjusted to zero. By making this adjustment, we are essentially telling the instrument that the two light segments are equal. We leave the blank solution in the reference cuvet and replace the blank solution in the sample cuvet with the solution to be measured. Thus, with this double-beam system, there is a constant comparison with the blank solution allowing immediate compensation for any variation intensity that affects both cuvets simultaneously. One cause for a variation in light intensity in both cuvets originates from the light source. Variations in intensity of light from the light source can be corrected by this double-beam system. These same variations in intensity from the light source cannot be corrected by a single beam-system resulting in errant results.

Another advantage of the double-beam system is seen when the reaction system we are monitoring by spectrophotometry changes color during the reaction. As our reagents sit exposed to air, certain reagents (or components of reagent systems) may become oxidized and show a slight color change. Since this color change would occur in both the sample and reference cuvets, the double-beam spectrophotometer would be able to correct for this while a single-beam system would not.

The ability to perform a **spectral scan** is an important function of a spectrophotometer. A spectral scan is developed by reading the absorbance produced by the compound of interest at each wavelength in the UV and visible region. This capability is very important in determining which wavelength to use when we perform our quantitative analysis of that compound. We would obviously want to use the wavelength where the instrument is the most sensitive, and, this is what we can determine from a spectral scan. Consider the spectral scan below.

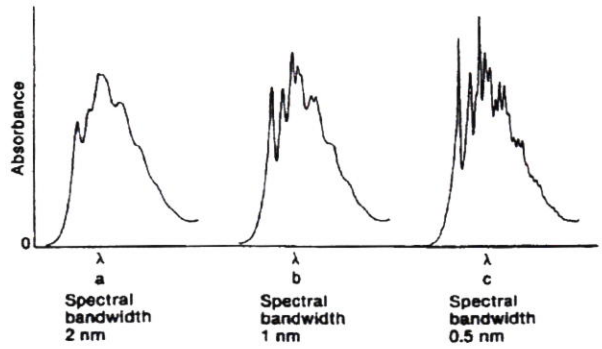


A common abbreviation for wavelength is λ. The wavelength that produces the greatest absorbance is known as λ_{max}. Considering the diagram above, λ_1 produces the greatest absorbance and is known as λ_{max}. This would be the wavelength of choice to perform our quantitative analysis.

Double-beam spectrophotmeters are ideally suited for making spectral scans because the instrument automatically corrects for the change in light transmission through the reference cuvet as the wavelength is changed. If a single-beam instrument were used, each change in wavelength would require an adjustment of the instrument to zero absorbance with a blank before reading the absorbance of the sample.

To produce a spectral scan, a computer is attached to a spectrophotometer. A drive motor is geared to the diffraction grating. It is turned slowly in the light path so that light of various wavelengths sequentially passes through the exit slit of the monochromator. The light is passed through the cuvet compartment in succession. The computer records the absorbance at each wavelength and prepares the plot shown above.

Spectral scans require adequate resolution for interpretation. To resolve absorbance peaks, the bandpass must be narrow. Often, a very sharp, narrow peak may be completely missed if a wide bandpass were selected. This is illustrated in the diagram below for the chemical toluene. Toluene was analyzed in three different spectrophotometers with different bandpasses.



Blanking the Spectrophotometer

Throughout this discussion reference has been made to blanking the spectrophotometer. **Blanking** refers to reading the absorbance of a solution that includes all light absorption not due to the desired compound of interest. Possible sources of extraneous absorption include absorption or reflectance of light by the cuvet, absorption or scattering of light by the solvent or the reagents, and absorption or scattering of light by color or turbidity in the sample. The two most common type of blank solutions that are utilized in the clinical laboratory are reagent blanks and specimen blanks. A **reagent blank** contains all the components of the reaction except the sample to be analyzed. The absorbance of the reagent blank includes absorbances due to the cuvet and all reagents. This type of blank is utilized when some component of the reagent may contribute unwanted absorption to the final product to be read by the spectrophotometer. Alternatively, a **specimen blank** contains the sample and all components of the reagent except the one component that is required for the reaction to occur. The absorbance of the specimen blank includes absorbances due to the cuvet, the sample's color and turbidity, and most of the reagents' absorbances.

As we have discussed above, both single-beam and double-beam spectrophotometers utilize blanks. In single-beam systems, the instrument is blanked first, then the blank solution is replaced with the sample of interest. In the double-beam system, the instrument is blanked with blank solution initially placed in both light paths, followed by replacing the blank solution with sample of interest in only the sample beam.

Bichromatic Analysis

Many laboratory analyzers in the clinical chemistry section that utilize spectrophotometry also employ bichromatic analysis. In these analyzers, the sample of interest is measured at two wavelengths. The first wavelength is λ_{max}. The second wavelength is close to λ_{max}, but at a wavelength that the compound of interest does not absorb light. For a given compound of interest, this second wavelength may be set at a wavelength where common interfering substances are known to have absorption. The problem is that the interfering compound may also absorb at λ_{max} for the compound of interest. Thus, when we measure at λ_{max}, we would get absorbance from the compound of interest plus the interfering compound. In this case, bichromatic analysis will allow the calculation of true absorption due to the compound of interest alone by taking the difference in absorbance at the two wavelengths.

A very common application of bichromatic analysis is in laboratory analyzers that have flow cells for spectrophotometric measurement. The problem is that protein buildup within the flow cell may begin to block light producing abnormally high absorbance readings. Here the absorbance measured at λ_{max} would be a composite of the absorbance due to the desired compound plus absorbance due to the light blocked by the protein buildup. With bichromatic analysis, the light blocked (read as absorbance) at the second wavelength would be subtracted from the absorbance at λ_{max} with the difference being the true absorbance of the compound of interest.

Quality Control Check of Wavelength Accuracy

It is sound laboratory practice to certify that spectrophotometers are functioning within specifications. One such check is for wavelength accuracy. A common method of checking wavelength accuracy involves the use of rare-earth glass filters such as holmium oxide and didymium. Holmium oxide has strong and sharp lines of absorption and is best suited for the calibration of narrow bandpass instruments. Didymium, on the other hand, has much broader absorption bands and is best suited for the calibration of broad bandpass spectrophotometers.