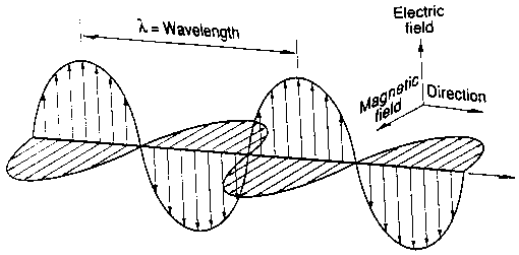
Absorption Spectrophotomety

Spectrophotometry is a versatile analytical tool. The underlying principle of spectrophotometry is to shine light on a sample and to analyze how the sample affects the light. Advantages of spectrophotometry are: 1) no destruction the sample, 2) it is selective (can be detected the sample without separation from solvent), 3) it has a short time interval of measurement. Spectrophotometric is a method which used for determining such important parameters for biomacromolecules (proteins, nuclear acids, et.al) as are: structure, composition and its environment.

Spectrophotometry Theory

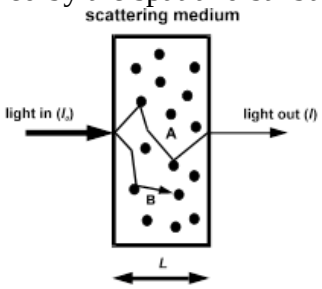
Light can be described as a wave. This wave has an electric component and a magnetic component which are perpendicular to each other (Figure).



Electromagnetic radiation exhibits a direction of propagation and wave-like properties (i.e., oscillations). The energy of electromagnetic radiation is defined as:

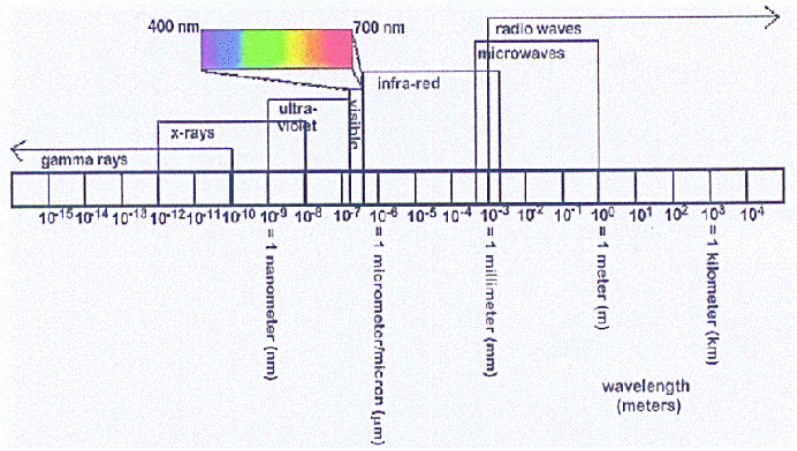
$$\mathbf{E = hc/\lambda = h\nu}$$

where **E** - energy, **h** - Planck's constant, **c** - the speed of light, **λ** - the wave length, and ν- frequency. Light behaves both as a wave and as a particle. The conceptual particle of light is called a photon and is represented by **hν**. Electromagnetic radiation exhibits a wide spectrum and specific ranges of wavelengths have names (Figure). The energy of electromagnetic radiation is inversely proportional to its wavelength. When a light wave encounters a particle, or molecule, it can be scattered (i.e., direction change d), absorbed (energy transferred), or unaffected. Molecules only absorb discreet packets of energy, or quanta, and absorption occurs when the energy of the photon corresponds to differences between energy levels in that particular molecule. These discrete energy levels, called electronic energy levels, are a property of the particular molecule and are determined by the spatial distribution of the electrons.



Absorption of the energy from the photon elevates the molecule to an excited electronic state (see picture) by causing an electron to move from one orbit to another. These electronic energy levels are further subdivided in to vibrational levels. The vibrational levels correspond to stretching and bending of various covalent bonds. The transitions to the excited 20 state can occur between different vibrational levels giving a range of energy that can be absorbed by the

molecule. A molecule or substance that absorbs light is called a chromophore. Chromophores exhibit unique absorption spectra (Figure) and can be defined by a wavelength of maximum absorption, or λmax, of a broad absorbtion band due to the vibrational levels. The absorption spectra can consists of several absorption maxi ma of various amplitudes. A large number of biological molecules absorb light in the visible and ultraviolet (**UV**) range.



The net affect of absorption is that the intensity of the light decreases as it passes through a solution containing a chromophore. The amount of light absorbed depends on the nature of the chromophore, the concentrate on of the chromophore, the thickness of the sample, and the conditions (eg., pH, solvent,etc.) under which absorption is measured.
Absorption is governed by the Beer-Lambert Law:

$$\mathbf{I = Io10^{-\varepsilon dc}\ or\ log(I/Io)=-\varepsilon dc},$$

where **I** - final light intensity, **Io** - initial light intensity, **ε** - molar extinction coefficient, **d** - thickness, and **c** - molar concentration. Absorption (***A***) will be defined by:

$$\boldsymbol{A = -log(I/Io) = \varepsilon dc}$$

The molar extinction coefficient (***ε***) is defined as the ***A*** of ***1 M*** of pure compound under standard conditions and reflects something about the nature of the chromaphore. The units of εare liter/cm·mole. However, the extinction coefficient can be expressed in other units. For example, it can be expressed in terms of ***mM*** concentration. The thickness of the sample (d) is almost always ***1 cm*** and therefore can be ignored in calculations. Sometimes, though, the extinction coefficient units is expressed in **cm2/mole** (by converting liters to cubic centimeters) and care should be taken in making calculations. In cases where the molecular weight of the substance is not known, or varies, ***E1%*** is used as the extinction coefficient. ***E1***% is defined as the ***A*** of a 1% (**w/v**) solution. It is important to precisely record the units of **ε**, when looking it up or determining it experimentally since these units will determine the concentration. It is also important to record the conditions (eg., **pH**, solvent, temperature, etc.) for an extinction coefficient (see below).

Instrumentation

Spectrophotometers produce monochromatic light and then accurately measure the light intensity. The major components of a spectrophotometer are the light source, a monochromator, sample holder, a light detector (phototube), and a meter. In most instruments a tungsten lamp is

used for the visible range and either high pressure $\mathbf{H_2}$ or $\mathbf{D_2}$ lamps are used for ***UV*** range. Monochromatic light is generated by either 1) a movable prism, 2) a diffraction gradient, or 3) filters. Monochromatic light is projected through the sample and then measured by a photomultiplier tube. A photomultiplier tube converts the energy of the light photons into electrons (see picture). The voltage resulting fromthese electrons is measured by a meter and converted to an absorbance value. The Io (initial intensity) is determined by calibrating the instrument with a 'buffer blank'. The relative difference in the light intensity between the blank and the sample is then expressed as the absorbance (***A***). Spectrophotometers often include accessories such as chart recorders or microprocessors for data analysis.