# Spectrophotometry

Spectrophotometry and colorimetry are conventional techniques for quantitatively determining substances encountered in biochemistry. All substances in solution absorb light of some wavelength and transmit light of other wavelengths. Absorbance is a characteristic of a substance just like melting point, boiling point, density and solubility. Absorbance can be related to the amount of the substance in solution, thus it can be used to quantitatively determine the amount of substance that is present.

## Light and Spectra

Light or electromagnetic radiation is composed of photons moving in a wave that oscillates along the path of motion. The wavelength of light is defined as the distance between adjacent peaks in the wave and can be further defined by the equation:

$$\lambda = c / \nu$$

where $\lambda$ is the wavelength, c is the speed of light, and $\varpi$ is the frequency or number of waves passing a certain point per unit time. Photons of different wavelengths have different energies that are given by:

$$E = hc / \lambda = h \nu$$

where h is Planck's constant. Thus, the shorter the wavelength, the greater the energy.

Electromagnetic radiation can be divided into various regions according to wavelength: the ultraviolet region has wavelengths 200-400 nm and the visible region has wavelengths of 400-700 nm. There are other regions such as infrared, radio wave, microwave and more, but you will not apply them in this course.

In the visible region, lights of different wavelengths have different colors: violet and blue in the low wavelength region and orange and red in the high wavelength region. When a substance in solution appears blue, it means that the substance is absorbing red light and transmitting blue light. A substance that appears red is absorbing blue light and transmitting red light. A substance is said to have absorption

spectra for a blue substance and a red substance.

Molecules possess both kinetic energy and the energy associated with their bonding electrons. The absorption of light does not directly affect the kinetic energy, but it can affect the energy of the bonding electrons. The energy of the bonding electrons can be further divided into three types: **electronic, vibrational, and rotational**. Absorption of electromagnetic radiation involves a change involves a change in one or more of these bonding energies. Each of the electronic, vibrational, and rotational energy components can have only certain definite values or energy levels. A molecule whose electronic, vibrational, and rotational energies are all at their lowest values are said to be in the ground state. When a molecule is irradiated by photons whose energies just correspond to the difference in energy between the ground state and some higher or excited state of the molecule, the photons are absorbed and the molecule is raised to a higher energy level.

Spectra arise when molecules absorb photons of specific energy. Transitions between different electronic levels give rise to spectra in the ultraviolet or visible regions; transitions between vibrational levels within the same electronic state give rise to spectra in the near-infrared region and transitions between rotational levels belonging to the same vibrational state give rise to spectra in the far-infrared region.

Bonding electrons do not absorb electromagnetic radiation of very long wavelengths (radio waves), but nuclei of specific kinds of atoms do and can give rise to nuclear magnetic resonance spectra.

Theoretically, since only discrete peaks of energy (specific wavelengths) are absorbed by the bonding electrons, the spectrum should consist of sharp lines. The existence of many possible vibrational levels or each electronic energy level, however, increases the number of possible transitions and gives rise to several distinct lines that together make up the broad peaks in the spectrum.

Whether any electronic transitions in a molecule can be brought about by the energy of a photon in the visible or ultraviolet region depends on the nature or the bonding electrons in the ground state. In general, the absorption of visible and ultraviolet light by organic compounds can occur only when there is some unsaturation in the molecule. A specific grouping of atoms having unsaturation and absorbing light is called a **chromophore**. The common chromophoric groups include carbon-carbon double and triple bonds and the carbonyl, carboxyl, amido, azo, nitrile, nitroso, nitro, imidazole, indole, purine, and pyrimidine groups. Any molecule containing one or more such groups will have an absorption band somewhere in the visible or ultraviolet regions. Conjugation of the unsaturated bonds will also contribute to specific absorption bands. The overlapping

molecular orbitals containing the electrons in a conjugated system give rise to delocalization of the electrons. The result is a decrease in the energy required for a transition and a spectral peak at higher wavelengths.

Once arrived at, an excited electronic, vibrational, or rotational state doesn't continue forever because the energy originally gained from photon absorption is lost by collisions with other molecules, such as solvent molecules. Eventually, the energy is transformed into kinetic or thermal energy and the molecule is returned to the ground state with the liberation of heat. Special arrangements of chromophoric groups can also result in the release of radiation from the energy of the absorbed photons, giving rise to the process known as fluorescence.

## The Lambert-Beer Law

The amount of light passing through a substance is called **transmittance**, T, or percent transmittance %T, and is defined by the following equations:

$$T = I / I_o$$
$$\%T = 100\% \ (I / I_o)$$

where $I_o$ is the intensity of the incident light and I is the intensity of the transmitted light. The amount of light of a specified wavelength absorbed by the substance depends on the length of the light path through the substance. The negative logarithm of the transmittance, the **absorbance**, A, is directly proportional to the amount of light absorbed and to the length of the light path and is described by the **Lambert law**, shown below:

$$-\log( T ) = -\log (I / I_o) = A = k_1 b$$

Here b is the length of the medium, usually a solution in a cell, and $k_1$ is a constant. A comparison of the scales for percent transmittance and absorbance may be used to convert percent transmittance into absorbance.

The negative logarithm of the transmittance is also directly proportional to the concentration of the absorbing substance c and is described by **Beer's law**, shown below.

$$-\log (I/I_o) = -\log T = A = k_2 c$$

Combining the two laws gives the **Lambert-Beer** law:

$$-\log (I/I_o) = -\log T = A = \varepsilon bc$$

where $\varepsilon$ is a constant called the **extinction coefficient** incorporating $k_1$ and $k_2$. The extinction coefficient is dependent on the wavelength of the light passing through the substance and on the chemical nature of the substance; b is the path length (cm), and c is the concentration of substance.

## Definition of Extinction Coefficients

If the extinction coefficient for a substance at the maximum absorbance is known and the path length is fixed, the concentration of the substance can be determined. The extinction coefficient may be obtained from the literature or determined by measuring the absorbance at different concentrations of the substance. A plot of the absorbance versus concentration should give a linear curve whose slope is the extinction coefficient when the cell length is 1.00cm.

The measurement of the amount of light absorbed may be either as percent transmittance (%T) or as absorbance (A). Absorbance is used more often than percent transmittance because this variable is linear with the concentration of the absorbing substance, whereas percent transmittance is exponential. The side-by-side absorbance and percent transmittance scales show that when the amount of light absorbed is greater than 50%, errors become magnified. The measurement of concentration, therefore, is best achieved between 0.05 and 0.30 absorbance and between 90 and 50% transmittance. The errors in measuring absorbance values of 1 or 2 could be very large.

When the extinction coefficient is known and a fixed path length established, the concentration of an unknown amount of the substance can be determined by measuring the absorbance of the substance and applying the Lambert-Beer law.

$$c = A / ( \varepsilon b )$$

The limit of sensitivity of a spectrophotometric analysis is determined by the value of the extinction coefficient. The higher value of the extinction coefficient, the lower is the

concentration that may be measured. If the molar extinction coefficient is 10,000 $Lmol^{-1}cm^{-1}$ and the minimum detectable absorbance is 0.01, then for a cell with a 1.00 cm path length, the minimum molar concentration that can be measured is $1.00x10^{-6}$ M.

$$a_M = 10{,}000\ Lmol^{-1}cm^{-1}$$
$$A = 0.01$$
$$b = 1\ cm$$
$$c = A / ( \varepsilon b ) = 0.01/((10{,}000\ Lmol^{-1}cm^{-1})(1\ cm)) = 1.00x10^{-6}\ M.$$

If the molar extinction coefficient, however, were 10 times greater, the minimum molar concentration that could be measured would be 10 times lower, or $1.00x10^{-7}$ M.

Substances that have very high extinction coefficients give high absorbance values, usually a desirable characteristic, as indicated above. To obtain reliable absorbance values between 0.10 and 0.30, however, the experimental parameters must be modified. Either the sample must be diluted or, if it is not desirable to dilute the sample, the path length must be decreased.

# Ultraviolet and Visible Spectrometry

## Instrumentation

The amount of light that is absorbed by a substance may be measured by spectrophotometers and colorimeters. These instruments have several parts, which include a light source, a monochromator or colored filter to give a selected wavelength, a variable slit, a sample holder, a photodetector, and a meter. Different types of light sources are required for the different spectral regions. Tungsten and deuterium lamps are used for visible (400-700 nm) and the ultraviolet (200-400 nm) regions, respectively. The wavelengths of light in these regions are selected by a monochromator in spectrophotometers and by colored filters in colorimeters. A monochromator is composed of prisms or diffraction gratings and gives light of a narrow band of wavelengths in both the visible and the ultraviolet regions. Colored filters give a relatively broad band of wavelengths exclusively in the visible region. The light then passes through an adjustable slit, which controls the intensity, and into the sample, from which it is detected and quantitated by a phototube or photomultiplier tube and measured by galvanometer and/or recorder.

## Applications of Ultraviolet and Visible Spectroscopy

The most common and obvious application of visible and ultraviolet spectroscopy is the determination of the concentration of a substance in solution if the extinction

coefficient is known and the Lambert-Beer law obeyed. This may be extended to the measurement of reactions if one of the reactants can then be determined by measuring the amount of loss of the reactant or the yield of the product. This approach has been applied especially to enzyme-catalyzed reactions to assay the effect of the enzyme.

An absorbance spectrum may be used, at least presumptively, to identify substances in a sample. For example, proteins and nucleic acids have characteristic absorbances at 280 and 260 nm, respectively. A measurement of the ratio of absorbance at 260 and 280 nm would give the relative amounts of nucleic acid and protein in a sample. Difference spectra can give the effects of solvent changes, helix-coil transitions, protein-protein association and dissociation, the number of hydrogen bonds, and the number of chromophoric groups on the surface or buried in a protein or other macromolecule.