Name of Unit:	UV Visible Spectroscopy and Fluorimetry
Course/Subject Name:	Instrumental Methods of Analysis
Course/Subject Code:	BP701T
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Learning Outcome of Module-I

LO	Learning Outcome (LO)	Course Outcome Code
LO1	To understand the interaction of matter with electromagnetic radiations and its applications in drug analysis.	BP701.1, BP701.2
LO2	To perform quantitative & qualitative analysis of drugs using UV spectroscopy	BP701.1, BP701.5, BP701.6
LO3	To perform quantitative & qualitative analysis of drugs using Fluorimetery	BP701.1, BP701.5, BP701.6

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Electromagnetic Radiation and Spectroscopy

The most challenging task of a chemist is to determine the chemical structure of an unknown compound. There are many ways by which we can identify the unknown substance. A person can use physical methods such as boiling point, melting point, spectroscopy as well as chemical methods such as functional group testing and others to determine the structure of compounds. Spectroscopy is one of the best methods to identify a substance, which may include UV, IR, NMR, Raman and others. Here we will discuss about the various aspects of different spectroscopic techniques and more specifically about UV-spectroscopy and its uses.

Electromagnetic spectrum covers a wide range of electromagnetic radiations, ranging from γ-rays having wavelength which are the order of a fraction of an angstrom, to radio waves having wavelength in meters or kilometers. The arrangement of all types of radiations in the order of their increasing wavelength or decreasing frequencies is known as electromagnetic (EM) spectrum. The electromagnetic spectrum includes radio and TV waves, microwaves, infrared, visible light, ultraviolet, X-rays, γ-rays, and cosmic rays, as shown in the ***Figure 1***.

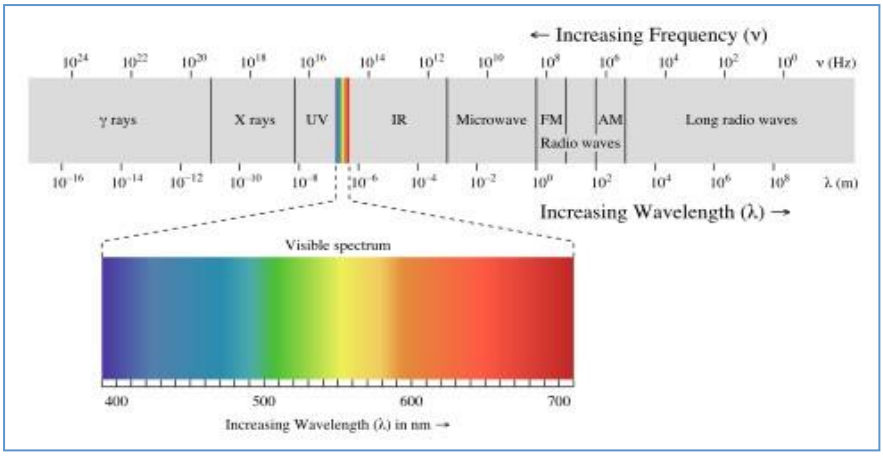


Figure 1: Electromagnetic spectrum

Spectroscopy: Spectroscopy is the study of interaction of electromagnetic radiations with matter. Electromagnetic radiations can interact with matter in various ways. Each interaction gives us insights about certain properties of the matter and use of electromagnetic radiations of different energies can give different information about the matter under study.

It is the motion of electrically charged particles that give rise to electromagnetic radiations. There are various forms of electromagnetic radiation e.g. radio waves, X-rays, gamma rays, infrared, visible, ultraviolet etc. All the types of radiations travel with the same velocity but differ from each other in terms of frequency and wavelength. They do not require any medium

for their propagation and can travel through empty space as well as through air and other substances. Each type of electromagnetic radiation has a dual nature- wave like nature and particle like nature. The particle nature has been established by the fact that the energy of particular radiation occurs in discrete packets or quanta known as photons. Each photon contains a certain amount of energy. The different types of radiation are defined by the amount of energy found in the photons. The energy associated with particular electromagnetic radiation is directly proportional to its frequency. The photons with the highest energy correspond to the shortest wavelengths.

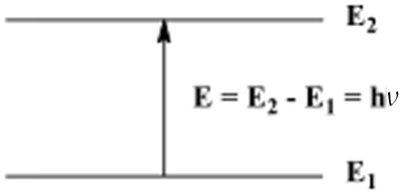
1. Absorption of Electromagnetic Radiations by Organic Molecules

The energy required for these transitions is quantized. Excited molecules are unstable and quickly come back to the ground state by releasing the energy they had received as electromagnetic radiation. The wavelength and intensity of the electromagnetic radiation absorbed or emitted can be recorded with the help of spectrometer to get a spectrum. The energy required for the transition

Types of Radiation	Spectral method	Energy Change involve
Gamma ray	Gamma spectroscopy	Ionisation
X-rays	X-ray Spectroscopy	Inner electrons
Ultraviolet	UV-Spectroscopy	Middle and valence shell electrons
Visible	Visible Spectroscopy	Valence shell electrons
Infrared	IR-Spectroscopy	Molecular Vibration and rotation
Microwave	ESR-Spectroscopy	Molecular rotation and spin orientation
Radio	NMR-Spectroscopy	Spin orientation of Nuclei (NMR)

Table 1: Energy changes depending on the type of Radiation

(E) from a state of lower energy (E_1) to state of higher energy (E_2) is exactly equivalent to theenergy of electromagnetic radiation that causes transition.



$$E_1 - E_2 = E = h\nu = hc/\lambda$$

Where E is energy of electromagnetic radiation being absorbed, h is the universal Planck's constant, 6.624 x 10^{-27} J sec and ν is the frequency of incident light in cycles per second (cps or hertz, Hz), c is velocity of light 2.998 x 10^{10} cm s^{-1} and λ = wavelength (cm)

Higher is the frequency, higher would be the energy and on the other side, longer is the wavelength, lower would be the energy. As we move from Gamma rays to ultraviolet region to infrared region and then radio frequencies, we are gradually moving towards regions of lower energies. However, almost all the parts of electromagnetic spectrum are used for understanding the matter, in organic chemistry we are mainly concerned with energy absorption from only ultraviolet and visible, infrared, microwave and radiofrequency regions.

Ultraviolet–Visible Spectroscopy

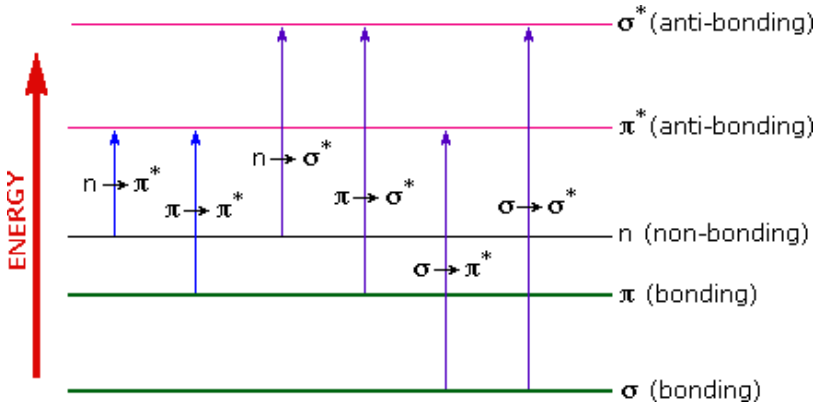
UV-Visible spectroscopy deals with the study of the electronic transitions of molecules as they absorb light in the UV (190-400 nm) and visible regions (400-800 nm) of the electromagnetic spectrum. The absorption of ultraviolet or visible radiation lead to transition among electronic energy levels, hence it is also often called electronic spectroscopy.

As a rule, the energetically favored electron promotion will be from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). The resulting species is said to be in an excited state. The wavelengths at which absorption occurs, together with the degree of absorption at each wavelength is recorded by **optical spectrometer**. A spectrum is obtained as a result. It commonly provides the knowledge about π-electron systems, conjugated systems, aromatic compounds and conjugated non-bonding electron systems etc.

Types of Electronic Transitions

The ground state of an organic molecule contains valence electrons in three principal types of molecular orbitals, namely -sigma (σ) orbitals, pi (π) orbitals and filled but nonbonding orbitals (n). Both σ and π orbitals are formed from the overlap of two atomic or hybrid orbitals. Each of these molecular orbitals therefore has an antibonding σ* or π* orbital associated with it. An orbital containing *n* electrons does not have an antibonding orbital because it is not formed from two orbitals. Sigma bonding orbitals have lower energy than π bonding orbitals, which in turn have lower energy than non-bonding orbitals. In the Electronic transitions, promotion of an

electron from one of the three Fig 2: Electronic Transitions in UV/VIS spectroscopy ground



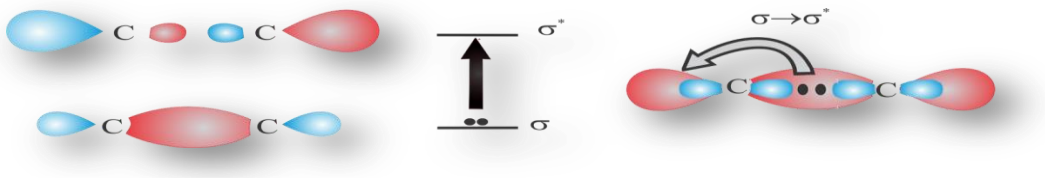
states (σ, π, or n) to one of the two excited states (σ∗, or π∗) takes place. As a result, there are six possible transitions- σ to σ∗, σ to π∗, π to π∗, π to σ∗, n to π∗, n to σ∗.

Fig 2: Electronic Transitions in UV/VIS spectroscopy

The most commonly observed transitions and their relative energies are summarized in **Figure 2**. The exact energy differences between the orbitals depend on the nature of atoms present and the nature of the bonding system. In each possible case, an electron is excited from a low energy, ground state orbital into a higher energy, excited state anti-bonding orbital (**Figure 2**). Each transition requires a defined amount of energy. The larger the gap between the energy levels, the greater the energy required to promote the electron to the higher energy level, resulting in electromagnetic radiation of higher frequency, and therefore shorter wavelength, being absorbed. The important modes of electronic transitions are described here.

1) σ to σ∗

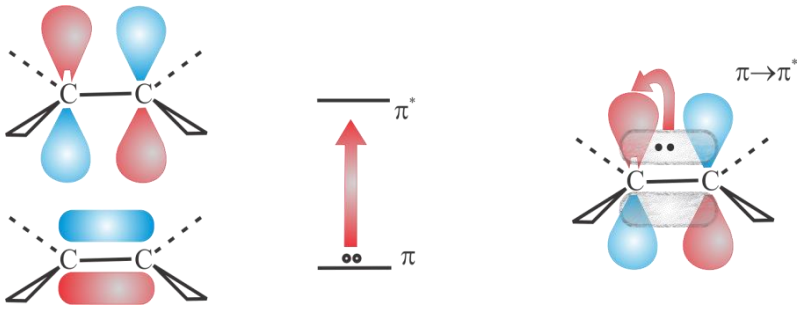
A transition of electrons from a bonding sigma orbital to the antibonding sigma orbital is designated as σ to σ∗ transition. These are high energy transitions as σ bonds are generally very strong. Thus these transitions involve very short wavelength ultraviolet light (< 150 nm) and usually fall outside the range of UV-visible spectrophotometers (200-800 nm). Alkanes can only undergo σ to σ* transitions. Methane and ethane undergo σ → σ^* transitions with an absorbance maximum at 122 and 135 nm, respectively. Study of such transitions is usually done in an



evacuated spectrophotometer (< 200 nm) since oxygen present in air absorbs strongly at 200 nm and below. Similarly nitrogen absorbs at ~150 nm and below.

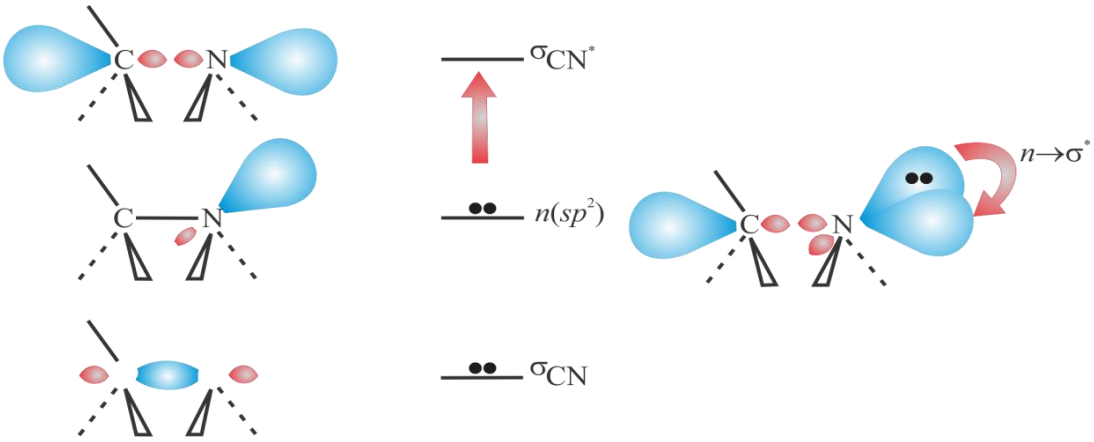
2) π to π∗

The transition of an electron from a π bonding orbital to a π∗ antibonding orbital is designated as π to π∗ transition. These types of transitions take place in compounds containing one or more unsaturated groups like simple alkenes, carbonyl, aromatics, nitriles, nitro etc. These transitions require lesser energy than n to σ∗ transitions. In non-conjugated alkenes, this type of transition occurs in the range 170-190 nm e.g. ethane shows absorption maxima at 171 nm. Similarly, π to π∗ transition in the range of 180-190 nm occurs in non-conjugated carbonyl compounds e.g. acetone shows absorption maxima at 188 nm.



3) n to σ∗

The transition of an electron from a non-bonding orbital to the antibonding sigma orbital is designated as n to σ∗ transition. Saturated compounds containing atoms with lone pairs (non-bonding electrons) like saturated alcohols, amines, halides, ethers etc are capable of showing n to σ∗ transitions.

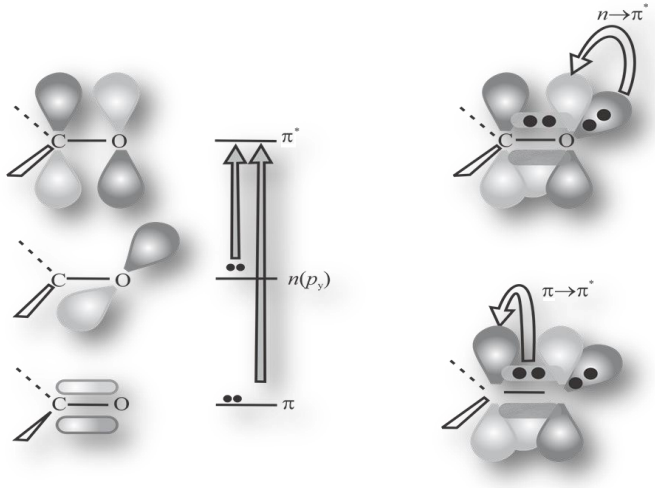


Energy required for these transitions is usually less than σ to σ∗ transitions. Such compounds absorb light having wavelength in the range 150-250 nm. For example the absorption maxima

for water, methyl alcohol, methyl chloride and methyl iodide are 167 nm, 174 nm, 169 nm and 258 nm, respectively.

4) n to π∗

The transition of an electron from a non-bonding orbital to a π∗ antibonding orbital is designated as n to π∗ transition. This transition involves the least amount of energy in comparison to all othertransitions and therefore gives rise to an absorption band at longer wavelength.



Saturated carbonyl compounds show two types of transitions, low energy n to π∗ (270-300 nm) and high energy π to π* (180-190 nm). The transition n to π∗ is of lowest energy but is of low intensity as it is symmetry forbidden. Thus the most intense band for these compounds is always due to π to π* transition.

Selection Rule

Some electronic transitions, which are otherwise theoretically possible, are generally not observed in the UV/VIS spectroscopy. Therefore there are some restrictions which govern the observable transitions.

1. Transitions, which involve change in the spin quantum number of an electron during the transitions do not occur, i.e. singlet-triplet transitions are not allowed.
2. Transitions between orbitals of different symmetry do not occur. For example, transition
3. n to π∗ is forbidden because the symmetry of n and π∗ do not match.

UV-Spectrophotometer

Spectrophotometer is a kind of spectrometer, which measures the transmittance or absorbance of a sample as a function of wavelength, when light of certain intensity and frequency range is passed through the sample. Unlike a spectrometer (which is any instrument that can measure the properties of light over a range of wavelengths), a spectrophotometer measures only the *intensity* of light as a function of its wavelength. The key components of a spectrophotometer are:

- Light source
- Monochromator
- Sample area
- Detector and Recorder

Light Source

The most suitable sources of light are

1. Deuterium lamp which emits the light in the UV-region (160-375 nm)
2. Tungsten lamp or tungsten-halogen lamp which emits radiation in the Visible and nearinfrared regions (350-2500 nm)
3. Xenon arc lamp which emits radiation in the range 190-800 nm
4. Light emitting diodes (LED) which emits radiation in the visible range 400-800 nm.

The instruments automatically swap lamps when scanning between the UV and UV-VIS regions.

Monochromator

The main function of the monochromator is to disperse the beam of light obtained from the primary source, into its component (**Figure 3**). The principle components of monochromator are

- An entrance slit
- A collimating lens
- A dispersing device
- A focusing lens
- An exit slit

The radiation emitted from the primary source (polychromatic radiation) enters the monochromator through the entrance slit. The beam is collimated and then strikes the dispersing

element (Prism or grit) at a particular angle. Two types of dispersion devices viz. prisms and holographic gratings (**Figure 4**) are commonly used in UV-visible spectrophotometers.

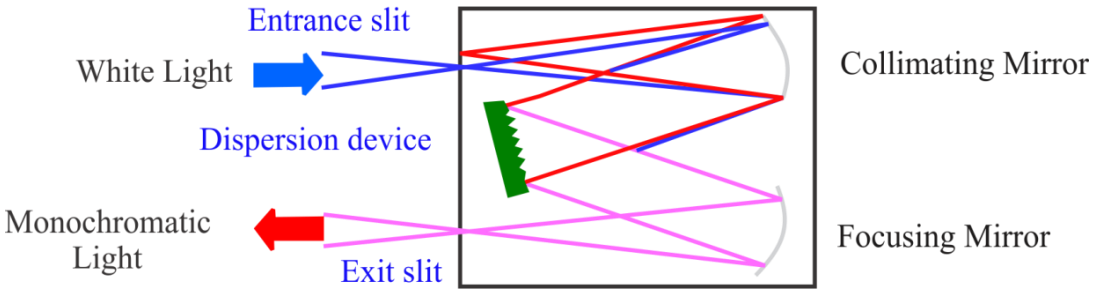


Figure 3: Functioning of Monochromator

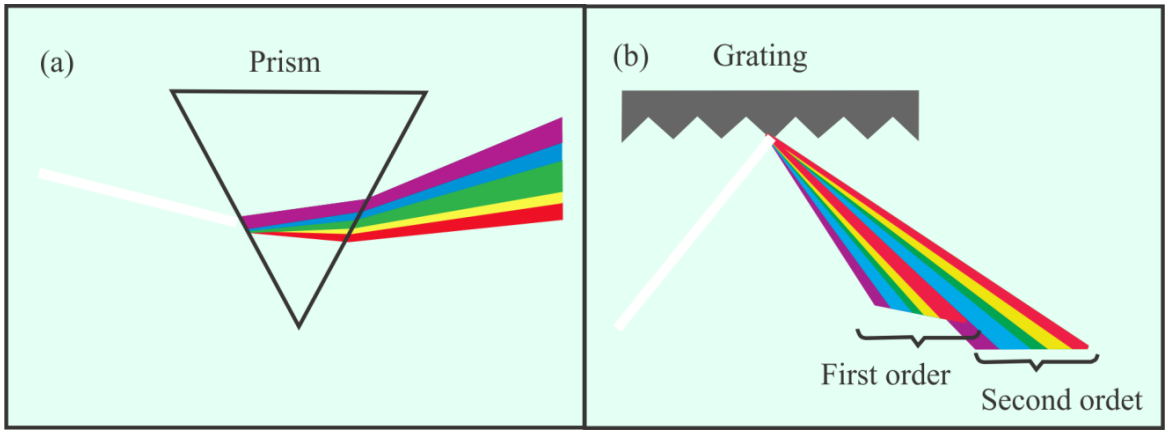


Figure 4: Dispersion of white light through prism and grating

Light falling on the prism is reflected at different angles, depending on the wavelength. The beam is split into its component colors. By moving the dispersing element or the exit slit, radiation of only a particular wavelength can be obtained, which leaves from the exit slit and can be used for the recording purpose (**Figure 3**). The beam selected by the slit is monochromatic and further divided into two beams with the help of another prism, which then passes through the sample andreference solutions.

Sample area

One of the two divided beams is passed through the sample solution and the other beam is passed through the reference solution. Although, the samples for recording spectra are most commonly liquids, but the absorbance of gases and even of solids can be measured. Both sample and reference solutions are placed in a transparent cell, known as cuvette. The cuvettes are rectangularin shape, and usually have an internal width of 1 cm (**Figure 5**).

Figure 5: A quartz cuvette

It is important that the material of cells must be transparent to the radiation throughout the region under study. The cells are usually made of glass, plastic as well as silica or quartz. Of these, glass cells cannot be used for the UV region as they absorb light in the UV region but can be used satisfactorily in the visible region. Quartz is transparent in all (200-700 nm) ranges and is the best choice and hence can be easily used in UV as well as visible region.

Detector and Recorder

A detector converts a light signal into an electrical signal. After the beams are passed through the sample under study as well as the reference cell, the intensities of the respective transmitted beams are then compared over the whole wavelength range of the instrument. Generally two photocells are used as detector in UV spectrometer to record the spectra. One of the photocell receives the beam from sample cell and second detector receives the beam from the reference. The intensity of the radiation from the reference cell is stronger than the beam of sample cell. This results in the generation of pulsating or alternating currents in the photocells.

Spectrophotometers consist of either a photomultiplier tube detector or a photodiode detector.

The commonly used detector in UV-Vis spectroscopy is **photomultiplier tube (Figure 6)**. It is mconsists of three components:

- a cathode which emits electrons when struck by photons of radiation known as **photoemissive cathode**
- several **dynodes** which emit several electrons for each electron striking them
- an **anode**

In its functioning; when a photon of radiation strikes the cathode, emission of several electrons

take place. These emitted electrons are then accelerated towards the many dynodes. The first dynode is 90V more positive than the cathode. The electrons strike the first dynode, causing the emission of several electrons for each incident electron. These electrons are then accelerated towards the second dynode, to produce more electrons which are accelerated towards dynode three and so on. Finally all the electrons are collected at the anode. Several dynodes are arranged between the anode and the cathode to produce an amplification effect. Each photon usually produces 10^6-10^7 electrons, resulting in the amplified current that can be measured.

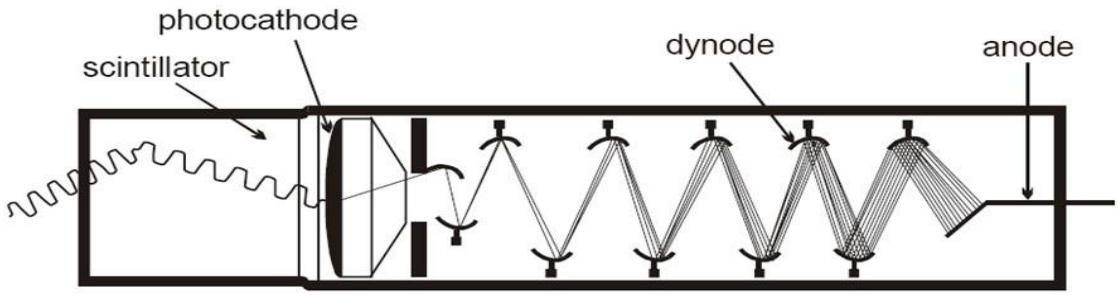


Figure 6: A photomultiplier tube

Photomultipliers are very sensitive to both UV and visible radiations and have fast response times. It is significant to note that Intense light may damage photomultipliers, hence they are limited to measuring low power radiation.

Photodiodes are increasingly being used as detectors in modern spectrophotometers. Photodiode detectors have a wider dynamic range and are more robust than photomultiplier tube detectors. In a photodiode, light falling on the semiconductor material allows electrons to flow through it, thereby depleting the charge in a capacitor connected across the material. The amount of charge that is required to recharge the capacitor at regular intervals is proportional to the intensity of the light. The limits of detection are approximately 170–1100 nm for silicon-based detectors.

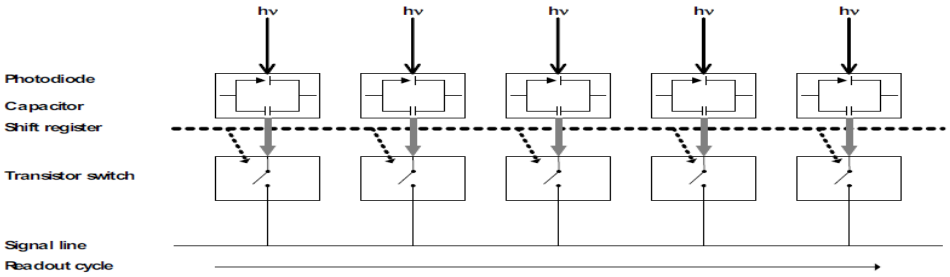


Figure 7: An array of photodiodes

Some modern spectrophotometers contain an array of photodiode detectors instead of a single detector (Figure 7). A diode array consists of a series of photodiode detectors positioned side by side on a silicon chip. Each photodiode is connected to a transistor switch via a charged capacitor When photons strike the diode, free electrical charge carriers are generated that discharge the capacitors. The capacitors are recharged at regular intervals. The amount of charge needed to recharge the capacitors is proportional to the number of photons detected by each diode, which in turn is proportional to the light intensity. The absorption spectrum is obtained by measuring the variation in light intensity over the entire wavelength range.

Types of UV/VIS Spectrophotometer

There are two types of spectrophotometers, namely, single beam spectrophotometer or double beam spectrophotometer (Figure 8). Each has its advantages and disadvantages.

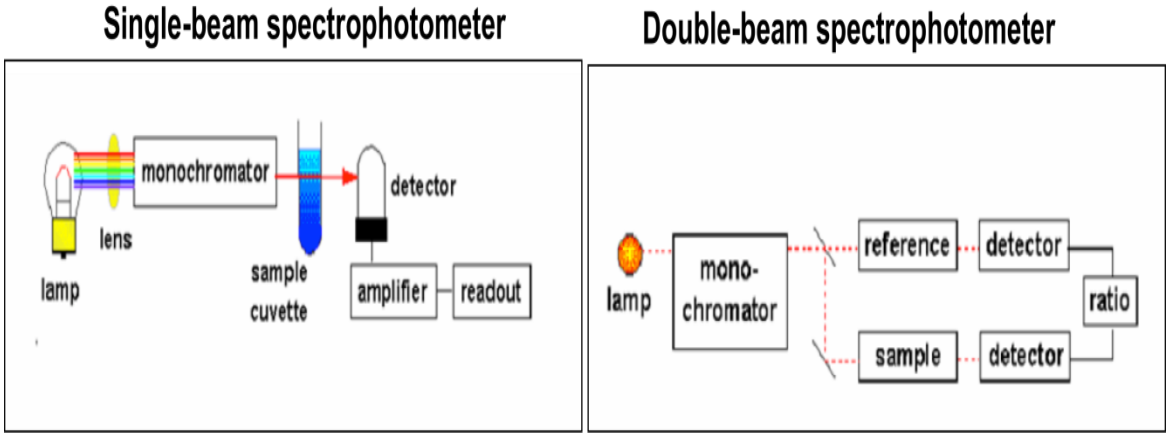


Figure 8: Types of Spectrophotometer

A single beam spectrophotometer has only one beam of light, which passes through the sample, therefore it requires taking reading for the reference and sampling separately. On the other hand, in a double-beam instrument, the light is split into two beams before it reaches the sample. The two beams move simultaneously, one passing through a reference solution and the other through the sample. The reference beam intensity is taken as 100% Transmission (or 0% Absorbance), and the measurement displayed is the ratio of the two beam intensities.

Of the two types of spectrophotometer, double beam spectrophotometers are faster to operate and in their performance. They provide more reproducible results because they perform an automatic correction for the loss of light intensity as the beam passes through the sample and reference solution.

Sample Preparation

The UV-Vis spectra are usually measured in very dilute solutions. Usually 1 mg of the compound of molecular weight of 100-200 is dissolved in 100 ml of suitable solvent and only a portion is used for recording the spectra. The solvent should not absorb radiation and must be transparent over the desired range of wavelength. The solvents, which do not contain conjugated system, are most suitable for recording the UV-spectra. The solvent should also be inert to the sample. Some commonly used solvents are water, 95% ethanol, methanol and hexane.

Absorption Laws

When the radiation passes through a solution, some amount of light is absorbed. Suppose I_0 is theintensity of the incident radiation and I, the intensity of the transmitted radiation.

The amount of radiation absorbed can be

measured byTransmittance $T = I/I_0$

% Transmittance = $T \times 100$

Absorbance $A = \text{Log}_{10}\ I_0/I =$

$\text{Log}_{10}\ 1/T$

If the entire light passes through a solution without any absorption, then

absorbance is zero.In this case, the percent transmittance is 100%.

If all the light is absorbed, then absorption is infinite and the percent transmittance is zero %.

Beer-Lambert Law: It gives a linear relationship between absorbance and concentration of an absorbing species. *This law states that the absorption is directly proportional to the concentration of absorbing substance and the length of the path of radiation through the sample*. It is independent of the intensity of the incident light and each successive unit layer absorbs an equal fraction of light incident on it.

$$\mathbf{A = \varepsilon cb\ Log_{10}\ I_0/I = \varepsilon cb}$$

Where **A** is the sample's Absorbance value at specific wavelength or frequency

ε is the molar absorptivity or the molar extinction coefficient of the substanceb is the path length of the sample in cm

c is the concentration of the solute in mol L^{-1}.

The value of ε absorptivity coefficient is constant for a particular material at a particular wavelength.

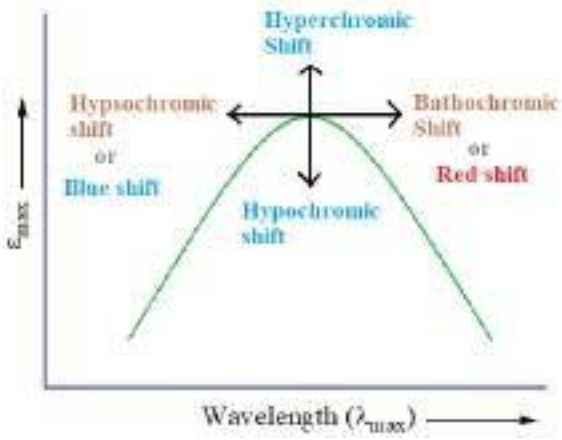
Limitations of the Beer-Lambert Law

Under certain conditions Beer-Lambert law fails to maintain a linear relationship betweenabsorbance and concentration of the solution.

a) Deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostaticinteractions between molecules in close proximity.
b) Scattering of light due to particles present in the sample.
c) Chemical deviations due to the specific chemical species of the sample under investigation.
d) Non-monochromatic radiation
e) Fluorescence or phosphorescence of the sample.

Absorption, intensity shift & UV spectrum

a) Bathochromic shift: It is also known as Red shift, in this case absorptionshift towards longer wavelength (λ_{max}).
b) Hypsochromic shift: It is also known as Blue shift, in this case absorptionshift towards shorter wavelength (λ_{max}).
c) Hyperchromic shift: Intensity of absorption maximum (ε_{max}) increases.
d) Hypochromic shift: Intensity of absorption maximum (ε_{max}) decrease.



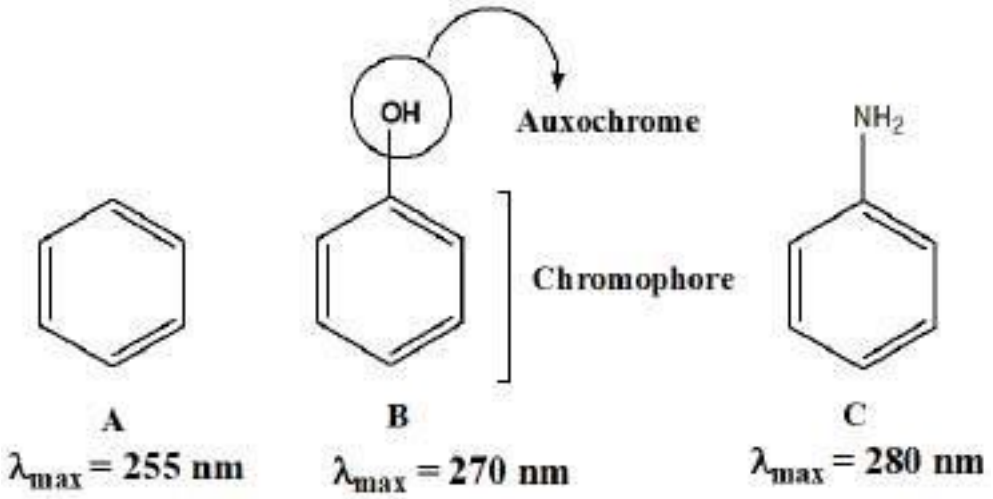
Absorption & intensity shift

Chromophores & Auxochromes

a) Chromophores: Chromophore is a covalently unsaturated group absorbed electromagnetic radiation in UV-visible region and impart color to the compound. For example, C=C,

C≡C, benzene, NO_2 etc.

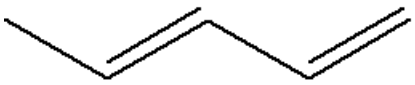
b) Auxochromes: Auxochrome is a saturated group (containing lone pair of electron), when auxochrome attach to the Chromophore absorption shift towards longer wavelength. For example, -OH, -SH, -NH etc.



Factor affecting absorbance & intensity

a) Conjugation

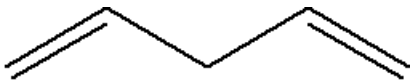
Absorption shift towards longer wavelength, if double bonds (chromophore)present in the molecule are in conjugation. For example,



λmax = 217 nm

conjugated

A



λmax = 175 nm

non-conjugation

B

In compound 'A', double bonds are in conjugation therefore 'A' possessing higher wavelength (λ_{max}) as compare to compound 'B' (non-conjugated derivative).

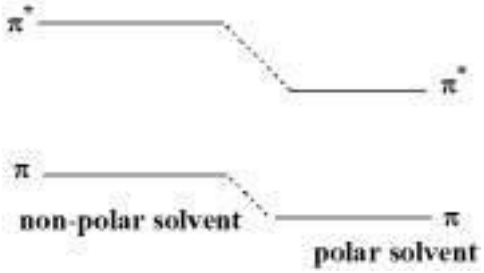
We know that,

$$E = \frac{hc}{\lambda}$$

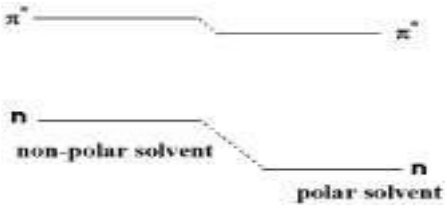
As the conjugation increase transition energy (E) between the orbitals is decrease and therefore wavelength (λ_{max}) increase.

b) Effect of solvent:

In polar solvent, $\pi - \pi^*$ transitions shift towards longer wavelength (Red shift), because dipole interactions with polar solvent molecules lower the energy of excited state (π^*) than that of the ground state (π^* orbital's are stabilized by hydrogen bonding with polar solvent).



Whereas in polar solvent, $n - \pi^*$ transitions shift towards lower wavelength (Blue shift), because dipole interactions with polar solvent molecules lower the energy of ground state (n) than that of the excited state (n orbital's are stabilized by hydrogen bonding with polar solvent).

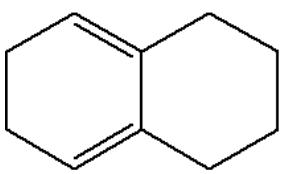


The Woodward-Fieser rules (Calculation of λ_{max} (nm) in conjugated dienes)

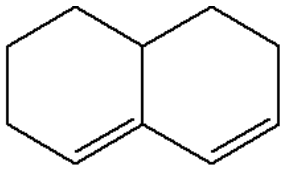
	λ_{max} (nm)
Base value:	
Acyclic or heteroannular	214
dienesHomoannular dienes	

Double bond extending conjugation	30
R alkyl substituent or ring residue	5
Exocyclic double bond	5
Polar groupings:	
$-OCOCH_3$	0
-OR	6
-Cl, -Br	5
$-NR_2$	60

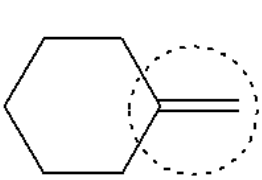
Explanation of terminology used in Woodward-Fieser rules

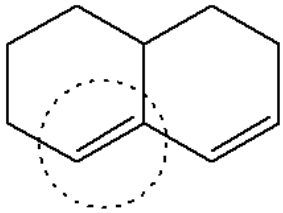


homoannular diene

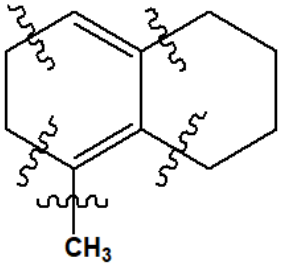


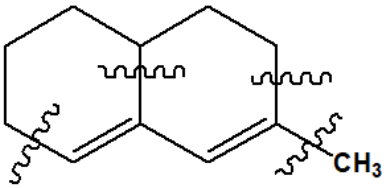
heteroannular diene



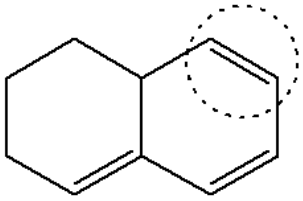


double bond exocyclic to the double bond

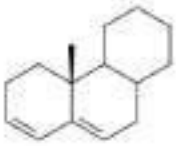




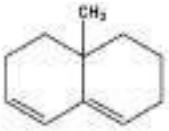
sign represent R alkyl group or ring residue



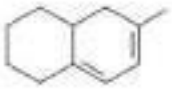
Double bond extending conjugation



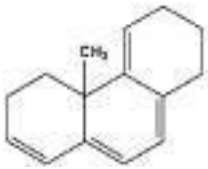
Base value:	214 nm
alkyl groups: 3x5	15 nm
Exocyclic double bond:	5 nm
calculated:	234 nm



Base value	214nm
Ring residue: 3 x 5	15nm
Exocyclic double bond	5nm
Calculated	234 nm



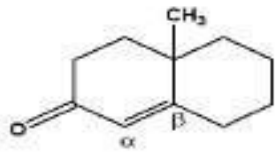
Base value:	253 nm
alkyl groups: 4x5	20 nm
Exocyclic double bond:	5 nm
calculated:	278 nm



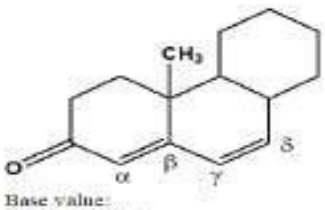
Base value:	253nm
Ring residue: 5 x 5	25nm
Exocyclic double bond: 3 x 5	15nm
double bond extending conjugation: 2 x 30	60nm
Calculated	353 nm

The Woodward-Fieser rules (Calculation of λmax (nm) in α,β-unsaturatedcarbonyl compounds)

	λ_{max} (nm)
Base value:	
Acyclic or 6-membered cyclic ketone	215
5-membered cyclic ketone	202
Aldehyde	39
Increments for:	30
Homocyclic diene component	$\alpha = 10, \beta = 12, \gamma$ & $higher = 18$
Double bond extending conjugationR alkyl substituent or ring residue Exocyclic double bond	5
Polar groupings:	
-OH	$\alpha = 35, \beta = 30, \gamma = 30$
	$\alpha = 35, \beta = 30, \gamma = 17$



Base value:	215 nm
β Ring residue: 2 x 12	24 nm
Exocyclic double bond:	5nm
Calculated	244 nm

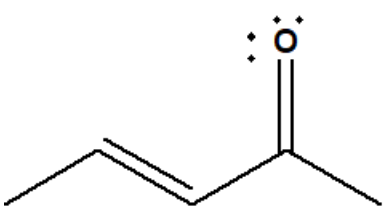


Base value:	215 nm
β Ring residue:	12 nm
δ Ring residue:	18 nm
Exocyclic double bond: 1 x 5	5nm
double bond extending conjugation: 1 x 30	30nm
Calculated	280 nm

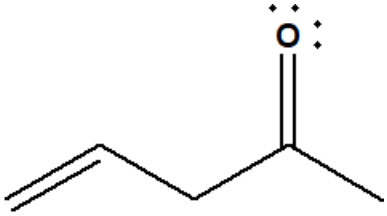
Application of UV spectroscopy

i) Extent of conjugation: As the double bond increase, absorption shift towards longer wavelength.

ii) Distinction between conjugated and non conjugated compounds:



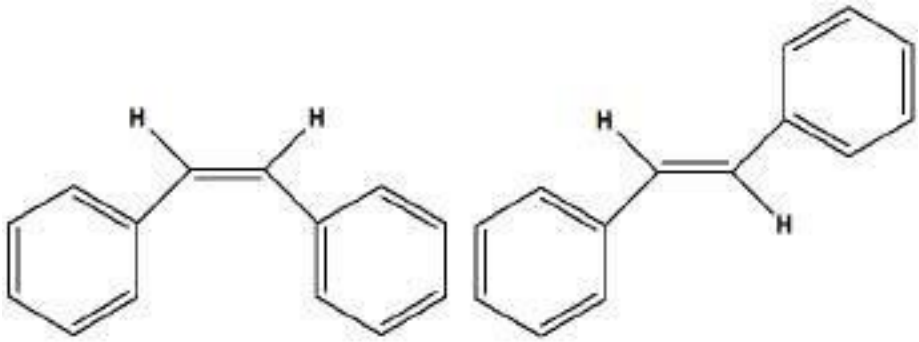
A



B

Both the above compound A & B are isomer of each other. Due to $n - \pi^*$ transition, compound A appear at longer wavelength than compound B.

iii) Study of geometric isomerism:



A (cis form)
$\lambda_{max} = 283$ nm

B (trans form)
$\lambda_{max} = 296$ nm

In isomer A, due to steric hindrance, absorption shift towards lower wavelength. We know that, steric hindrance destabilize the compound means energy of the molecule increase which decreases the wavelength.

iv) Used in the detection of impurities: Due to presence of impurities in the compound, give additional peaks are observed in UV spectrum.

Fluorimetry

Molecular luminescence spectroscopy is an analytical method which studies two types of luminescence processes viz., fluorescence and phosphorescence.

.Luminescence refers to the emission of light by a substance (not resulting from heat which means it is a form of cold body radiation). This means that only the luminescent analyte can be analyzed by molecular luminescence spectroscopy.

Fluorimetry is an analytical method which measures the fluorescent light radiated by the unknown sample and comparison is done with relation to fluorescent light radiated by the known sample

The terms flourimetry and fluorometry are used interchangeably in the chemical literature. Similarly, phosphorimetry is an analytical method which measures the phosphorescence radiated by the unknown sample and comparison is done with relation to phosphorescence radiated by the known sample.

Principle

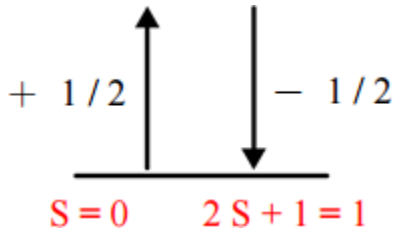
Collectively, fluorescence and phosphorescence are called photoluminescence methods. The photoluminescence methods are the ones which result from absorption of photons. The absorption of photon results in the excitation of molecule which results in luminescence (as when the excited molecules or species revert back to the ground state, they give off light) and this type of luminescence is therefore referred to as "photoluminescence'.

Before learning the principle of photoluminescence phenomena, let us first learn and understand the terminology that we will use for excited states that lead to fluorescence and phosphorescence.

Singlet and Triplet States: When we talk about molecular systems, we refer to movement of electrons on absorption of photons (A photon is a single quantum of electromagnetic energy). And the electrons in molecular orbitals are paired, according to Pauli's exclusion principle (no two electrons in the same quantum state can have all four quantum numbers similar; they differ in their spin – as you all know paired electrons in a molecular orbital have opposite spins). The electrons are initially in their ground state (which is the most stable energy state where the electrons ar paired). When an electron absorbs energy (i.e., when photons strike), it gets excited to a higher energy state keeping its spin orientation intact. These excitations are not stable and the excited electrons have a tendency to revert back to the stable ground state. And this reverse route from excited state to ground state gives rise to the phenomena fluorescence and phosphorescence.

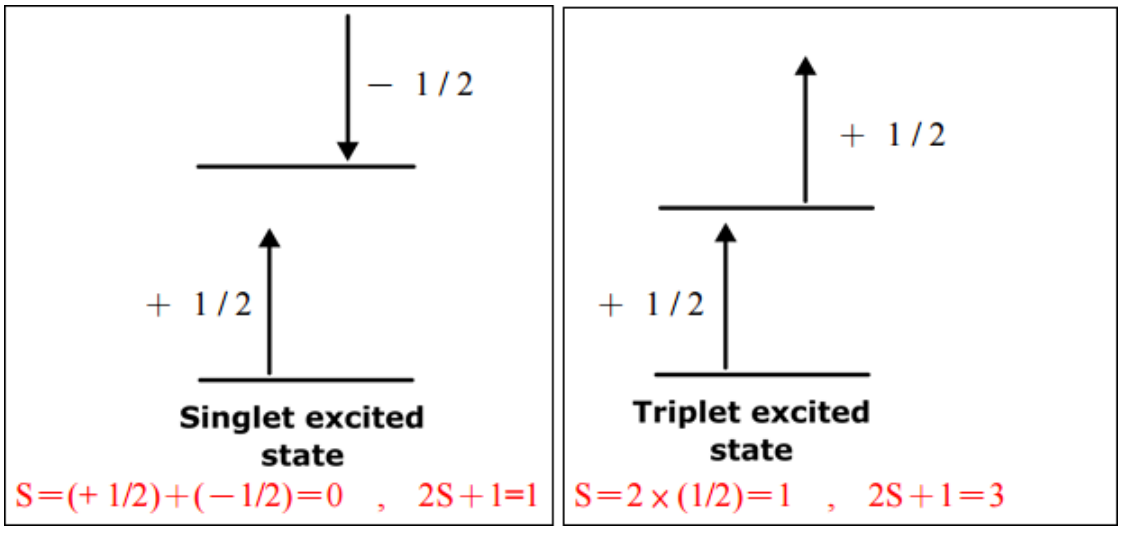
Let us now look into the electronic picture of this process:

Ground electronic state: It is the one where the electrons are paired with opposite spins.



The molecular electronic state in which the electrons are paired is referred to as Singlet State ($Multiplicity$ = $2S$ + 1 = 1; $diamagnetic$). So, the ground state is always spin paired and is a singlet state.

Excited electronic states: (a) If the spins remain paired in the excited state, the molecule is in an **excited singlet state** ($Multiplicity$ = $2S$ + 1 = 1; $diamagnetic$). (b) If the spins become unpaired, the molecule is in an **excited triplet state** ($Multiplicity$ = $2S$ + 1 = 3; $paramagnetic$)**.**



After learning the singlet and triplet terminology, let us now look into the energy level diagram for photoluminescence molecules and understand the processes that take place on absorption of photons. This diagram is referred to as Jablonski Diagram.

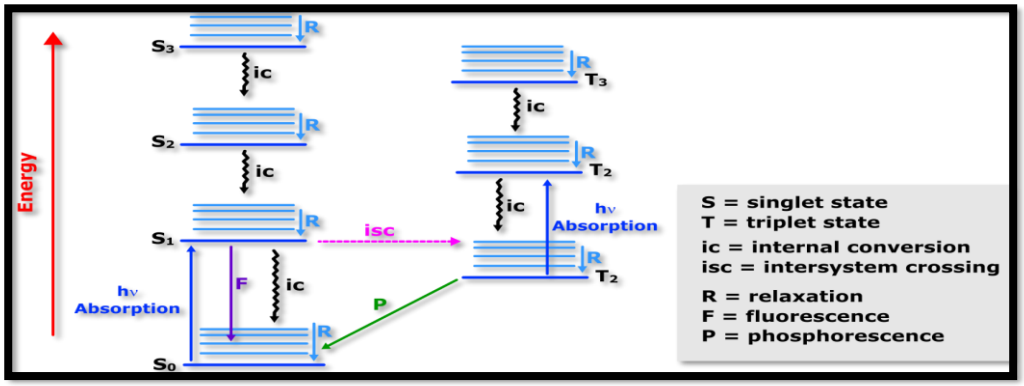


Figure 1: Jablonski diagram representing absorption, fluorescence and phosphorescence

Jablonski Diagram is named after physicist Aleksander Jablonski. It is a diagram used in molecular spectroscopy for illustrating the electronic states in a molecule and the transitions that occur between them. The electronic states are arranged vertically by energy and grouped horizontally by spin multiplicity.

When an electron is transferred from a singlet energy level into a triplet energy level, by a processcalled inter-system crossing, it is accompanied by a flip spin.

Also, the probability of a singlet to triplet transition is much lower han a singlet to singlet transition.Therefore, the intensity of the emission from a triplet state to singlet state is much lower than the emission intensities from a singlet state to a singlet state.

Absorption of UV-Vis radiation is necessary for exciting the molecules from the ground state to one of the excited states. At room temperature, essentially all electrons in a molecule occupy the ground state energy level (S_0). This absorption of light populates number of vibrational energy levels of excited electronic states (S_1, S_2, ……..). After an electron has been excited to higher energy levels, the energy is eventually dissipated to the environment and the electron reverts to the ground state. This energy dissipation can take place in a number of ways and is responsible for fluorescence and phosphorescence. The dissipation of absorbed energy can occur by a number of electronic process which can be categorized as radiation-less transitions and radiative transitions.

Radiation-less transitions (Heat)	Radiative transitions (Photoluminescence)
a. Vibrational relaxation **b.** Internal Conversion **c.** Inter-system crossing **d.** Energy transfer during molecular collisions (External Conversion)	**a.** Fluorescence **b.** Phosphorescence **c.** Delayed fluorscence

Both fluorescence and phosphorescence processes are basically nothing but relaxation of an excited state via light emission. Let us learn now more about these processes.

The processes that take place on absorption of photons are explained below in the table which also explains the Jablonski diagram.

Term	Process	Effect
Absorption	Analyte molecule absorbs photons (very fast ~ 10^{-14} – 10^{-15} s). An electronic transition in molecules after absorbing photons from an excitation beam of light in the UV-Visible range. The transition is typically from the lower energy level that is the lowest vibrational level of the ground singlet state (S_0). The electronic transition is from the low to high energy level, i.e. excited singlet state (S_1, S_2, …..). Slightly different wavelengths excite to different vibrational energy levels.	Excitation
Vibrational Relaxation	Collisions of excited state analyte molecules with other molecules □ loss of excess vibrational energy and relaxation to lower vibrational levels (within the excited electronic state).	Deactivate, Radiationless
Internal conversion	Molecule passes to a lower energy state – vibrational energy levels of the two electronic states overlap and molecules passes from one electronic state to the other.	Deactivate, Radiationless

Fluorescence	Emission of a photon via a singlet to singlet transition (short – lived excited state ~10^{-7} – 10^{-9} s).	Deactivate, Emission of h□
Intersystem Crossing	Spin of electron is reversed leading to change from singlet to triplet state. Occurs more readily if vibrational levels of the two states overlap. Common in molecules with heavy atoms (e.g., I or Br). In inter-system crossing, the molecules transit from the forbidden transition state to an overlapping triplet state. In	Deactivate, Radiationless
	the triplet state, the molecules may go through vibrational relaxation from the excited state to the lowest vibrational level of T1 or may return to S0 by intersystem crossing	
External Conversion	Collisions of excited state analyte molecules with other molecules □ molecule relaxes to the ground state without emission of a photon.	Deactivate, Radiationless
Phosphoresc ence	Emission of a photon via a triplet to single transition (long– lived excited state ~ 10-4 – 101s)	Deactivate, Emission of h□

So now with this understanding of Jablonski diagram, we can talk about fluorescence and phosphorescence.

Fluorescence: This photoluminescence phenomenon is observed when an electron relaxes from its first singlet electronic excited state to its singlet electronic ground state. The return to ground

state produces a photon emission of longer wavelength. Fluorescence is observed in simple as wellas in complex gaseous, liquid, and solid chemical systems. It is of two types:

Resonance fluorescence is the one where the absorbed radiation is remitted without a change in frequency (emitted □ = excitation □).

Molecular fluorescence is the one where the bands are found centered at wavelengths that arelonger than the resonating wavelengths. This shift toward longer wavelengths, or lower energies,is termed the *Stoke's shif*

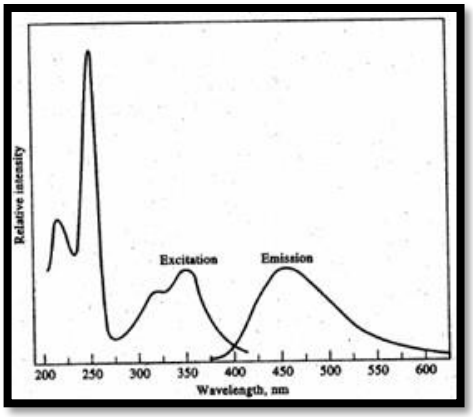
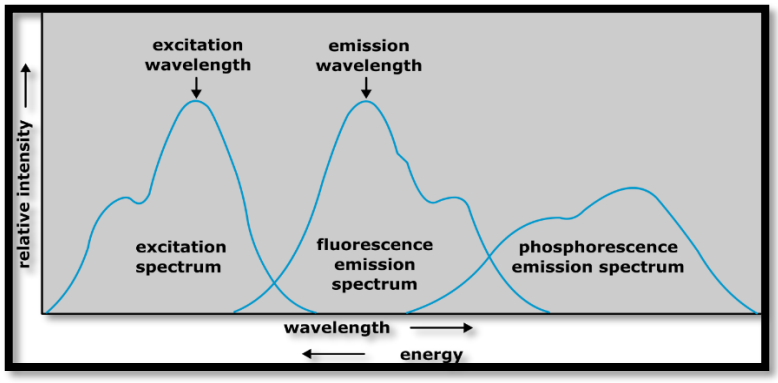


Figure 2: Fluorescence excitation and emission spectra for a solution of quinine

Phosphorescence: This photoluminescence phenomenon is observed when an electron relaxes from its first triplet electronic excited state to its singlet electronic ground state. It occurs via conversion of an excited singlet state to an excited triplet state through the process of intersystem crossing (facilitated by presence of non-bonding electrons as well as heavy atoms). The return to ground state produces a photon emission of longer wavelength in comparison with fluorescence. Phosphorescence is not



common in solutions at room temperature but it can be easily observed at liquid nitrogen temperature or

in solid state. This kind of emission is ordinarily observed only at low temperatures, in highly viscous media or by molecules that are adsorbed on solid.

Delayed fluorescence: An electron in the excited triplet state can cross back to the singlet excited state and can result in a photon as fluorescence but at a much longer time than prompt fluorescence. This process is referred to as delayed fluorescence and has similar characteristics as prompt fluorescence except for longer lifetime. Actually, the corresponding lifetime is intermediate between fluorescence and phosphorescence.

> The difference between fluorescence and phosphorescence is that phosphorescence occurs at longer wavelength because of the triplet stage as it represents a lower energy level in comparisonto the overlapping excited singlet stage.

Now, before taking up the instrumentation for fluorimeter and phosphorimeter, let us learn about luminescence spectra.

Luminescence spectra

The data obtained from a luminescence spectrophotometer is represented in the form of excitation or emission scan. The excitation scan is a spectral profile of excitation wavelengths which are produced at a fixed emission wavelength whereas the emission scan is a spectral profile of emission wavelengths which are produced by a fixed excitation (i.e. absorbance) wavelength.

The transition from the lowest vibrational level in the electronic ground state to the lowest vibrational level in the first excited electronic state, called the 0 - 0 transition, is common to both the excitation and emission process. Thus, the emission spectrum would be expected to overlap the excitation spectrum at the wavelength corresponding to the 0 - 0 transition and the rest of the emission spectrum to be of lower energy and hence a longer wavelength. In practice the 0 - 0 transition for the excitation and emission spectra of a molecule rarely coincide exactly. This difference is representative of the small loss of energy through the interaction of the absorbing molecules with the surrounding solvent molecules

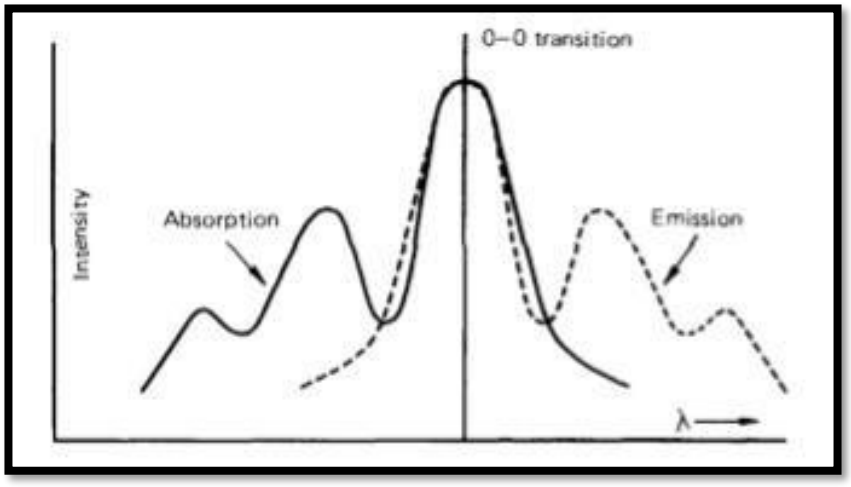


Figure 3: 0 - 0 transition overlap in the excitation and emission process

Let us now briefly look into the factors that affect fluorescence and phosphorescence phenomena. Both molecular structure and chemical environment influence whether a substance will or will not luminesce; these factors also determine the intensity of emission when luminescence does occur.

Factors affecting fluorescence and phosphorescence:

- **Solvent-** Modification in polarity of the solvent will have substantial effect on the fluorescent behavior of the sample. Apart from solvent polarity, the viscosity of the solvent also effects the fluorescence and phosphorescence. Increase in viscosity will increase the fluorescence because chances of deactivation is less.
- **Temperature-** As the temperature changes the viscosity of the medium also changes which causes change in collisions of sample molecules present in solvent, which results in escalation in chances of deactivation due to vibrational relaxation and internal conversion. Therefore, increase in temperature will result in decrease in fluorescence and vice-versa.
- **Dissolved oxygen-** It is a paramagnetic substance which reduces fluorescence and will cause intrusion in fluorimetry analysis. Dissolved oxygen stimulates intersystem crossing. Dissolvedoxygen also influences phosphorescence.
- **pH-** Any change in pH effects the intensity of fluorescence and spectral features of fluorescence. Presence of basic or acidic functional groups in molecules effects the pH of the medium which changes the degree of ionization of functional groups. This will result in changes in molecules in terms of conjugation and aromaticity that affects fluorescence.

Apart from these factors adsorption, intensity, oxidation also affects fluorescence and phosphorescence.

Spectro-fluorometer

Fluorescence is measured by Spectro-fluorometer and Fluorometer. Following are the basiccomponents of Spectro- fluorometer and Fluorometer-

- Excitation source
- Excitation Monochromator
- Cuvette
- Emission Monochromator
- Detector

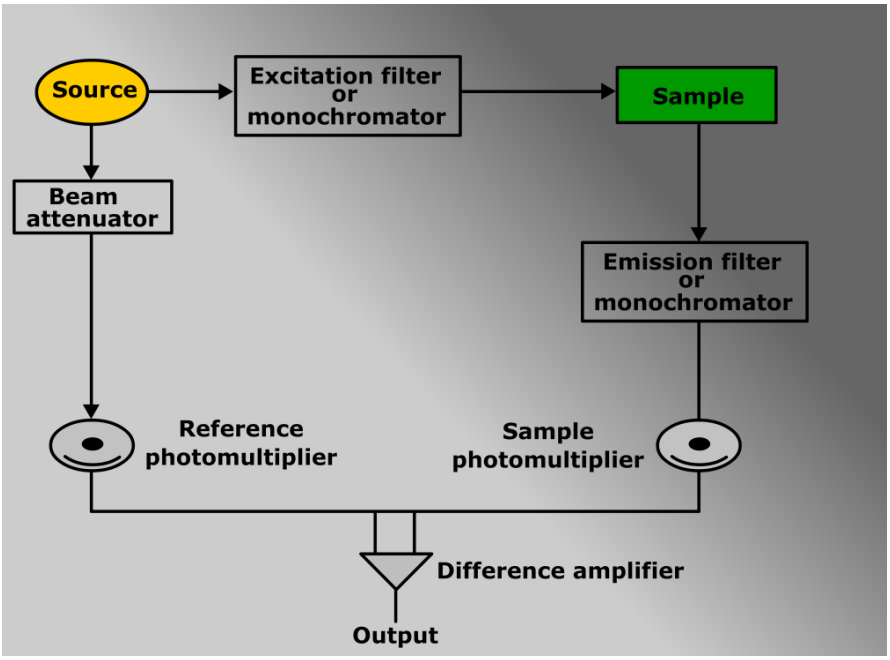


Figure 3- Flow diagram of fluorometer

- **Excitation source:** 300-700 nm is the range of interest for most of the fluorescent compounds. The excitation sources used in Spectro- fluorometer and fluorometer include xenon lamps, lasers, mercury arc lamps and quartz halogen lamps. Xenon lamps are one of the known excitation source and it provides continuum high intensity radiant energy in 250-800 nm spectral region. Because of high energy output of xenon lamp, stability of lamp flashes and higher Ultra-violet and visible spectral productivity, it is widely used in fluorometer. Lasers are also used as an excitation source because of its high intensity and is well focused.

- **Excitation and Emission Monochromator:** In fluorometers and spectro-fluorometers, the monochromator consist of filters (interference and colored glass filter), prisms and gratings. Colored glass filters selectively absorbs particular wavelength, this type of filter is generally used for both excitation and emission selection of wavelength. But the only limitation is that colored glass filters are vulnerable for transmitting stray light and undesirable fluorescence. Grating monochromators are used for isolation of selective regions of UV-Visible spectrum.

  The spectral resolution of the light passing from the slit is a function of width of the slit and grating resolution. To obtain higher excitation intensity the spectro-fluorometers generally uses large slit width. Grating monochromators are of great use because it provides selective emission and excitation wavelengths. The grating rotations are digitally controlled. For elimination of extra order of emission, laser is used as both monochromator and excitation light source.

- **Cuvette:** Cuvettes are used to hold the sample to be examined. The shape of cuvettes used in fluorescence instruments are generally of rectangular and square shape and the material used for constructing these cuvettes allows the excitation and emission light to pass through it. Glass and plastic materials are used for visible light and for Ultra Violet light, quartz is used. Glass and plastic is not used for UV light because instead of passing the UV light it absorbs the UV light. The position of the cuvette and the excitation beam is important for establishing the optical geometry for the fluorometers. Right angle detector approach is used by the majority of modern fluorometers and spectro- fluorometers because of its property of minimizing background signal used for limiting analytical sensitivity. To accommodate various types of geometries, the sample cell is placed at different angles according to the excitation source and detector.

- **Photodetector:** Detectors used in spectro- fluorometers and fluorometers are same as detectors used in spectrophotometers, for instance- photomultiplier tube (PMT) and charged couple detectors (CCD). Visual observation is also used.

- **Photomultiplier Tube (PMT):** Photomultiplier tube is commonly used in spectrofluorometers and fluorometers for quantitative analysis. The most important characteristics of PMT for

fluorescence analysis are

i. Wide range of spectral responses

ii. Highly sensitive

iii. Quick photon response time

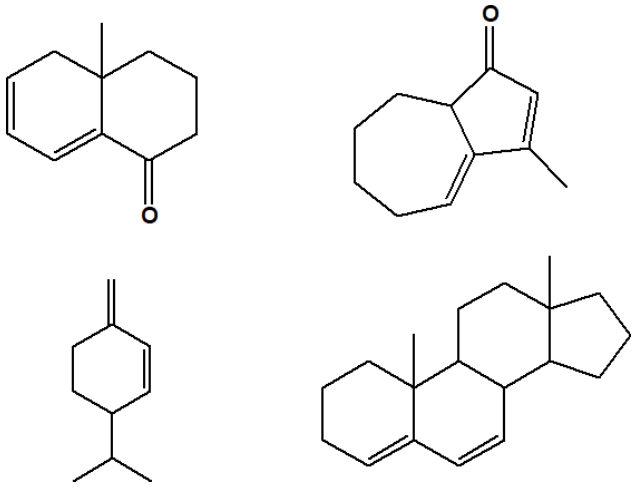
At high light intensities, analog techniques are used for measuring PMT current, then this analog signal is converted into digital signal for computer use and displaying the result. At low light level, at cathode of PMT spikes are counted. The number of pulses generated per unit time is directl

roportional to intensity of fluorescent light emitted by the sample which are striking the PMT. This techniques is called as photon counting.

- **Charged couple detectors (CCD):** These are multichannel devices which have vast range and a signal to noise ratio, which is higher in comparison with photomultiplier tube. These are solid state devices which are composed of photon detecting shift registers and these registers reads horizontally and vertically. They are used for fluorescence applications because they can detect very low intensity of light and can measure low concentration of fluorescent molecules. A 30W mercury lamp is used when fluorometer is used with charged couple detectors.

Long answer type Questions (10 Marks)

1. Find out the λ_{max} of following molecules



2. Give principle and detailing of various components of double beam UV-visible Spectrophotometer.
3. Explain various electroic transitions taking place in UV Spectroscopy.
4. Discuss the instrumentation of UV Spectrophotometer
5. Explain Beer's law Describe factors responsible for deviation from Beer's law.
6. Describe various detectors used in UV spectroscopy.
7. Write a note on the various factors affecting Flouresence
8. What are the structural requirements of flourophore? What are the factors affecting Flouresence?

Short answer type questions (5Marks)

1. Write the short note on electronic transitions.
2. Write notes on application of UV-visible spectroscopy.
3. Explain the term Hypsochromic shift and Bathochromic shift. What structural features may lead to these shifts in organic molecules?
4. Explain the effect of polar solvent on $\pi - \pi^*$ and $n - \pi^*$ transition.
5. How 1,3-pentadiene and 1,4-pentadiene can be distinguished by UV spectroscopy?
6. Why aniline behaves like that of benzene in acidic medium?
7. Two isomeric dienes 'A' and 'B' having the molecular formula C_5H_8 absorb at λmax 225 nm and λmax 180 nm respectively. Write the structure of the two isomers.
8. Discuss the applications of Flourimetry
9. Describe the factors affecting fluoresence intensity of compound in flourmetric analysis.

Very Short answer type questions (2 Marks)

1) What is electronic spectroscopy?
2) What is Chromophore? How does it differ from auxochrome?

3) Discuss how conjugation affects the intensity of absorption band.

4) Enlist elements of process validation.

5) Write the limitations of process validation.

6) Name the parameters for calibration of GC.

7) Name the parameters for calibration of HPLC.

8) Name the parameters for calibration of UV Visible spectrophotometer.

9) What is quenching?

10) Explain triplet excited state of molecule giving diagram

11) Define electronic transition.

12) Give the principle involved in Flourimetry?

13) Difference between flouresence and phosphorescence?