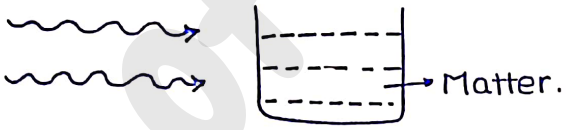
Unit I

Instrumental method of Analysis

- UV Visible Spectroscopy
- It is the study of interaction between EMR (Electromagnetic radiation) and matter.
- Matter = Biological molecule (DNA, RNA, Protein) or Chemical compound.



- EMR is a wave which show Electric and Magnetic Property
- E.g. of EMR ⟶ UV rays, X-rays, γ-rays.

Here we use,

UV as a EMR so, it is called UV visible Spectroscopy.

- If, we arrange the wave of EMR according to the wavelenght then a spectrum is obtained called Electro magnetic spectrum.

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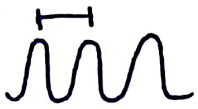
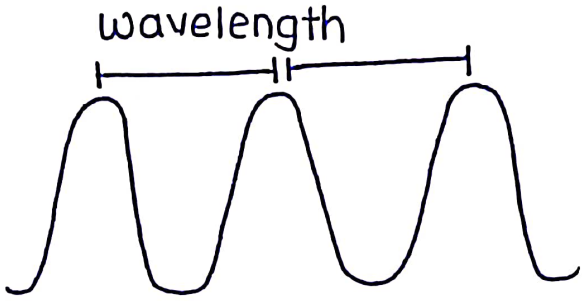
- Shortest wavelength → Gamma rays (r rays)



- Longe Wavelength
  OR
  Longest Wavelength → Radio Waves



- Highest frequency → Gamma rays (Energy ↑)
- Lowest frequency → Radio waves (Energy ↓)

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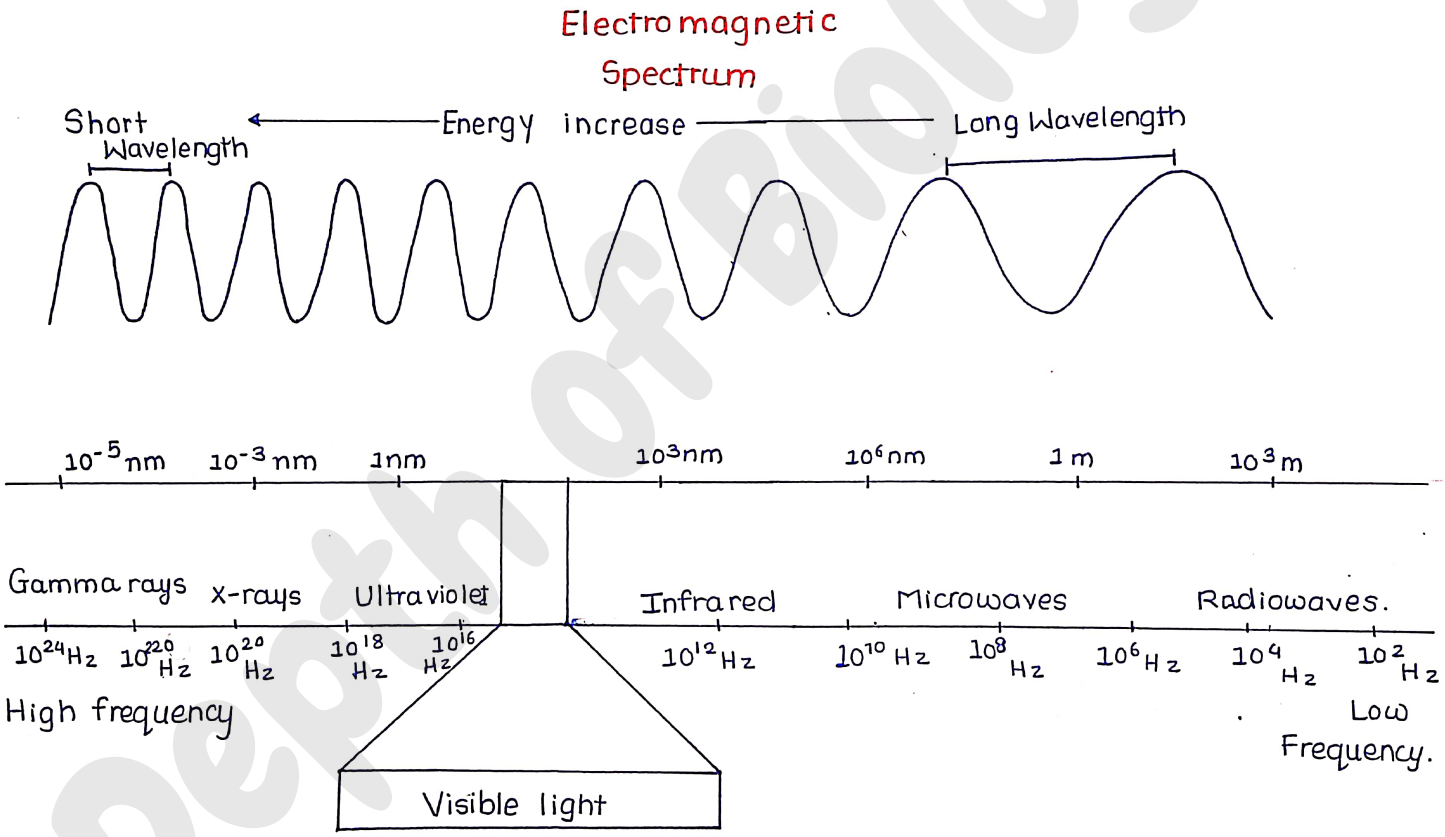
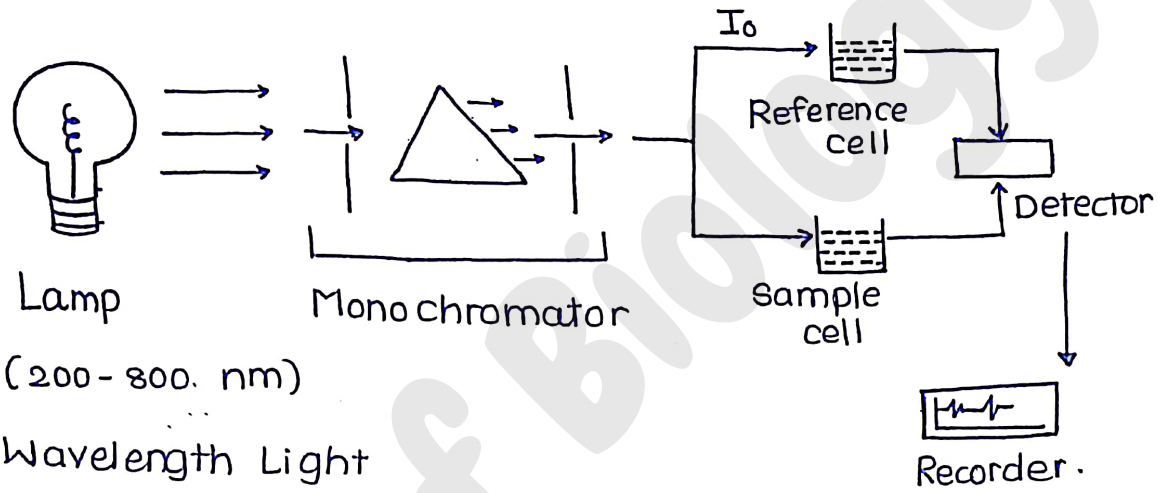


• Instrumentation of U.V Visible.



(200 - 800 nm)

Wavelength Light is produced

↓

UV = 200 - 400 nm (Wavelength)

Visible = 400 - 800 nm (Wavelength).

Lamp produce light of Wavelength (200 - 800nm) in which UV ranges from (200 - 400nm) and Visible light ranges from (400 - 800nm), So, it is called UV Visible light.

⇓

The light falls on Monochromator (Monochromator consist of 2 slit and one Prism).

- Role of first slit of monochromator is to fall the Photon particle of light which is produced by Lamp parallely on the monochromator.
- Now, Prism convert the UV visible light into different wavelength of light and shows different colours V. I. B. G. Y. O. R.
- From the second slit a specific wavelenght of light is passed, and this specific wavelenght of light converted into two rays of equal intensity.
- The first ray of light
  ↓
  goes to the reference cell (Reference cell is a Cuvette) → It is made up of Glass or Quartz.
  ↓
  Container for Holding
  Liquid sample in spectrophotometer.

Reference cell (Cuvette) only contain buffer solution or Blank sample due to this the intensity of first ray of light will be same even after it passes from Reference cell. [because reference cell does not contain any sample.]

- Second ray falls on the sample cell (e.g → Protein).
- Protein are long chain of Amino acids and they absorb light or second ray.
- Protein contain some Aromatic amino acid (Tryptophan, Tyrosine) ~> This type of aromatic amino acid contain double bond and Rings and they use the energy / Light for electronic Transitions.
- The e⁻ (electron) of amino acid moves from lower energy state to Higher energy state after the use of energy and the intensity of light passes from the sample cell is decreases.
- Absorbtion of light depends on two factor
  - (a) Protein concentration
  - (b) Path length of Cuvette.

(a) Protein concentration

- If protein concentration is more in sample cell (Cuvette) then absorption of light is more.

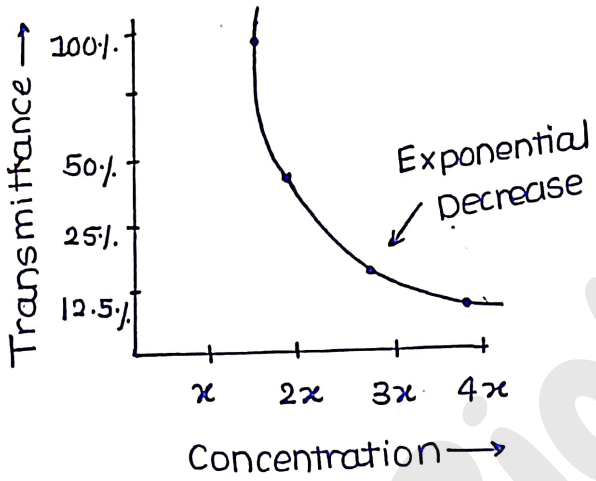
(b) Path length of Cuvette

- If more the path length of cuvette more will be the absorption of light.
- This both Absorption factor → Protein Concentration, Path length of Cuvette is explained by Beer Lambert's Law.

$$A \propto C L$$

where,
A is absorbance
C is described by Beer
L is described by Lambert.

- So, after passes the light from reference cell intensity of light will be same.
and after passes the light from sample cell intensity of Light will decrease.
- Now, detector find (or detect) the ratio of transmitted light and send it to the recorder generate reading inform of Absorbance with the helpof this we can detect the Protein concentration & Absorbtion of Light.



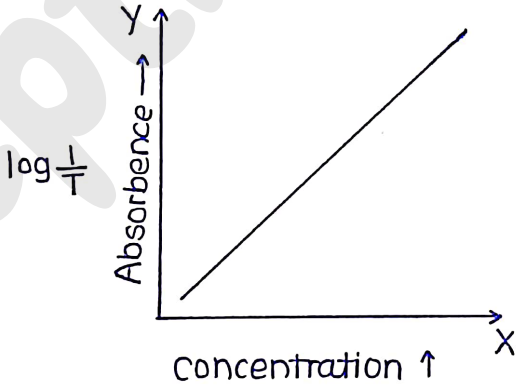
- As the concentration of Protein or sample increase the transmittance is decreased.

When transmittance is converted into

Absorbence → $\left[\log \frac{1}{T}\right]$

where,

T = Transmittance.



Here we check concentration on the basis of Absorption of light. So, this type of spectroscopy is called Quantitative Spectroscopy./Quantitative U.V. Visible Spectroscopy.

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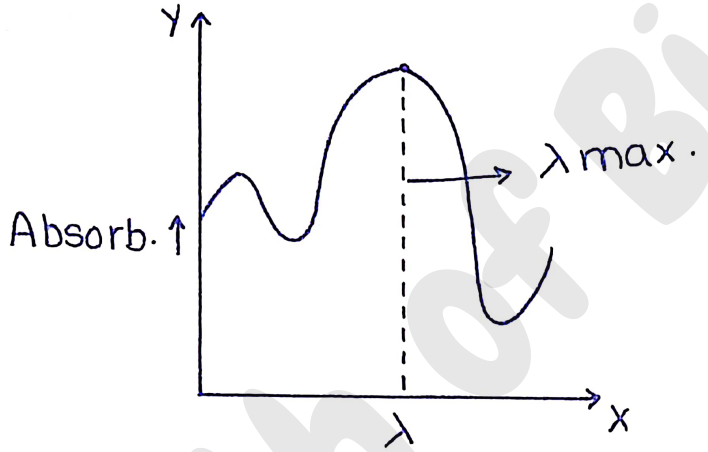


λmax = wavelength at which absorbtion is maximum

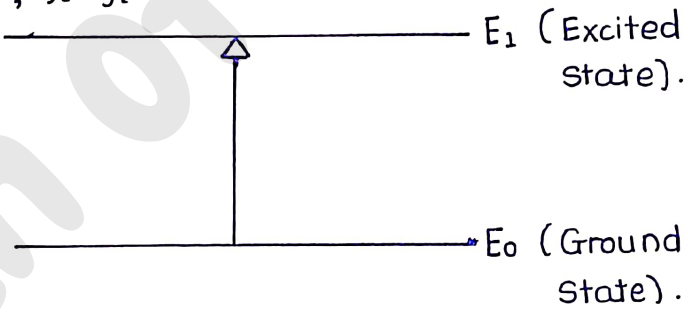
OR means our sample absorb maximum light at λmax.

Here we check the Quality of sample on the basis of λmax.

So, it is called Qualitative Analysis.

Electronic Transition

- Electronic transition refer to the process when electron jump from one energy level to another.
- When a molecule absorb EMR (U.V visible light) the electron get promoted from ground state to Higher Excited State which results in promotion. from a bonding / non-bonding orbitals to an anti-bonding orbitals
  i.e $n-\pi^*$, $\pi-\pi^*$



1. Sigma (σ) electron

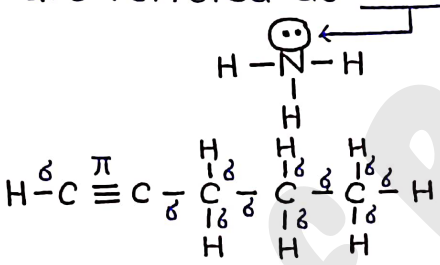
- These electron are involved in saturated σ-bonds. (Each sigma bond consist of two e^-)

2. π electron

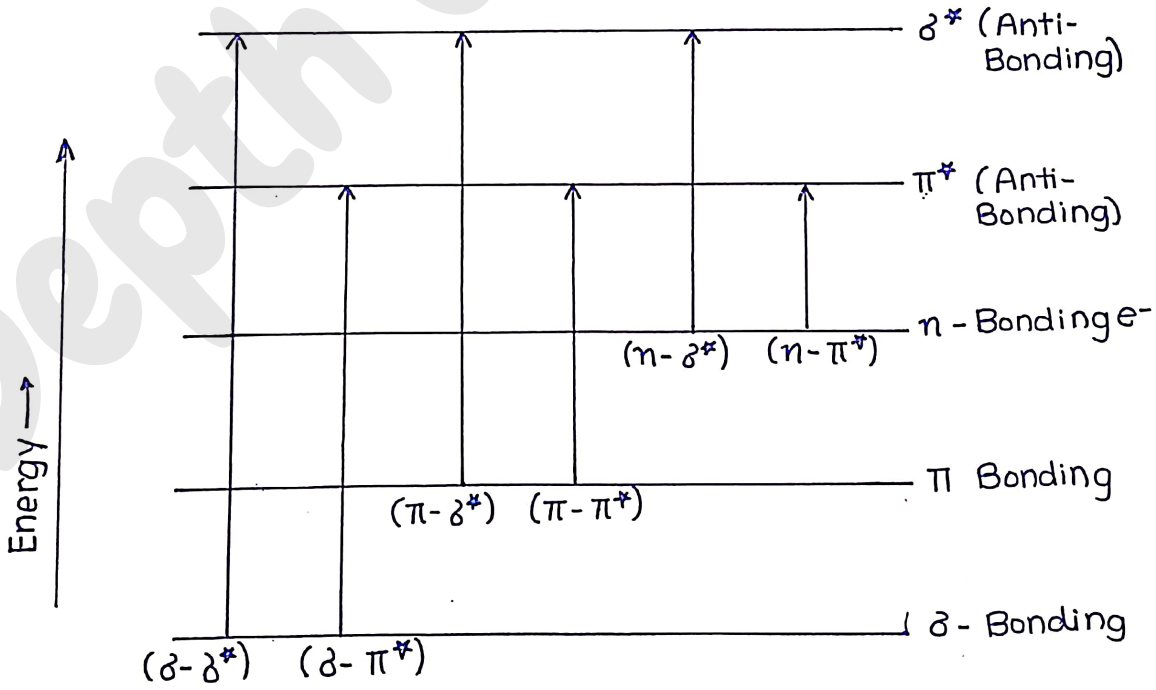
- These are the electrons which involved in Unsaturated Hydrocarbons. (Double bond or Triple bond).

3. Non-Bonding electron

- Electron which are not involved in chemical bonding. (or does not participate in bonding with other atom).
- They are reffered as lone pair.



* Possible Electronic Transitions Graphically shown



σ, π bonding and n-bonding → Occupied level.

σ^*, π^* → Unoccupied level.

- Here, e^- are shifted from lower energy to higher energy Level or from Highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) by absorbing the EMR.

1. $\sigma - \sigma^*$ Transition :

- A transition of an electron from a sigma bonding orbital to the Higher energy anti-bonding sigma orbital.

2. $\sigma - \pi^*$ Transition :

- A transition of an e^- from a sigma bonding orbital to the Antibonding π orbital.

3. $\pi - \sigma^*$ Transition :

- A transition of an electron from a π bonding orbital to the Antibonding Sigma orbital.

4. $\pi - \pi^*$ Transition :

- A transition of an electron from a π bonding orbital to the Antibonding π orbital.

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5. $n - \sigma^*$ Transition:

- A transition of an electron from a non-bonding orbital to the antibonding sigma orbital.

6. $n - \pi^*$ Transition:

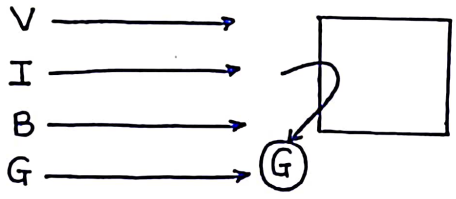
- A transition of an electron from a non-bonding orbital to the anti-bonding π orbital.

Chromophore and Auxochrome

Chromophore

- Chromo = colour
- Phoros = bearer

- Group in the molecule which is responsible for colour.
- Chromophore is a group in the molecule responsible for absorption of visible light (400-800nm). and UV light or we can say chromophore absorb light in the UV visible region and responsible for imparting colour / reflect a certain colour to the compound.
- The chromophore containing compound is Chromogen.



- The wavelength reflected by the molecule is what we experience as colour.

e.g. → $-N=O$, $>C=C<$, $>C=O$, $-N(=O)-O^-$

* Nitro compound ⇒ Yellow colour.

Colorless Benzene + $-N=O$ ⟶ PALE YELLOW COLOR.

Chromophore

@ Independent Chromophore

- A single chromophore is responsible for reflecting a certain colour in the compound.

Example : $>C=C<$ & $-N=O$

Groups	λmax
C-C	1350
C=C	1900
C=O	1900-2800
NO_2	2800
C_6H_5	1950 2500

(b) Dependent Chromophore

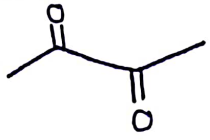
- More than one chromophore is required to produce Colour in compound.

Example : C=O group.

- Acetone having one ketone group is colourless

$CH_3-\overset{\overset{O}{\|}}{C}-CH_3$

- Diacetyl having two ketone group is yellow in colour.



Auxochrome

↳ Colour enhancing group

- A saturated / Unsaturated group with non-bonding e^- when attach to chromophore altering (↑se) both wavelength as well as intensity of Absorption.
- Example : OH , NH_2, COOH, NHR , Cl.

Colourless Benzene + -N=O chromophore ⟶ Pale yellow colour + Hydroxyl group (Auxochrome) ↓ Dark yellow colour obtained

Auxochrome increases the intensity of absorption.

Absorbance ↑ = Wavelength ↑

Eg.

Benzene

Aniline

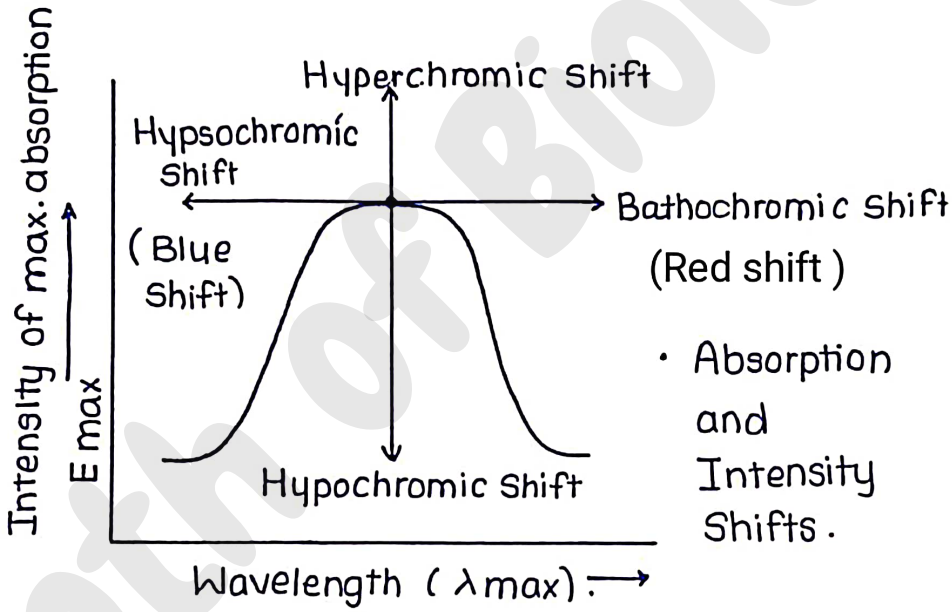
λ max → 255 nm

λ max → 280 nm

Spectral Shifts

VIBGYOR
Blue ⟷ Red

- The position of E max. (Absorb maximum) and Absorption intensity can be modified by change in solvent.



- Absorption and Intensity Shifts.

1. Bathochromic Shift :

- Also known as Red Shift.
- This type of Shift occurs due to Auxochrome or by changing the Polarity of Solvent. Here Absorption maximum is shifted towards the longer wavelength.

2. Hypsochromic Shift :

- Also known as Blue Shift.
- This shift occurs due to removal of Auxochrome or by change in polarity of solvent.
- Here Absorption maximum shifted towards shorter wavelength.

3. Hyperchromic Shift :

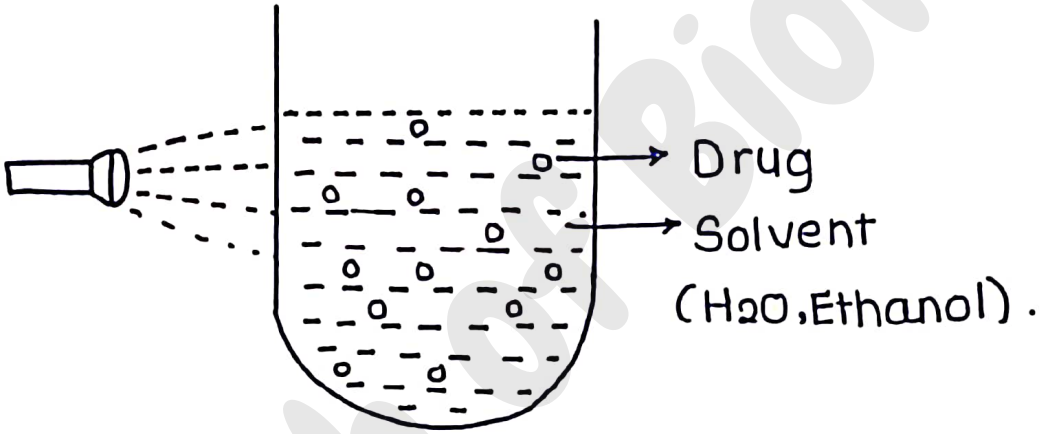
- This shift occurs due to Auxochrome.
- In this shift the Intensity of Absorption maximum (E.max). increases.

4. Hypo chromic Shift:

- It is defined as an effect due to which the intensity of absorption maximum decreases.

- Solvent Effect on Absorption Spectra
- If we change the solvent of our solution then it affect absorption and wavelength.

- We can say absorption and wavelength alter by changing solvent.



- Polarity of solvent α $\frac{1}{\text{wavelength}}$ & absorbance.

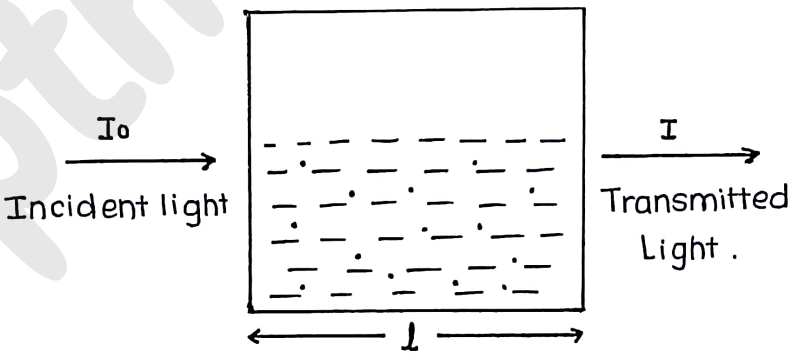
H_2O > Methanol > Ethanol > Benzene > Hexane

← POLARITY

BEER & LAMBERT'S LAW

- Beer's Law
- Absorbance is directly proportional to the concentration of solution.
- [Absorbance α Concentration]

- Lambert's Law
- Absorbance is directly proportional to path length of Cuvette.
- [Absorbance α Path length]



$[I_o > I]$

$A \alpha Cl$

$A = \varepsilon Cl$

ε = Molar Absorbtion Coefficient.

C = Concentration of solution.

l = Path length of Cuvette.

- **Derivation**

According to Beer Lambert's Law when a monochromatic Light is passed through a solution containing the absorbing substance, the decrease in the intensity of light with path length is proportional to the concentration of solution and the intensity of light.

$$\left[-\frac{dI}{dl} \propto IC\right]$$

where,
C = concentration
I = Intensity of light
l = Path length.

$$\frac{-dI}{dl} \propto I.C$$

$$-\frac{dI}{dl} = KIC \qquad (K = \text{constant})$$

Interchange

$$-\frac{dI}{I} = Kc.dl$$

Integration both side

$$-\int_{I=I_0}^{I=I} \frac{dI}{I} = Kc \int_{l=0}^{l=l} dl$$

$$-\ln \frac{I}{I_0} = KCl.$$

$$\ln \frac{I_0}{I} = KCl$$

Multiplying on both sides by 2.303

$$2.303 \times \ln \frac{I_0}{I} = 2.303 \times KCl$$

$$\log_{10} \frac{I_0}{I} = \varepsilon Cl \quad \text{—— (1)}$$

↳ new constant

Transmittance (T) → Amount of light emitted after absorption.

$$T = \frac{I}{I_0}$$

$$\text{Absorbance} \propto \frac{1}{\text{Transmittance}} \left[A \propto \frac{1}{T}\right]$$

Now,

$$A = \underset{\downarrow 1.}{\log_{10}} (1/T) \quad \text{—— (2)}$$

$$A = \log_{10} \left(\frac{1}{I/I_0}\right)$$

$$\left(A = \log_{10} \frac{I_0}{I}\right)$$

According to equation (i) and (iii)

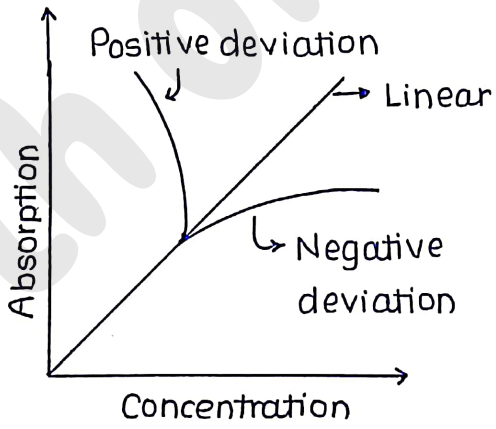
$(A = \varepsilon c l)$

where,

A = Absorption
ε = Molar Extinction Coefficient
C = Concentration
l = Path length.

- **Deviation**

Absorb. α concentration of Solution



- **Non-Linearity (Positive and Negative deviation).**
- Three types of deviation

1. Real deviation / True deviation
2. Chemical deviation
3. Instrumental deviation / Spectral deviation.

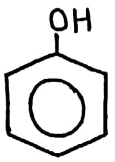
1. Real deviation

- This type of deviation occur when concentration of molecule are high in solution.

2. Chemical deviation

- This type of deviation occurs due to chemical changes such as pH, Association, Dissociation, etc. in Absorbing medium.

Eg.

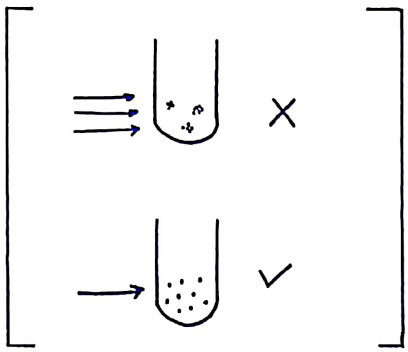


Phenol (270 nm wavelength).

Phenoxide anion (287 nm wavelength).

3. Instrumental deviation

- This deviation occur due to polychromatic radiation and it leads to negative deviation (absorbance ↓)
- Monochromator are used to prevent this.



INSTRUMENTATION

- Spectrophometer

- It is an instrument that measure the amount of photons (the Intensity of light absorbed after it passes through sample solution.
  and with the help of this we can also detect the concentration of chemical substance /drug.

- Essential Parts of Spectrophotometer are

1. Source of Radiation
2. Wavelength Selector (monochromater)
3. Sample cell or Cuvette
4. Detector
5. Recording system.

1. Source of Radiation

- It should provide continuous radiation.
- The light which is more stable, more intense are the best.

- Source of radiation must given range of spectrum 180-700 nm (200,400, 400-800)

a. Hydrogen discharge lamp

- In this lamp a pair of electrodes is enclosed in a glass tube filled with hydrogen gas under relatively high pressure.
- High voltage current is passed through electrode

↓

Discharge of electron occur.

↓

Excitation of Hydrogen molecule.

↓

Cause Emission of U.V radiation

- It is a continuous source.
- Covers a range 160-375nm (stable and widely used).

b. Deuterium Lamp

- It is used when high Intensity is required.
- Similar to Hydrogen discharge lamp.
- Deuterium is filled in place of Hydrogen.
- The intensity of radiation emitted is 3-5 times the intensity of Hydrogen Lamp.
(More Expensive then Hydrogen Lamp).

c. Tungsten Lamp

- Similar in its functioning to an electric bulb. (continaus source of light).
- It provides a supply of radiation in the wavelength range of 320-2500 nm.
- Electric current is pass, Tungsten filament is heated and the light is produce.
- The glass bulb enclosing the filament contains a low pressure of Inert gas (usually Argen(Ar)].
- Small amount of Halogen like Iodine is added to improve the intensity (Tungsten-Iodine Lamp).

d. Xenon Discharge Lamp (Continuous source)

- Xenon gas is store in lamps at 10-30 atm pressure.
- It contain 2 tungsten electrode that are separated by a distance of about 8 mm.
- When current pass through Xenon cause Thermal excitation.
- It produce greater radiation then Hydrogen Lamp.

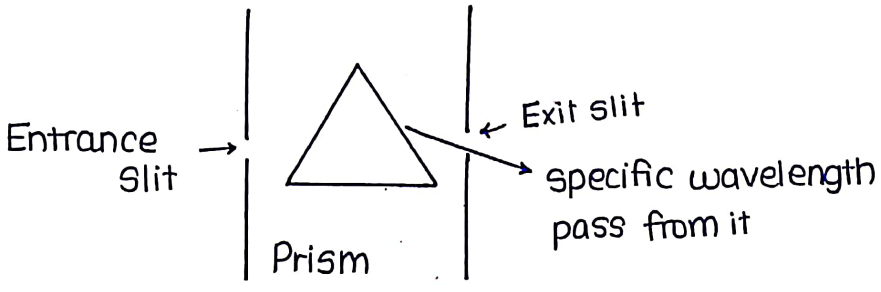
e. Mercury Arc Lamp

- In this Hg Vapour is stored under High pressure and the excitation of Hg atom is done by electrical discharge.

2. Wavelength Selector

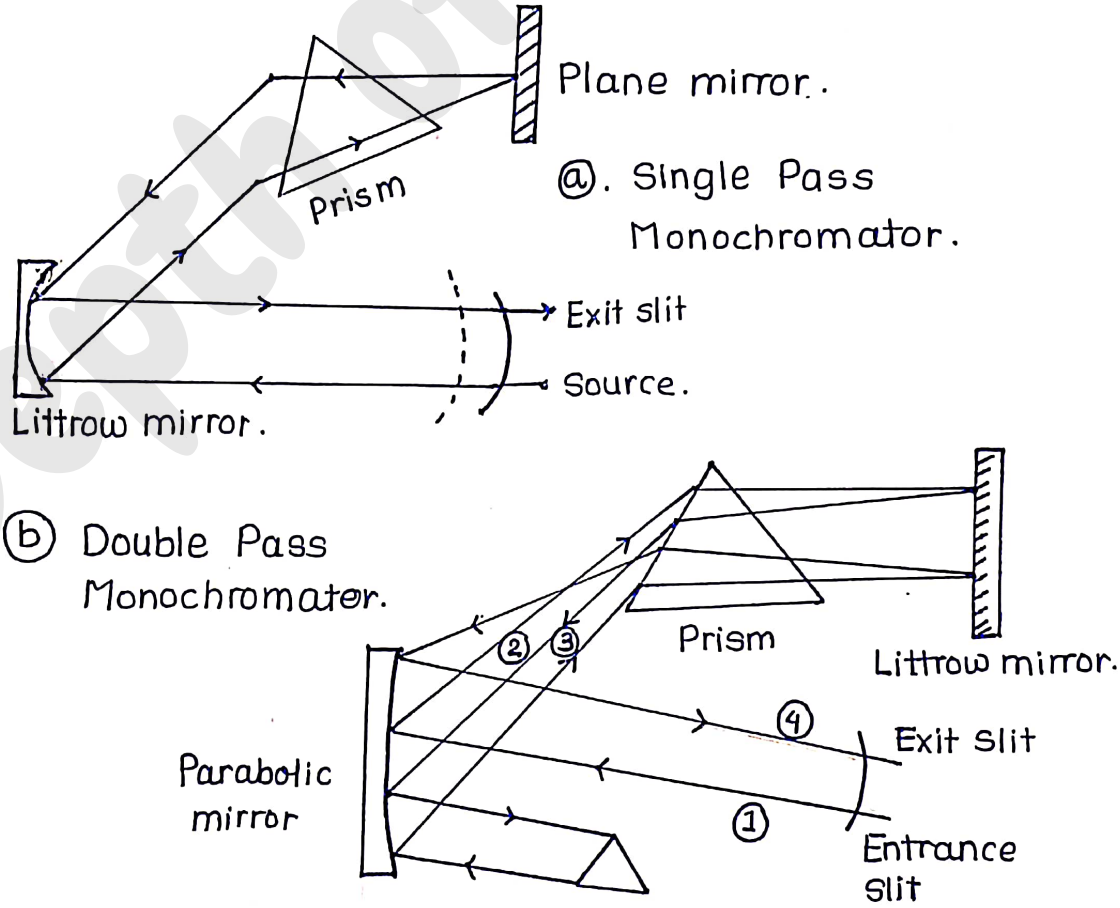
- Converts polychromatic light to monochromatic Light.
- Filters and monochromator are used for this purpose.
- Filters remove wide bands of radiation from a signal.

- Monochromator
- Monochromator is an optical device that separate polychromatic Light (Sunlight or light coming from a lamp) into a range of individual wavelength (Monochromatic Light).
- Monochromator consist of Entrance Slit, Exit slit & prism.



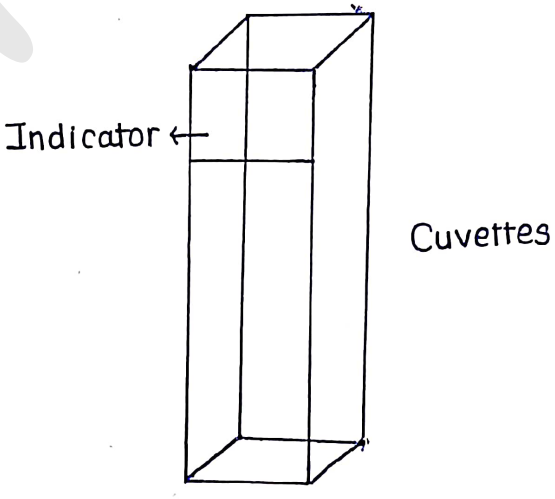
- Prism
- Made up of Glass, Quartz or fused Silica.
- Quartz and silica is the choice of material of U.V spectrum.
- Exit Slit
- Passed the single radiation of desired wavelength.
- Prism monochromator → Two types

a. Single pass monochromator.
b. Double pass monochromator.



3. Cuvettes

- Also known as sample cell.
- The cell or cuvettes are used for handling Liquid samples.
- The cell may either be rectangular or cylindrical in nature.
- For study in U.V region the cells are prepared from Quartz or fused silica.
  whereas colour corrected fused glass is used for visible region.
- Cleaning is carried out washing with distilled water or with dilute Alcohol, Acetone.
- The surface of cell / Absorption cell / Cuvettes must be clean.
  No fingerprint present on cell.



4. Detector

- Device which converts light energy into electrical signal, that are displayed on readout device.
- The transmitted light falls on the detector which determines the intensity of radiation absorbed by sample.
- The followed types of detectors are employed in instrumentation of absorption spectrophotometer.

  a. Barrier Layer cell / Photovolatic cell.

  b. Phototubes / Photo emissive tube

  c. Photomultiplier tube.

- Requirements of Ideal Detector.

ⓐ It should give Quantitative response.

ⓑ It should have high Sensitivity and low Noise level.

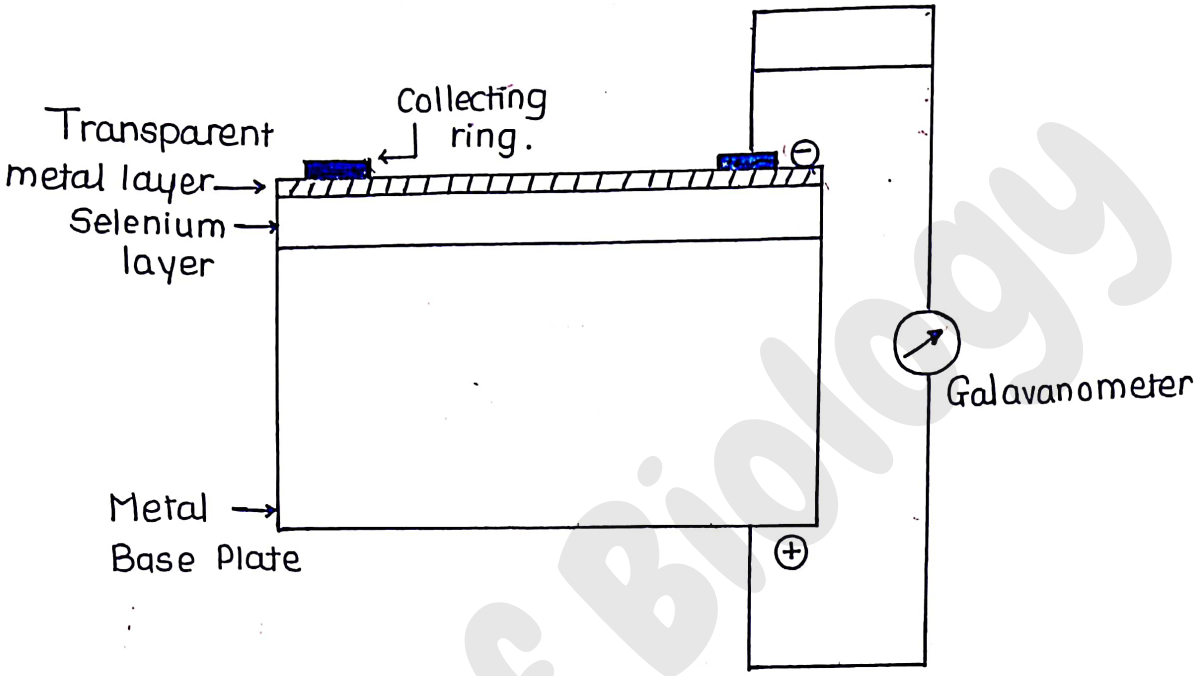
ⓒ It should have a short response time.

ⓓ It should provide response Quantitative to Wide spectrum of radiation received.

- Detector used in UV-Visible Spectrophotometer can be called as Photometric detector.

1. Barrier Layer Cell / Photovolatic cell

- Also known as photronic cell.
- The detector has a thin film metallic layer coated with silver or Gold and act as another electrode.
- It also has a metal Base plate which act as another electrode.
- These 2 layers are separated by a semiconductor layer of Selenium.
- This creates a potential difference between two electrodes and cause the flow of current.
- When it is connected to galvanometer, a flow of current observed which is proportional to the intensity and wavelength of light falling on it.
- When light radiation falls on Selenium layer electrons become mobile and are taken up by transparent metal layer.



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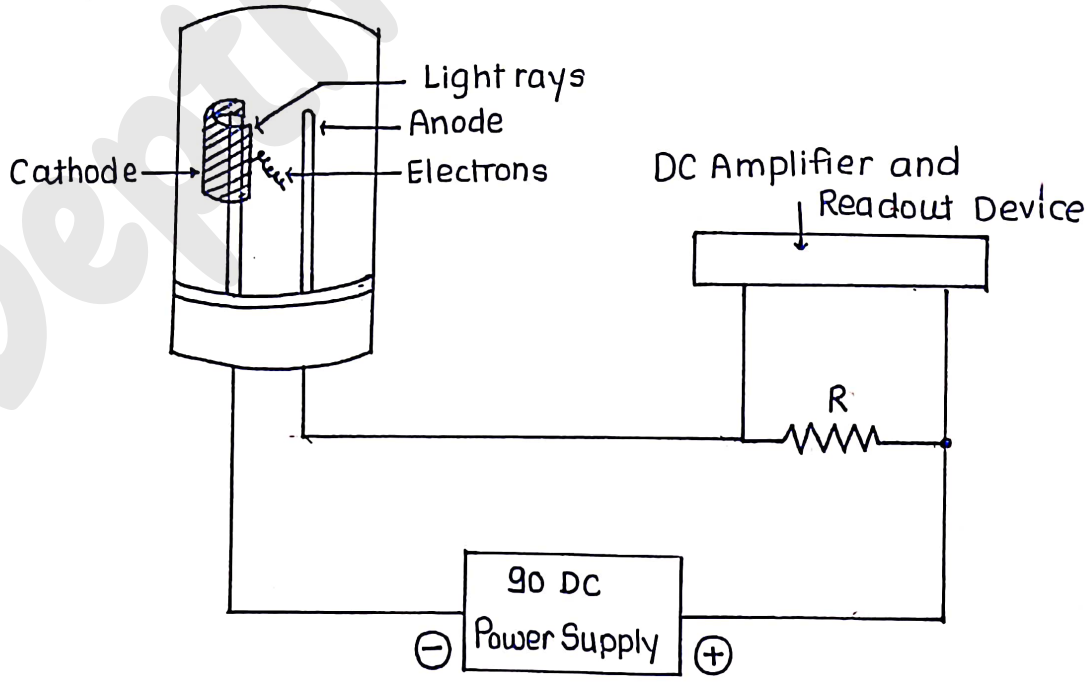
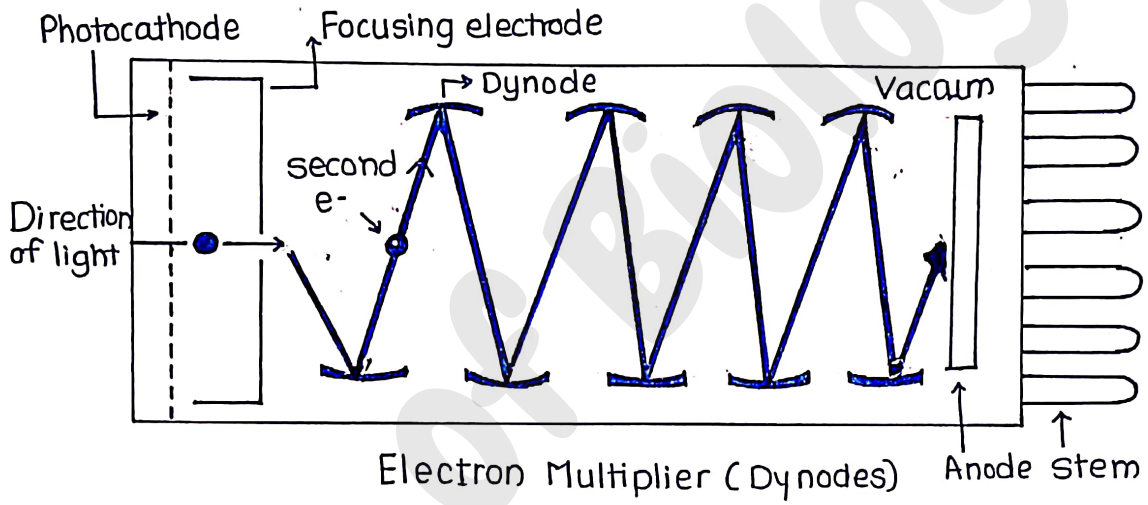


- Phototubes / Photoemissive Tubes.
- Consists of a evacuated glass tube with a photocathode and collector anode.
- The surface of photocathode is coated with a layer of elements like Cesium, Silver Oxide or mixture of them.
- When radiant energy falls on photosensitive cathode, electrons are attracted to anode causing current to flow.
- More sensitive compared to barrier layer cells & therefore widely used.

3. Photo multiplier Tubes

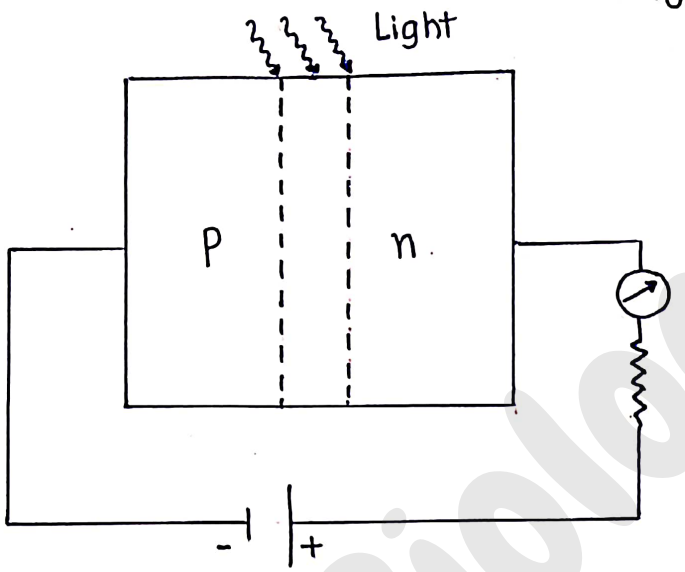
- The principle employed in this detector is that, Multiplication of photoelectrons by secondary emission of electron.
- In a vaccum tube a primary photo-cathode is fixed which receives radiation from the sample.
- Eight to ten diode are fixed each with increasing potential of 75-100 higher than proceeding one.

- Photomultiplier is extremely sensitive to light and is best suited.
- Anode is fixed near the last dynode



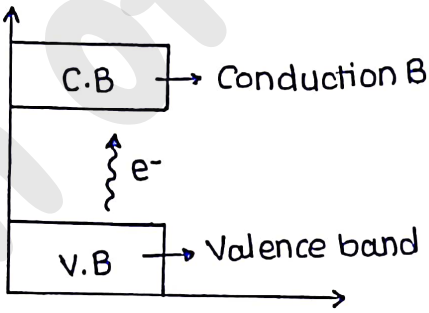
4. Silicon Photodiode

- A photodiode is a PN- Junction diode that converts light energy to produce Electric Current.
- Sometimes it is also called as photodetector, A light detector and photo sensor.
- The diode is very sensitive to light so when light falls on the diode it easily change light into electric current.



- Incident light must have more energy then energy Band gap.

- e⁻ generated in depletion layer or P-N junction



- When incident light have more energy than Band energy
  ↓
  Then e- moves from valence Band to Conduction Band.

- When the photodiode is illuminated with (photon) and light energy is greater than the energy gap of the semiconductor then electron hole pair

are generated due to the absorption of photons.

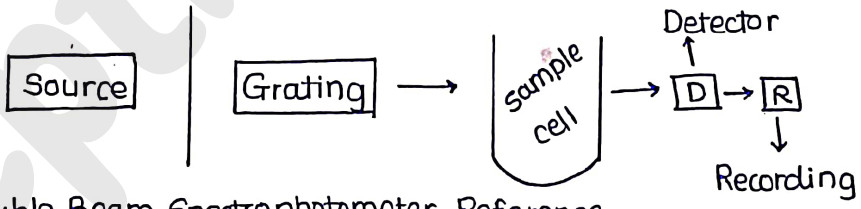
- Photoiodide only used / always used in Reverse Bias it means that the p side of Photodiode is associated with the negative terminal of the battery and n side is connected to the positive terminal of the battery.

- UV Visible Spectrophotometer.

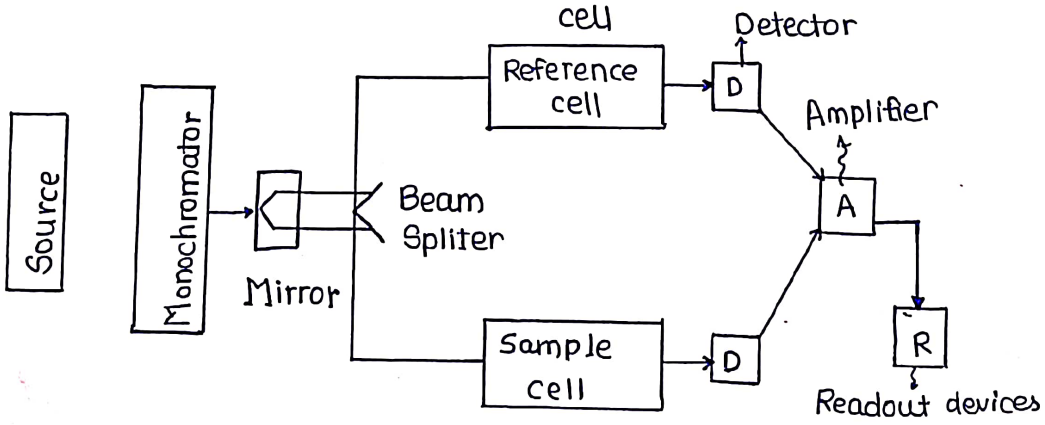
ⓐ Single beam Spectrophotometer

ⓑ Double beam Spectrophotometer

a. Single Beam Spectrophotometer.



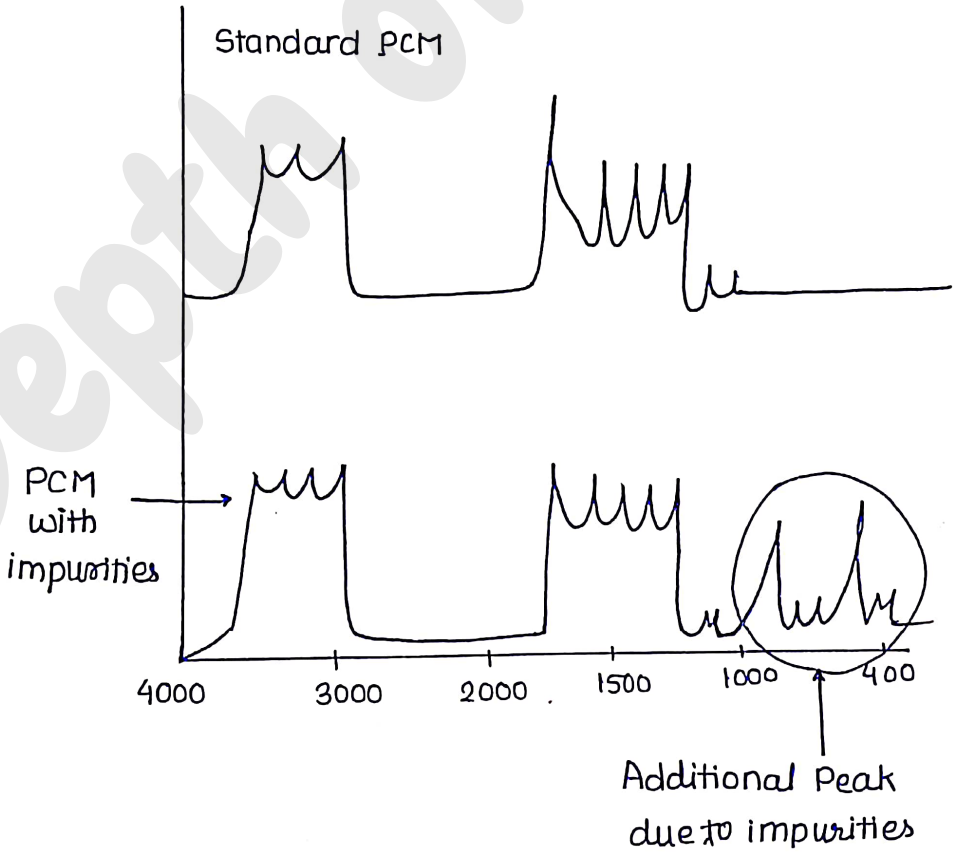
b. Double Beam Spectrophotometer.



Application of UV Visible Spectroscopy.

1. Detection of Impurities.

- U.V spectroscopy is one of the best method for determination of impurities in organic molecule.
- Additional peak can be observed due to impurities in the sample and it can be compared with that of standard raw material.



2. Structure elucidation / Clarification of Organic compound.

- U.V spectroscopy is useful in the structure elucidation of organic molecule (The presence or absence of unsaturation, the presence of Heteroatom).

- From the location of Peak and combination of Peak it can be concluded that whether the compound is saturated or unsaturated Heteroatom are present or not.

3. Quantitative Analysis

U.V absorption spectroscopy can be used for the Quantitative determination of compounds that absorb UV radiation.

- This determination is based on Beer's Law.

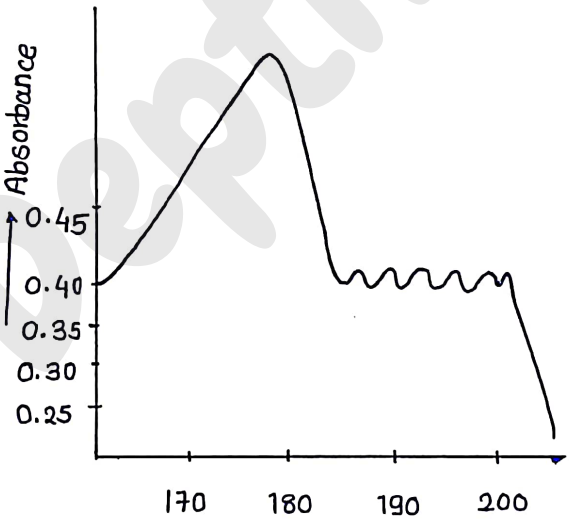
4. Qualitative Analysis

U.V Absorption spectroscopy can characterize those type of compound which absorb U.V radiation.

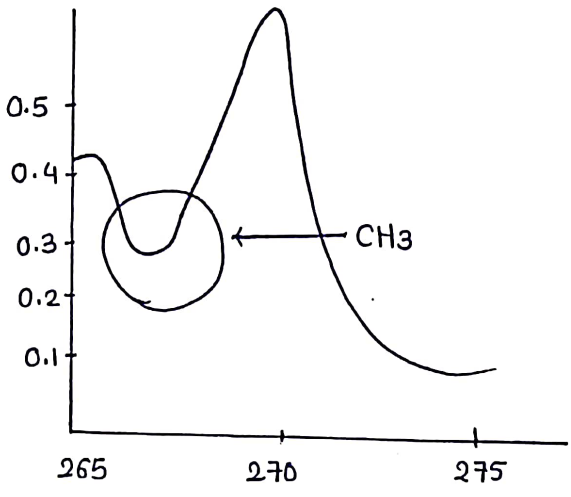
Identification is done by comparing the absorption spectrum with the spectra of known compounds.

4. Detection of Functional Group

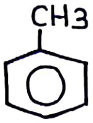
- This technique is used to detect the presence or absence of functional group in the compound.
- Absence of Band at Particular Wavelength regarded as an evidence for absence of particular group.



- Benzene UV-Visible Spectrum.



- Toulene



6. Quantitative Analysis of Pharmaceutical Substance.

- Many drugs either in forms of raw material or in the form of formulation.
- They can be assayed by making a suitable solution of the drug in solvent and measuring absorbance at specific wavelength.

Eg. Diazepam tablet can be analyzed by
0.5% H_2SO_4.
in methanol at wavelength 284 nm.

7. Examination of Polynuclear Hydrocarbon.

- Benzene and polynuclear Hydrocarbon have Characteristic spectra in UV and visible region.
- The identification of polynuclear Hydrocarbon can be made by comparison with the spectra of known polynuclear compound.

8. Molecular Weight determination.

- Molecular weight of compound can be measured spectrophotometrically. by preparing the suitable derivative of compound.

  Eg. If we want to determine the Molecular Weight of Amine then it is converted into Amine picrate.

9. As HPLC Detector.

- UV Visible Spectrophotometer may be used as a detector for HPLC (High Performance Liquid Chromatography).

Quantitative Analysis.

i. Spectrophotometric titration.

ii. Single Component Analysis.

iii. Multi- Component Analysis.

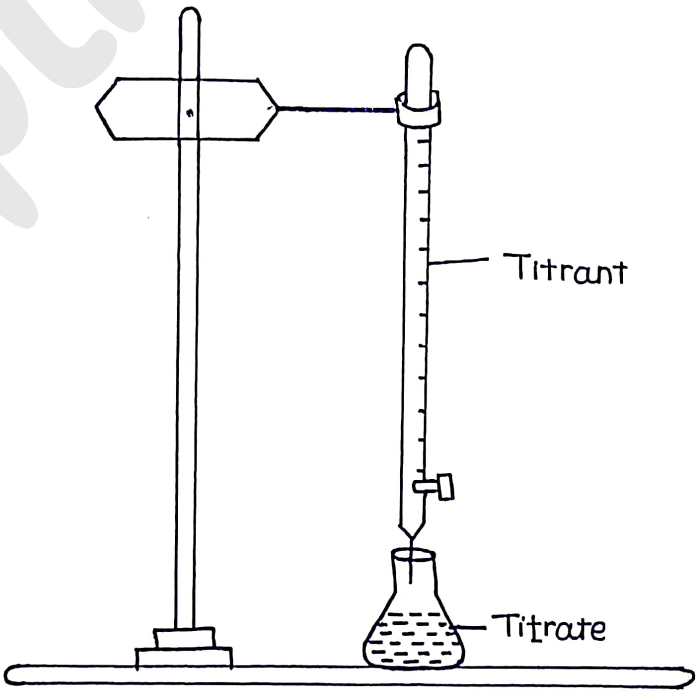
i) Spectrophotometric Titration

- It is the process of determining the Quantity of a sample by adding titrant Until the end point.

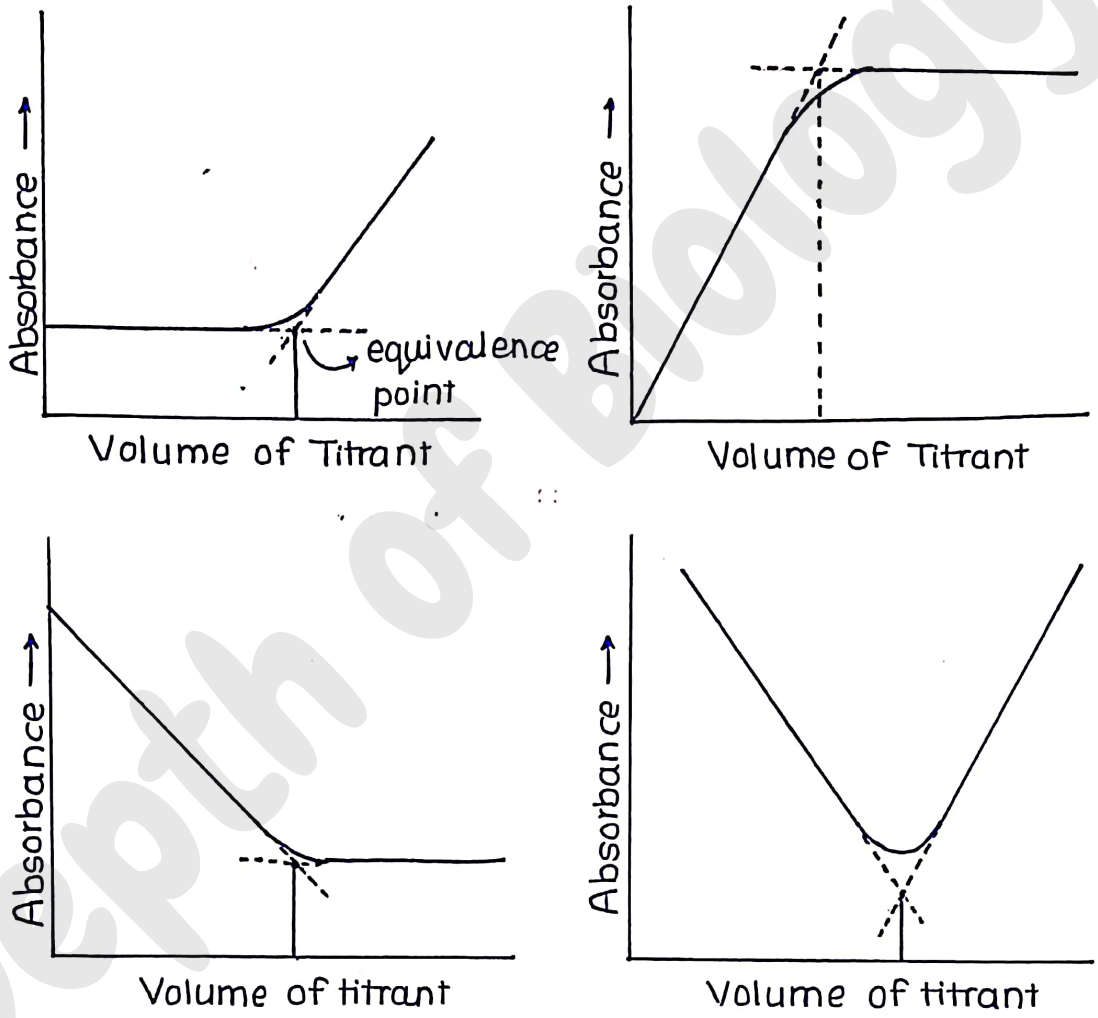
- The end point is where the graph is discontinuous

- The titration are based on Beer's Law [$A \propto c$].

- Titration curve is plot of Absorbance Vs Volume of Titrant.



• Titration Curves.



ii) Single Component Analysis.

If sample is consists of drug and excipient but only drug absorb radiation and excipient does not absorb radiation.

iii. Multi- Component Analysis

- If sample has more than one drug and all absorb at same wavelength then this analysis is required.

- In case when sample carry excipient, impurities and absorb at same wavelength then this is required.

Methods used
- (a) Simultaneous equation method.
- (b) Dual Wavelength method.
- (c) Absorptivity factor method.

Derivative Spectroscopy

It is a modified version of normal UV spectroscopy.

When you do normal UV spectroscopy, you get a smooth curve (called the absorbance spectrum). But sometimes:

Two peaks are very close together.

Or one peak is hidden under another.

So, it becomes hard to see or measure them clearly.

👉 To solve this, we use derivative spectroscopy.

-In derivative spectroscopy, we take the slope or change of the absorbance curve to sharpen the peaks and see hidden details.

🔢 Types (Even Simpler):

Order	Meaning	Looks Like
0th Order	Normal absorbance spectrum	A smooth hill
1st Order	Shows the **change** (slope) of that hill	A curve that crosses zero
2nd Order	Shows the **change of the slope**	Sharp M or W shapes

🎯 Why Use It? (Advantages)

✅ Helps you:

Separate overlapping peaks

Detect hidden peaks

Get more accurate results

🧪 Example:

Let's say two drugs A and B absorb light at similar wavelengths. In normal spectroscopy, you'll see just one wide peak.

But in 1st or 2nd derivative, you will see two separate peaks. That helps you identify both drugs.

💊 Example: Paracetamol + Ibuprofen

When you do normal UV spectroscopy (0th order),

The absorbance peaks of Paracetamol and Ibuprofen are very close— they overlap.
So, it looks like one big peak.

👉 You can't tell how much of each drug is present.

Solution: Use Derivative Spectroscopy

When you apply 1st derivative, the curve now crosses zero at the Amax of each drug.
When you use the 2nd derivative, the curve becomes sharper.

Now you can clearly see two peaks — one for Paracetamol, one for Ibuprofen.

O So you can measure the concentration of both drugs separately, even though their peaks overlapped before.

📘 Summary:

Drug	Peak in UV (nm)	What Happens
Paracetamol	~243 nm	Overlaps with Ibuprofen
Ibuprofen	~222 nm	Overlaps with Paracetamol
Derivative Used	1st/2nd order	Separates both clearly