Course : PG Pathshala-Biophysics

Paper 10 : TECHNIQUES USED IN MOLECULAR BIOPHYSICS II (Based on Spectroscopy)

Module 06 : *UV-Visible Spectroscopy: Beer-Lambert Law, instrumentation*

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Introduction

Atoms and molecules undergo electronic transitions in visible and ultraviolet region. This makes measurement of absorption or reflectance in this region an important tool for understanding electronic structures of molecules and their interactions. The technique has number of applications in the area of: biophysics, biochemistry, medicinal chemistry, molecular biology, structural biology, drug designing, protein engineering, agriculture, forensic sciences, industry etc. UV-Vis spectroscopy refers to measurement of absorption or reflectance in ultra violet, visible and near infra red (IR) region, as a function of wave-length λ of absorbed/reflected electromagnetic radiation (EMR). Absorption in this region directly affects colours of chemicals and is governed by Beer-Lambert Law. Every biochemical, medical and structural biology laboratory is usually equipped with UV-Vis spectroscopy and special equipments based on it. The design and exact set up may vary and depend on the ultimate objectives, resources and man power available.

Objectives

The objective of the present module is to:

a) Describe basic principle of UV-Vis –spectroscopy,
b) Discuss Beer-Lambert law,
c) Explain deviation from Beer-Lambert law,
d) Give theory of UV-Vis-and near IR spectroscopy,
e) Discuss basic features of UV-Vis spectrophotometer,
f) Discuss problems associated with UV-Vis measurements,
g) Describe special instruments based on UV-Vis absorption.

6.1 Basic principle of UV-Vis- absorption and reflection spectroscopy

All spectroscopic techniques are based on absorption or reflection of light. Latter is dependent on the energy levels within the system. A simplified picture is shown in figure 6.1.

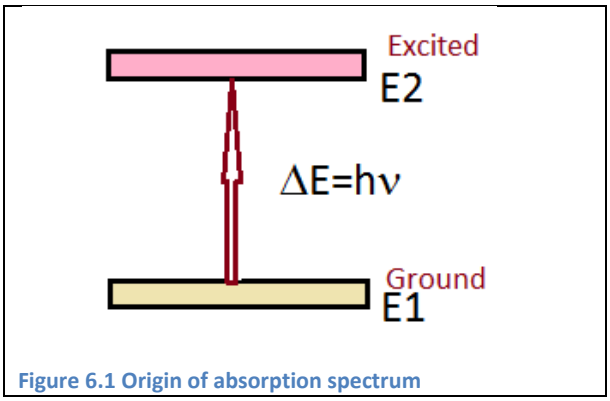

Figure 6.1 Origin of absorption spectrum

Nucleus, electron, atom, and molecules all have discrete energy levels. When there is absorption of a light with energy ΔE equal to the energy gap between the two levels, the energy gets deposited on the electron and it goes to excited state E2, from the ground state E1 with the frequency of the absorbed radiation given by Planck's principle $\Delta E = E2 - E1 = h\nu$.

Although the spectral lines appear to be sharp, these have definite line widths. This is because the energy levels are not as sharp and have characteristics widths (figure 6.1). Typical energy levels for excited and ground state of a system are shown in figure 6.2. Transition between these levels is possible only under certain fixed conditions (selection rules). Energy of absorbed or reflected beam is also not sharp like a Dirac's distribution δ function, but has distribution. A typical line shape is depicted in the figure 6.3. Latter usually has Gaussian or Lorentzian shape and can be defined in terms of position (wave length), multiplicity (number of peaks), absorption (amplitude) and line width (line shape). Line widths are important and can be related to detailed structure and dynamics of the system.

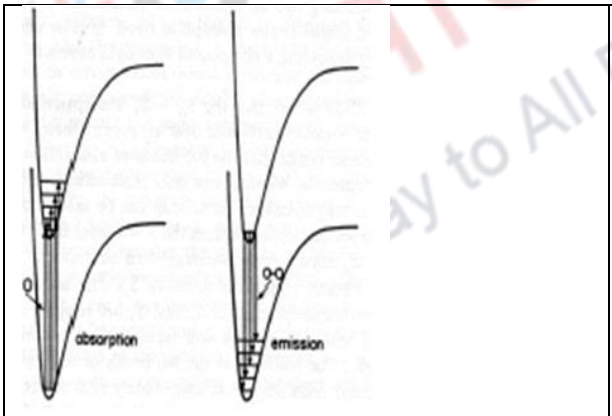

Figure 6.2 Energy levels leading to absorption or emission of light.

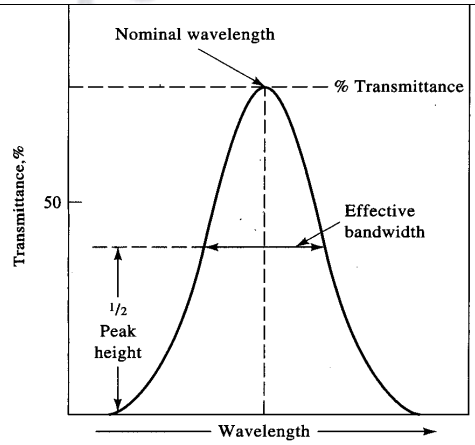

Figure 6.3 Spectral line shape

At any wave length, the area under the curve which can be equated to peak height under special circumstances, is related to number of absorbing centers as well as absorbing power of individual centers ε (extinction coefficient). A typical read out from spectrophotometer is shown in figure 6.4.

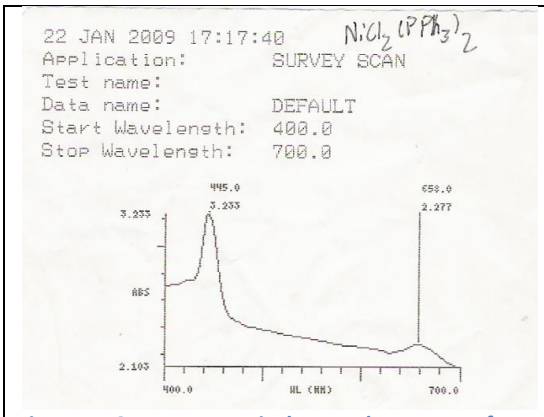

Figure 6.4 A typical read out of spectral measurement. Figure from https://en.wikipedia.org/wiki/Ultraviolet%E2%80%93visible_spectroscopy

The absorption follows Beer Lambert law.

6.2 Beer Lambert Law

6.2.1 The law

The law for absorbance was originally discovered by Pierre Bouguer in 1729 and attributed to Jahanne Heinrich Lamberts. In 1852 August Beer discovered attenuation relation which is later known as Beer-Lambert law. The modern derivation of the Beer–Lambert law combines the two laws and correlates the absorbance to both the concentrations of the attenuating species as well as the thickness of the sample material.

Consider a beam of power I_0 traveling a distance b through an absorbing solution of concentration c. The amplitude of the out coming beam can be expressed as:

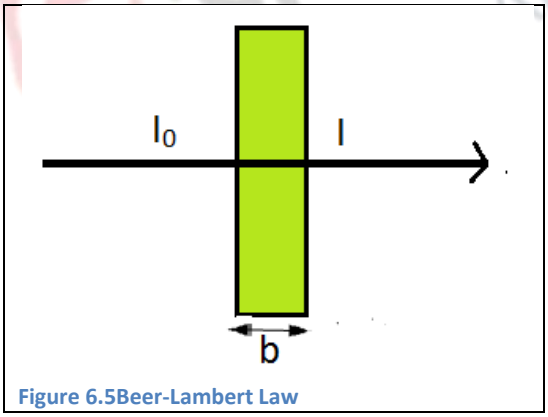

Figure 6.5Beer-Lambert Law

$$I = I_0 \exp(-c\varepsilon L) \tag{6.1}$$

Where L= path length = b, ε-molar extinction coefficient, and c-concentration.

6.2.2 Derivation of Beer Lambert Law

It is more common to calculate optical density (OD). For this we write the number of molecules undergoing transition (*dI*) as proportional to intensity of the beam *I*, given by relation

$$dI = -K.n.dL.I \tag{6.2}$$

Where *K*- is a constant equal to $P.\sigma$, where the *P* is the probability that the molecule is met by radiation, σ-is the scattering cross section for the radiation, *n*-the number density (number of absorbing molecules per unit cross section) and *L*- the path length. Integrating equation (6.2), we get

$$\log I = -Pn\sigma L \tag{6.3}$$

Or

$$I = I_0 \exp(-\varepsilon cL) \tag{6.4}$$

in the same form as equation (6.1)

Where $\varepsilon = P\sigma$ and n- is expressed in terms of concentration *c*.

Optical density OD is defined as:

$$OD = \log_{10}(I_0/I) \tag{6.5}$$

Substituting transmittance $T = (I/I_0).100$ (6.6)

$$OD = 2 - \log(T) = -\varepsilon CL \tag{6.7}$$

For monochromatic radiation, absorbance *A* is directly proportional to the path length *b=L* through the medium and the concentration *c* of the absorbing species.

$$A = abc \tag{6.8}$$

If c= 1 mole/liter, b=1 cm , A=a= ε (6.9)

The constant *a* is a proportionality constant called the *absorptivity coefficient*. It is also sometimes called the *extinction coefficient* and is characteristics of the sample.

NOTE: The units of *a* must be such that *A* is unit less**.**

When concentration is expressed in moles per liter and the cell length in centimeters, the absorptivity is called the *molar absorptivity* or molar extinction coefficient and is given a new symbol ε.

The absorptivity has lots of factors built in and is wavelength dependent. It depends on the absorption efficiency of the sample, scattering losses as the light passes through the sample, reflection losses as the light strikes the cell interfaces etc.

6.2.3 Example of Beer Lambert law

A typical example of Beer-Lambert's law is given below. We show in table 6.1 absorbance of a sample with four different concentrations. One can plot this data in Excel work sheet and find out the concentration of a sample with absorbance 1.52 (figure 6.6). Most instruments are in built with recorders and a computer monitor.

Figure 6.6 Plot of intensity vs wave length

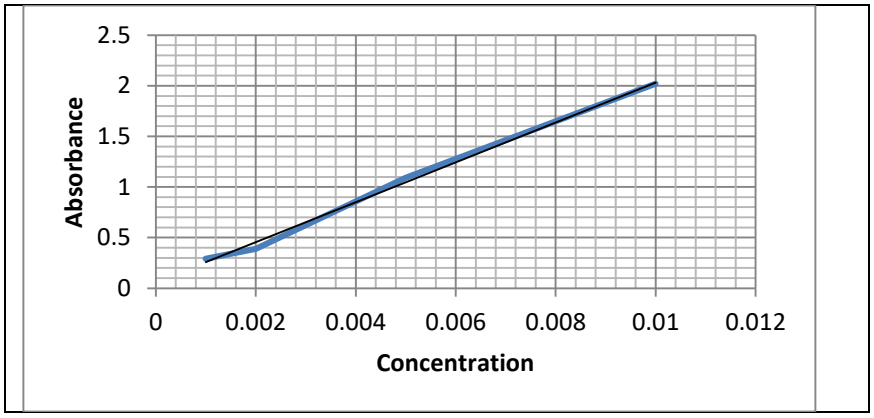

Table 6.1

Concentration (Mole/L)	Absorbance
.001	0.21
.002	.39
.005	1.01
.01	2.02
	1.52

6.2.4 Limitations of Beer Lambert law

Under certain conditions Beer Lambert law fails to give linear relationship between the concentration of analyte and attenuation. These can be i) real: fundamental deviation in the law itself, ii) chemical or iii) instrumental due to way in which attenuation is measured.

There are six basic conditions which have to be fulfilled in order that Beer Lambert Law is valid. These are;

1. The attenuators must act independently of each other,
2. The attenuating medium must be homogeneous in the interaction volume,
3. The attenuating medium must not scatter the radiation—no turbidity unless this is accounted for,
4. The incident radiation must consist of parallel rays, each traversing the same length in the absorbing medium,
5. The incident radiation should preferably be monochromatic or have at least a width that is narrower than that of the attenuating transition. Otherwise a spectrometer as a detector for wave power is needed instead of a photodiode which has not selective wavelength dependence.
6. The incident flux must not influence the atoms or molecules; it should only act as a non-invasive probe of the species under study. In particular, this implies that the light should not cause optical saturation or optical pumping, since such effects will deplete the lower level and possibly give rise to stimulated emission.

Thus Beer's law is valid only at low concentrations. At high concentration the average distance between the absorbing molecules becomes small. The charge distribution is affected by the close proximity of the neighbors, altering the ability of the molecules to absorb a given wavelength. This phenomena causes deviations from the linear relationship between absorbance and concentration.

When the analyte dissociates, associates, or reacts with a solvent to produce a product having a different absorption spectrum from the analyte eg

$$HIn_{color\ 1} = H^{+} + In^{-}_{color\ 2} \qquad (6.10)$$

The reaction causes the color to be pH dependent (indicators for instance). We must buffer our solution to a constant pH to eliminate pH related chemical deviations.

Beer's law is followed only with truly monochromatic radiation. Radiation that is restricted to a single wavelength is seldom practical. If the radiation comes from a line source, then the output will not be adjustable. If the radiation comes from a continuum source and passed through a device to isolate only certain portions, the output is a symmetric band of wavelengths around the desired one. Using a polychromatic beam typically causes non-appreciable deviations from Beer's law provided the radiation does not encompass a spectral region in which the absorber exhibits large changes in absorption as a function of wavelength. The non absorbed stray radiation will increase the power striking the detector. The absorbance will be affected through the relationship. Table 6.2 lists wave length ranges for Biological useful spectroscopy.

Table 6.2 Biological useful spectroscopy range

Wave length λ cm	EKcal/mole	Region
10^{-11}	$3x10^{8}$	γ(gamma)-ray
10^{-8}	$3x10^{5}$	X-ray
10^{-5}	$3x10^{2}$	Vacuum UV
$3x10^{-5}$	10^{2}	Near UV
$6x10^{-5}$	$5x10^{1}$	Visible
$10x10^{-3}$	$3x10^{0}$	IR
$10x10^{-1}$	$3x10^{-2}$	Micro waves
10	$3x10^{-4}$	Radio waves

6.3 Theory of UV-Vis spectroscopy

Theory of absorption or emission of radiation has direct link with the electronic energy levels and structure of atoms and molecules. This is quite important for understanding of atomic and molecular structures. However, the major use of UV-Vis spectroscopy is for quantitative determination of components in a chemical/biochemical reaction. One can follow these reactions as a function of time, temperature, composition of constituents etc.

6.3.1 Atomic spectroscopy

Atomic spectroscopy is the study of absorption or emission of light by atomic electrons. Since unique elements have unique spectral characteristics both electromagnetic spectra and mass spectra can be used for determination of elemental composition. Mass spectroscopy usually gives better results. However optical spectroscopy is more common because of cost effectiveness and performance adequate for many routine tasks.

Theory of atomic spectroscopy is theory of atomic energy levels which can be understood on the basis of classical mechanics or quantum approach which is already discussed in the module 5. Transition between energy levels is governed by definite selection rules.

6.3.2Molecular Spectroscopy

Molecular spectroscopy is the study of absorption of light by molecules in the gas phase at low pressure. Molecules exhibit absorption in narrow spectral widths which are typical characteristics of the molecules, as well as temperature, pressure and environment. Molecular spectroscopy belongs to three different classes:

Electronic spectroscopy - involving electronic energy levels of the molecule in UV or visible region,

Vibrational spectroscopy - or IR spectroscopy in IR region- This involves vibrational energy levels,

Rotational spectroscopy or Radio frequency spectroscopy- Involves rotational energy levels of the molecule in radio frequency range.

Dealing all the three phenomena simultaneously either theoretically or experimentally is an impossible task. Theory does not permit to involve all the three motions and their inter dependence simultaneously. Experimental conditions such as: light emitting source, sample holders, optics, detectors and sample preparation techniques for such a wide range of frequency differ significantly. We take advantage of the fact that electronic, vibrational and rotational energy levels are well separated from each other so that, each of them can be treated separately assuming that the other two really do not alter during the period of their measurement. We shall focus here our attention on molecular electronic spectroscopy which is in UV-Vis region.

6.3.3 Molecular electronic spectroscopy

The theory of molecular electronic spectroscopy is more complex than atomic electron spectroscopy because of the fact that molecules constitute non-centro-symmetric system. One cannot use the approach used for the study hydrogen like atoms discussed earlier in module 5. One can also not use the extension of the method to other atoms using Hartree-Fock-Slater approximation with self consistent- field (SCF) theory approach. The interpretation of molecular electronic energy levels is based on quantum chemical methods known as molecular orbital (MO) methods. Most of these methods originate from Roothan's equation which is an extension of Hartree-Fock equation for non-centro-symmetric system. It uses wave function in the form of linear combination of atomic orbitals (LCAO). These are called 'basic sets' and can be either 'Slater type orbitals' or simply 'exponential functions' (discussed elsewhere). A wave equation is written under set of approximations and solved by self consistent field (SCF) method. Different MO techniques differ in: choice electrons considered for calculation, choice of basic set of atomic orbitals, choice of two electron integrals, method of computation of two electron and Coulomb integrals which form part of Hamiltonion etc. These methods usually take into consideration only valence electrons. Some methods use selected valence electrons.

The simplest methods are: π (pi) electrons theory by Hückel and σ (sigma) electron method by Del Re. Slightly more complex is Extended Hückel Theory (EHT) of Rudolf Hoffman for all valence electrons. For π electrons we have improvised methods as: PPP (Pariser-Paar-Pople) or VE-PPP (Variabale Electro-negativity Parriser-Paar-Pople) method. There are more complex all valence electrons methods as: (CNDO-II) (Complete Neglect of Differential Overlap), INDO (Intermediate Neglect of Differential Overlap) which neglect differential overlap between the electrons at some stage of calculations and *ab initio* techniques which calculate wave functions using exponential orbitals and do not neglect any integrals. In between these two extreme approaches there are series of

methods as: NDDO, MINDO, MINDO/3, AM1, PM3 and SAM1 which are discussed elsewhere (Kothekar 2004). Readers are recommended to refer to specific references given in quadrant III. Some basic features of MOs are discussed in the next paragraph.

6.3.4 Bonding and antibondig orbitals

Molecular electrons are arranged in various MOs characterized by their composition and specific energy levels. The MO's are composed of atomic orbitals (AO's) characterizing different atomic (valence) electrons in the constituent atoms. These are written as linear combination of atomic orbitals as:

$$\Psi_i = \sum_j C_{ij} \cdot \Phi_j \qquad (6.11)$$

The summation *j* goes over all the atomic orbitals. In case, there is only one atomic orbital eg Φ_1 and Φ_2 per atom one can write equation 6.11 as:

$$\Psi_s = \Phi_i(1)\Phi_j(2) + \Phi_i(2)\Phi_j(1) \text{ symmetric} \qquad (6.12)$$

$$\Psi_a = \Phi_i(1)\Phi_j(2) - \Phi_i(2)\Phi_j(1) \text{ anti-symmetric} \qquad (6.13$$

Ψ_s is called bonding orbital because it has lower energy compare with individual atomic orbitals Φ_1 and Φ_2. Ψ_a Is called anti-bonding orbital as it has energy higher than individual atomic orbitals . We show in figure 6.7 bonding and anti-bonding orbitals constituted from two 1s orbitals.

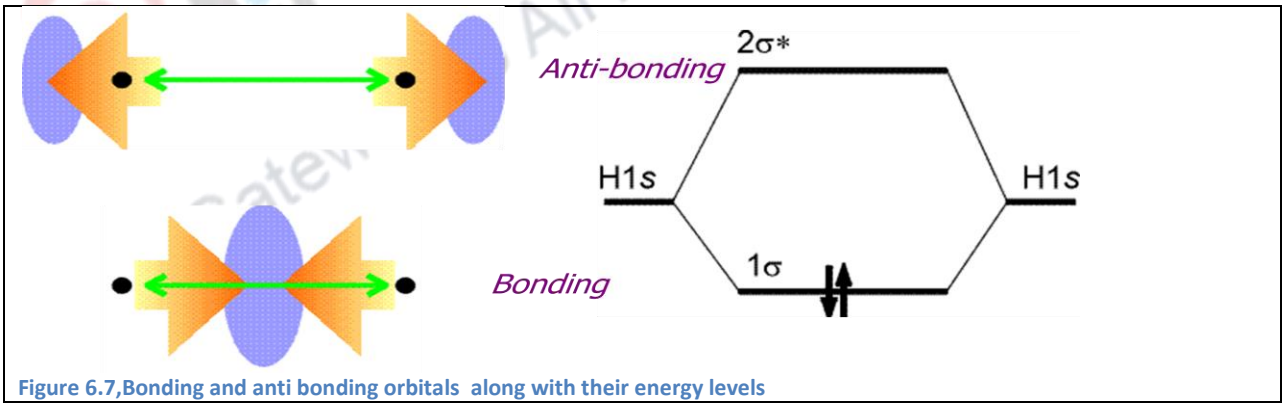

Figure 6.7,Bonding and anti bonding orbitals along with their energy levels

Figure 6.8 shows how 1s orbitals from two atoms overlap each other in the shaded region when these are added together (equation 6.12). This is called constructive interference.

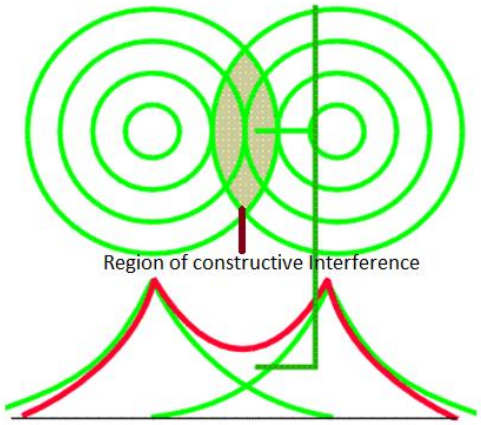

Figure 6.8 Formation of bonding σ MOs from two AOs

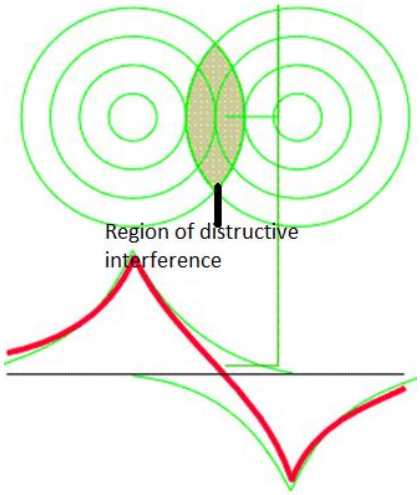

Figure 6.9 Formation of antibonding σ* MO from two AOs

The energy of the MO in this case is lower compared to the sum of energy of two atomic orbital due to exchange interaction between them. These are called bonding orbitals as these hold together two atoms. Reverse is observed (figure 6.9) when two 1s atomic orbitals are subtracted from each other (equation 6.13). These are called anti-bonding orbitals and usually denoted by * as a superscript with them.

Presence of anti-bonding orbitals is extremely important for electronic transitions. Electrons doubly occupy MOs starting from the lowest energy level. These are stable orbital. MOs with higher energy are empty or partially occupied. These are called excited states. Electrons when absorb energy go to excited states depending upon availability of vacancy in the excited state.

The overlap between electrons depends on their shapes (figure 6.10) which are dependent on: principle quantum number (n), orbital/azimuthal quantum number (l) and magnetic quantum numbers (m). When n=1 as in the case of hydrogen and helium atoms *l* can be only 0. It is called 1s orbital which is spherical. When *n*=2, *l*=0 or 1. When *l*=0, *m*=0 and the orbital is spherical. However when *l*=1, *m* can be equal to 0, 1, or -1. These are called *p* orbitals (Px, Py and Pz) with *m*=1,-1 and 0.When *n*=3, *l* can be equal to 0, 1 and 2. When *l*=2 there are five possible orbitals for *m*=0, ±1, ±2.

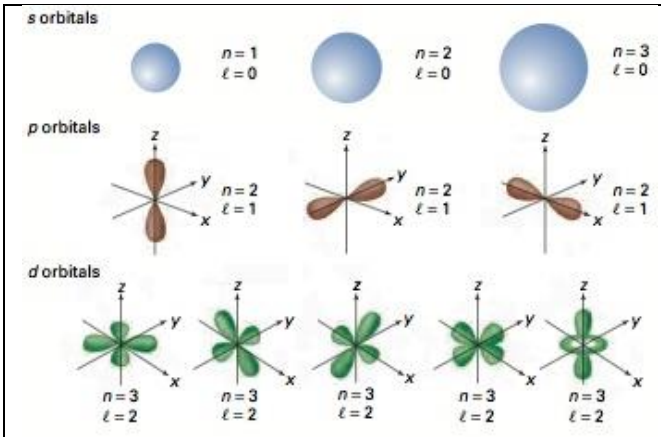

Figure 6.10 Shapes of atomic orbitals. Figure from https://socratic.org/questions/what-are-the-shapes-of-the-atomic-orbitals

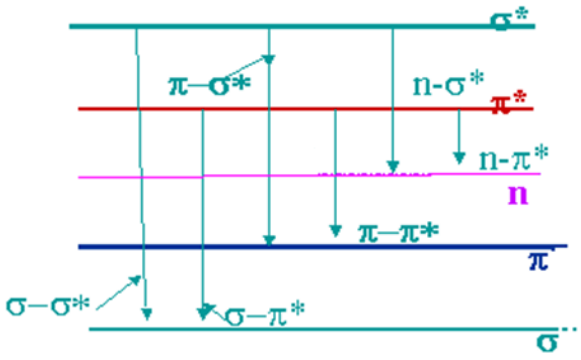

Figure 6.11 Energy level diagram showing positions of bonding and anti-bonding σ,σ*, n, π,π*orbitals

The σ orbitals are in the direction of the bond. These are formed by the combination of atomic orbitals with m=0 (s, Pz and dz2)(Note: the axial system here is attached to the bond such that the direction of the bond is taken as Z axis).. The π orbitals are formed due to overlap in the direction perpendicular to the bond due to combination of atomic orbitals with m= ±1 (Px, Py, dxz, dyz etc). The energy levels for σ, σ*, π and π* are shown in figure 6.11 . We see one additional energy level *n*. This is because all the electrons in the valence shell do not participate in bonding. For example in NH3 two *2s* electrons of nitrogen rotate in the same orbit and are called *lone pairs of* electrons. These are denoted by *n*. Energy wise σ-orbitals have lowest energy, next to it are π orbitals. σ* have highest energy. Next to it are π*. The lone pair *n*- lies in the centre. Transitions amongst these levels take place as per selection rule Δl=0 or ±1. The possible transitions are also depicted in the figure 6.11.

In the presence of chemical interactions spectral lines can change intensity or shifts. These effects are named as: Bathchromacity- Red shift, Hypsochromacity- Blue shift, Hyperchromicity-Increase in absorbance, Hypochromicity– reduction in absorbance.

Wavelength of π -electron transition depends on the size and delocalization of the - electron. Peptide π– π* electron transition is at 190 nm, in DNA it is at 260 nm, in Hemoglobin at 400 nm. In going from non polar to polar solvent n-π* transition shows - Hypsochromacity (blue shift) while π–π* shows Bathochromic effect (red shift). There is a tendency of the electron system to interact via dispersion interaction. Melting of DNA shows hyperchromacity. σ–σ* transition is in far UV range and useful for structural analysis, π–σ* transition occurs >180 nm. The π–π* transition occurs around 260 nm. If the compound does not absorb in 200-800 nm, it means that it does not have a benzoid, aldehyde or keto group. Aminoacid backbone has λ < 230 nm, Side chains >250nm. His = 210, Cys +250, Tyr and Trp =280 and nucleic acids at 260 nm.

6.4 Instrumentation

UV-Vis spectrophotometer typically measures the intensity of light passing though a sample and compares the same with the intensity before it passed through the sample. The ratio is called transmittance and referred as %*T*. $T = (I/I_0)\ x100$.

The UV-VIS spectrophotometer can be configured to measure reflectance (used generally in case of opaque sample such as tiles). It measures the light reflected from the sample and compares it with the intensity of incident beam I_0. The ratio is called reflectance and measured as % R.

A typical setup necessary for spectroscopic measurement is shown in the figure 6.12. Latter consists of three major components: a source of monochromatic light, a sample holder and an analyzer. Usually it is more common to use a light source which has large band-width with a monochomator for selection of a particular wave length. Most common light sources are Tungsten filament (300-2500 nm), a deuterium arc lamp which is a continuous source over 170-350 nm, a Xenon Arc lamp for 200-1200 nm or light emitting diodes in (LED) in the visible region. Inert solids heated to 1500-2000K make common IR sources.

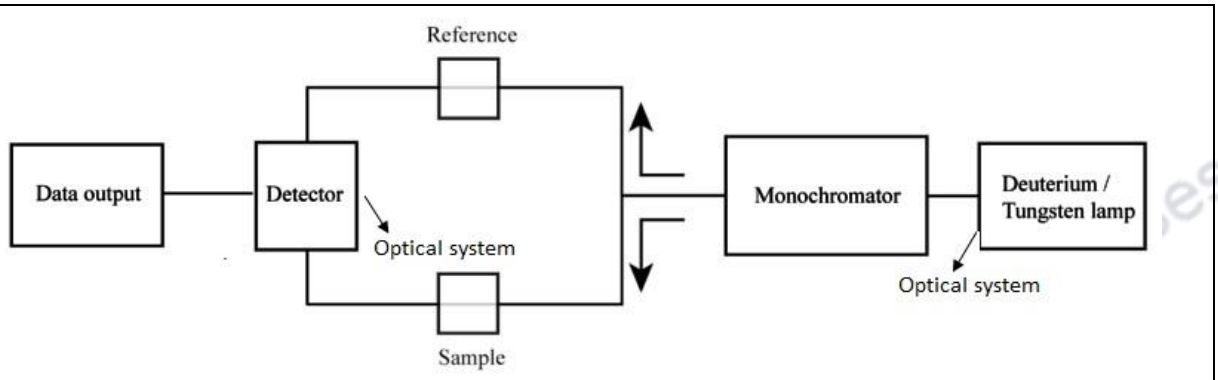

Figure 16.12 Schematic representation of a spectroscope

Line source: Mercury and sodium vapor lamps provide a few sharp lines in the UV and Vis regions.

Laser Sources: Lasers (Light Amplification by Stimulate Emission of Radiation) are highly useful sources in analytical instruments because these have high intensities, narrow bandwidths, and coherent output.

The detector is a photo multiplier tube, a lead sulfide cell (360-3500nm in infra red region), a photo diode, a photo diode array or a charged coupled device (CCD). A single photodiode and photo multiplier tubes are used with scanning monochromators which filters the light such that only a light of a single wave length reaches the detector. The scanning monochromator moves the diffraction grating through each wavelength so that its intensity may be measured as a function of wavelength. Fixed monochromators are used with CCDs and photodiode arrays. As both of these devices consist of many detectors grouped into one or two dimensional arrays, they are able to collect light of different wavelengths on different pixels or groups of pixels simultaneously (Ultra Violet Visible spectroscopy.

Spectrophotometers can use a single beam or a double beam. In a single beam instrument as in Spectronic 20 all light passes through the sample cell and it has to be moved for measuring reference intensity. This is more common in teaching labs. In the double beam instrument light is split into two beams before it passes through the sample. Some double beam spectrophotometers have two detectors and sample and reference beams are measured simultaneously. Others use beam choppers which blocks one beam at a time and detector alternates between the two beams. There may be a dark interval. In this case the measured beam has to be corrected for by measuring the intensity in the dark interval.

The sample is usually a liquid although one can use solid or gaseous sample. Samples are usually placed in a transparent cell called cuvetts. These are rectangular in shape with internal diameter 1cm Smaller cuvetts are available for some specialized experiments. The sample holder often has a chamber to accommodate two cuvetts with one serving as a reference against which the beam passing through the sample is compared. Although one can use pyrex glass cuvetts in the visible region, cuvetts are usually made up of fused silica or quartz glass, because of their transparency to UV light. The quantitative measurements are carried out by detector producing data output.

The sample chamber can be heated and one can do measurements at a particular temperature etc. There are many specialized instruments for special purpose which measure absorbance at a fixed wave length but different temperature as in the case of DNA melting measuring instruments. These also can be used for carrying special interactions within a cell to measure kinetics and rate constants of chemical interactions. One can measure equilibrium constant of a reaction by measuring at definite intervals. A typical example of mercury *dithizonate* is given below.

One can shine light on the sample to turn the solution blue, then run a UV/Vis test every 10 seconds (variable) to see the levels of absorbed and reflected wavelengths change over time in accordance with the solution turning back to yellow from the excited blue energy state. From these measurements, the concentration of the two species can be calculated.

The spectrophotometer can be attached to a telescope to measure spectrum emitted by astronomical objects. Also it is possible to do microspectrophotometry by attaching it to microscope.

6.4.1 Wave length selectors and spectral bandwidth:

Radiation that consists of a limited, narrow, continuous group of wavelengths is required for most spectroscopic analyses. A better wavelength selector will have a narrower effective bandwidth. Latter is measured as the width of the triangle formed by the intensity spike at one half of the intensity. If the width is of the same order as the absorption line, measurements would be erroneous. In the reference sample instrument bandwidth is kept sufficiently small. Reducing the spectral bandwidth reduces the energy passed to the detector and will, therefore, require a longer measurement time to achieve the same signal to noise ratio.

6.4.2 Wave length changer

In liquids, the extinction coefficient usually changes slowly with wavelength. A peak of the absorbance curve (a wavelength where the absorbance reaches a maximum) is where the rate of change in absorbance with wavelength is smallest. Measurements are usually made at a peak to minimize errors produced by errors in wavelength in the instrument, that is errors due to having a different extinction coefficient than assumed.

6.4.3 Stray light error of the monochromator

Purity of the incoming beam is important. The amount of stray light should be kept minimal. The detector used is always a broad band and responds to all wave lengths that reach it. Stray light will reduce the efficiency of the detector. As a rough guide, an instrument with a single monochromator usually has stray light level about 3 Absorbance Units (AU). This makes measurement of absorbance above 2AU problematic.

6.4.4 Use of Polarizers

Polarizers can select radiation in a particular plane. We can measure asymmetry in absorption in parallel and perpendicular beam for helical molecules (DNA or protein α helix) using relation

$$A' = \log \frac{P_0 + P_s}{P + P_s} \qquad (6.14)$$

Figure 6.13 Beckman's UV-Vis spectrophotomter figure from By TimVickers - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=4375121

6.4.5 Optics

Most important in any instrument is optics. Monochromator and analyzers use either a diffraction grating or a prism to diffract the beam. The prism/ grating are usually mounted on a rotating table and their movement is controlled by a motor. The wave lengths are directly calibrated and linked to the movement of motor which is set at the factory. Quality of mechanical movement of parts is important. It is always a good idea to check the calibration of the wave lengths. The material used for optics and sample cuvetts is of prime importance as the transparency level of the material is different. We show in the figure 6.13 a conventional UV-Vis spectrophotometer by Beckman.

Quadrant II

Summary

We have introduced the reader to basic features of UV-Vis spectroscopy. The parameters such as: absorbance, line shape and line multiplicity are discussed. Beer-Lambert's law is given. Its derivation, importance and limitations for quantitative measurement are discussed.

We have given a brief introduction to the theory of molecular spectroscopy on the basis of molecular orbital (MO) method. Overview of different MO methods is given. We have enumerated basic concepts such bonding and anti-bonding orbitals, described shapes of atomic orbitals (s, p and d), gave rules for forming MOs, selection rule for transitions etc. We have explained formation σ, π and δ bonds, energy level scheme for the molecules and allowed transitions.

Basic instrumental set up necessary for spectrophotometric measurement is described. We have also discussed problems associated in quantitative estimations using spectrophotometric technique. Lastly we have discussed spectrophotometers for special purpose.