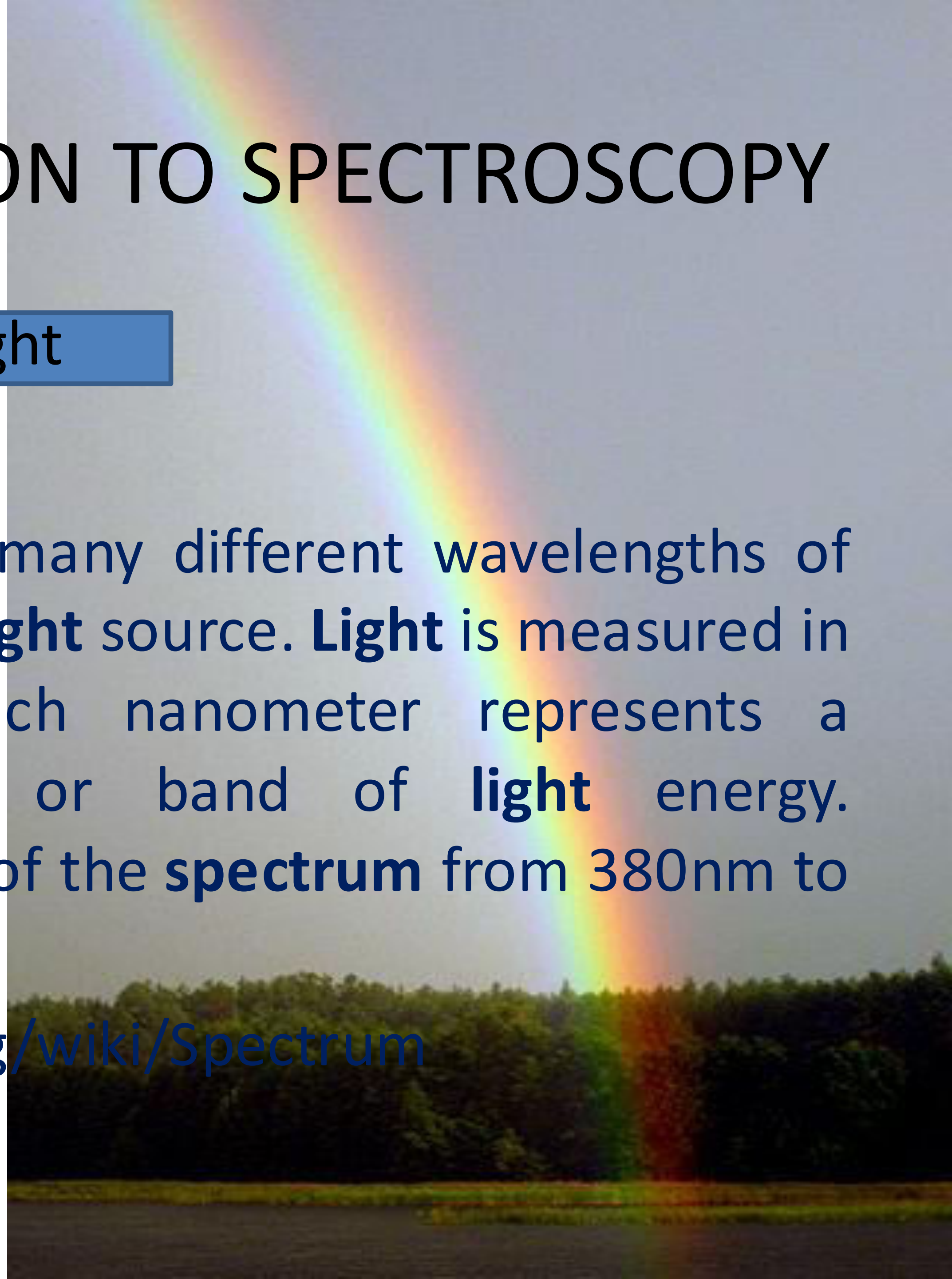
INTRODUCTION TO SPECTROSCOPY



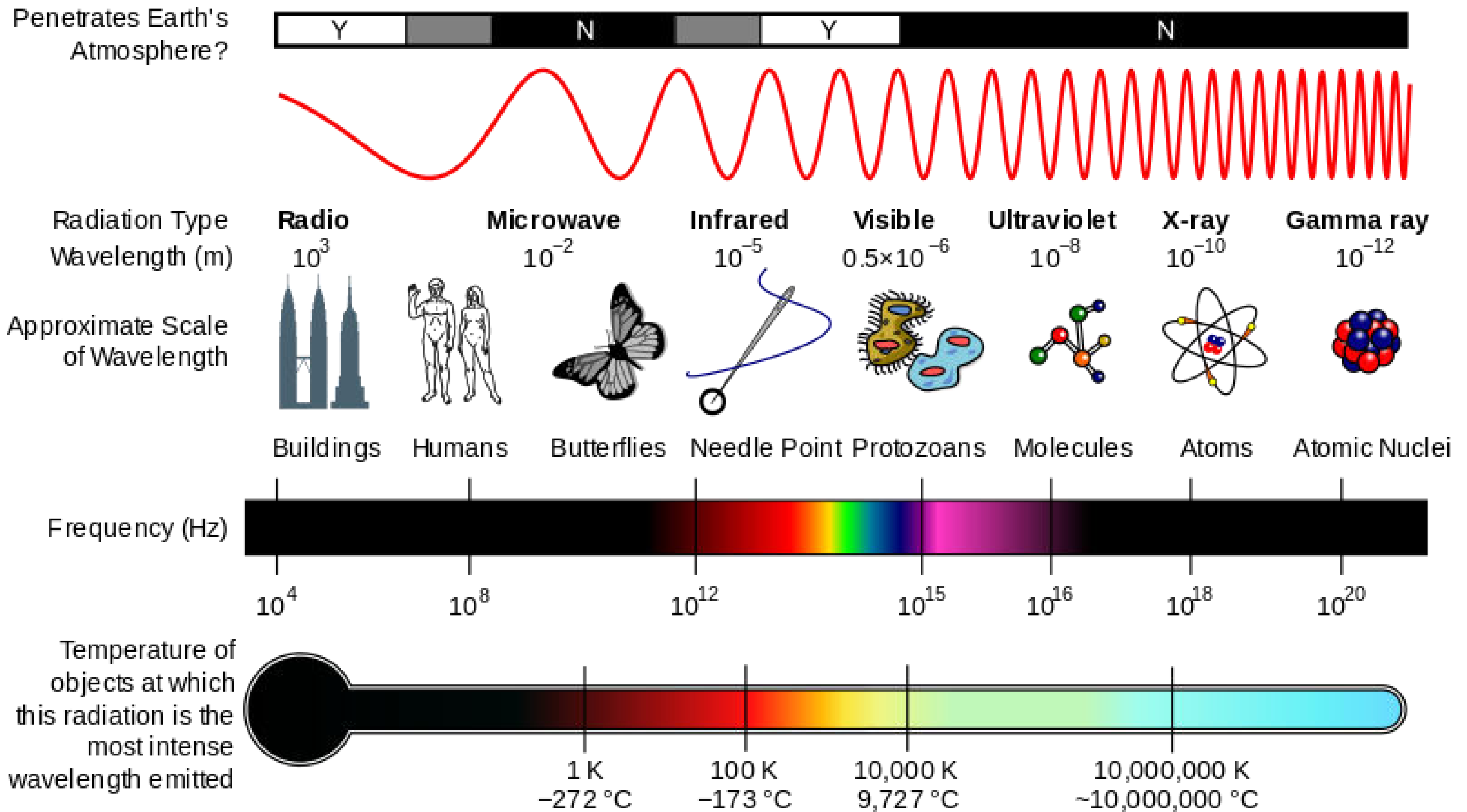
Light & Spectrum of light

Sir Isaac Newton:

Light spectrum is the many different wavelengths of energy produced by a **light** source. **Light** is measured in nanometers (nm). Each nanometer represents a wavelength of **light** or band of **light** energy. Visible **light** is the part of the **spectrum** from 380nm to 780nm.

https://en.wikipedia.org/wiki/Spectrum

ELECTROMAGNETIC SPECTRUM



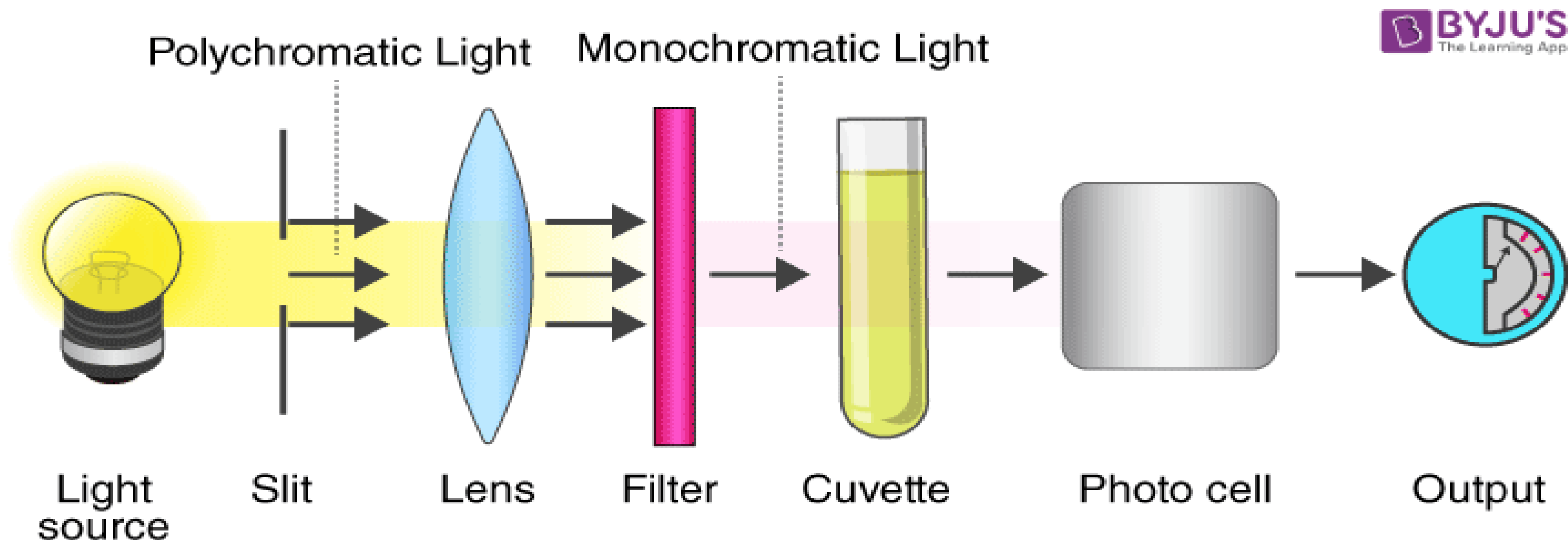
BASICS OF SPECTROSCOPY IS A COLORIMETER

A colorimeter is a device that is used in Colorimetry. It refers to a device which helps specific solutions to absorb a particular wavelength of light. The colorimeter is usually used to measure the concentration of a known solute in a given solution with the help of the Beer-Lambert law. The colorimeter was invented in the year 1870 by Louis J Duboscq.

Principle of Colorimeter

- It is a photometric technique which states that when a beam of incident light of intensity I_o passes through a solution, the following occur:
- A part of it is reflected which is denoted as I_r A part of it is absorbed which is denoted as I_a
- Rest of the light is transmitted and is denoted as I_t Therefore, $I_o = I_r + I_a + I_t$
- To determine I_a the measurement of I_o and I_t is sufficient therefore, I_r is eliminated. The amount of light reflected is kept constant to measure I_o and I_t.
- Colorimeter is based on two fundamental laws of photometry. We have discussed them below:
- Beer's law: According to this law the amount of light absorbed is proportional to the solute concentration present in solution. $\log 10\ I_o/I_t = a_s c$
- where, a_s is absorbency index, c is the concentration of solution
- Lambert's law: According to this law the amount of light absorbed is proportional to the length as well as thickness of the solution taken for analysis.
- $A = \log 10\ I_o/I_t = a_s b$
- Where, A is the test absorbance of test, a_s is the standard absorbance
- b is the length / thickness of the solution

Colorimeter



SPECTROPHOTOMETRY/SPECTROSCOPY

- Spectroscopy is the study of the interaction of electromagnetic radiation with matter. When matter is energized (excited) by the application of thermal, electrical, nuclear or radiant energy, electromagnetic radiation is often emitted as the matter relaxes back to its original (ground) state.
- The spectrum of radiation emitted by a substance that has absorbed energy is called an emission spectrum.
- The resulting spectrum from the substance contains the original range of radiation with dark spaces that correspond to missing, or absorbed frequencies.
- This type of spectrum is called an absorption spectrum. In spectroscopy the emitted or absorbed radiation is usually analyzed, i.e., separated into the various frequency components and the intensity is measured by means of an instrument called spectrometer.

- The resultant spectrum is mainly a graph of intensity of emitted or absorbed radiation versus wavelength or frequency.
- There are in general three types of spectra: continuous, line, and band.
- The sun and heated solids produce continuous spectra in which the emitted radiation contains all frequencies within a region of the electromagnetic spectrum.
- A rainbow and light from a light bulb are examples of continuous spectra.
- Line spectra are produced by excited atoms in the gas phase and contain only certain frequencies, all other frequencies being absent. Each chemical element of the periodic chart has a unique and, therefore, characteristic line spectrum.
- Band spectra are produced by excited molecules emitting radiation in groups of closely spaced lines that merge to form bands.
- These categories of emission and absorption spectra contain tremendous amounts of useful information about the structure and composition of matter.

- Spectroscopy is a powerful and sensitive form of chemical analysis, as well as a method of probing electronic and nuclear structure and chemical bonding. The key to interpreting this spectral information is the knowledge that certain atomic and molecular processes involve only certain energy ranges.
- Much of the scientific knowledge of the structure of the universe, from stars to atoms, is derived from interpretations of the interaction of radiation with matter.
- The regions of the electromagnetic spectrum and the associated energy transitions that occur in atomic and molecular processes is given in the following figure

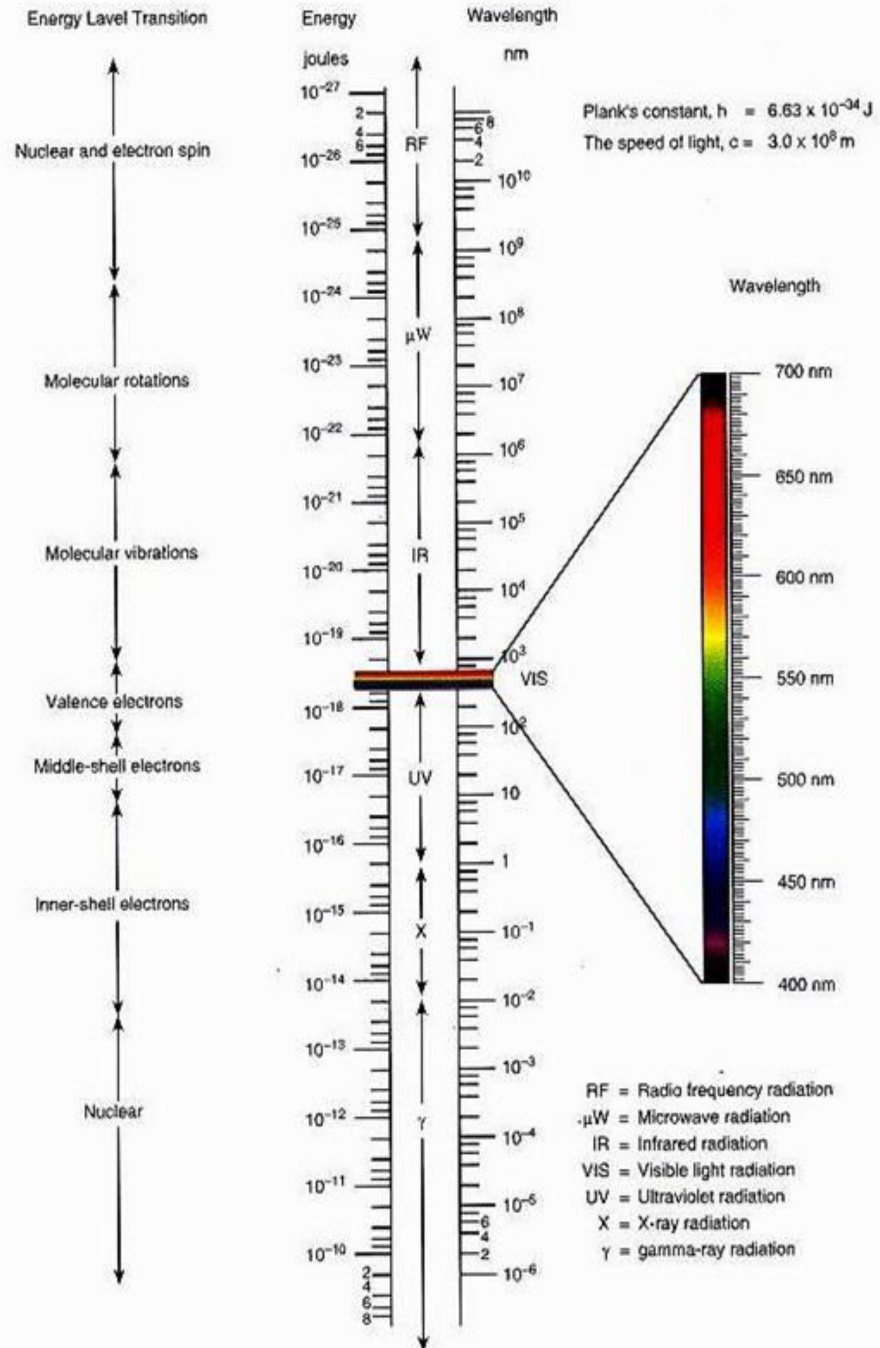


Fig. 12.1: The electromagnetic radiation ruler

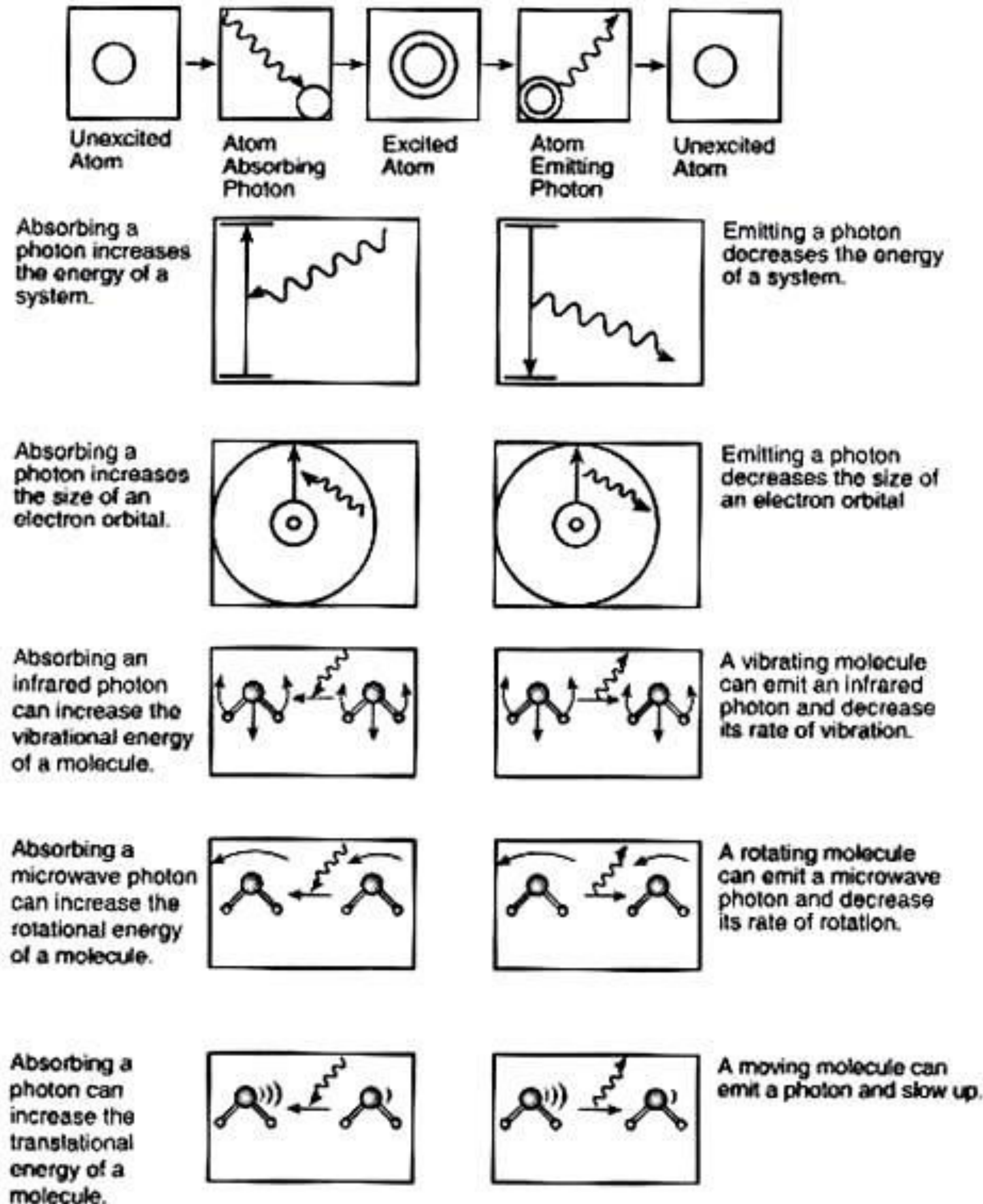


Fig. 12.2: Fundamental processes during spectroscopy

Types of Spectroscopy

- Spectroscopic techniques have been classified mainly on two parameters,

A) Type of radiation to be measured :

i. Electromagnetic spectroscopy involves interactions with electromagnetic radiation, or light. Ultraviolet-visible spectroscopy is an example.

ii. Electronic spectroscopy involves interactions with electron beams. Auger spectroscopy involves inducing the Auger effect with an electron beam.

iii. Mechanical spectroscopy involves interactions with macroscopic vibrations, such as phonons. An example is acoustic spectroscopy, involving sound waves.

iv. Mass spectroscopy involves the interaction of charged species with a magnetic field, giving rise to a mass spectrum. The term "mass spectroscopy" is deprecated in favour of mass spectrometry, tor the technique is primarily a form of measurement, though it doe- produce a spectrum for observation.

B) Measurement procedure employed:

Either atomic or molecular (based on whether or not they apply to atoms or molecules).

Spectroscopy based on Nature of interaction

i) Absorption spectroscopy uses the range of the electromagnetic spectra in which a sub stance absorbs. This includes atomic absorption spectroscopy and various molecular techniques, such as infrared spectroscopy in that region and nuclear magnetic resonance (NMR) spectroscopy in the radio region.

ii) Emission spectroscopy uses the range of electromagnetic spectra in which a substance radiates (emits). The substance first must absorb energy. This energy can be from a variety of sources, which determines the name of the subsequent emission, like luminescence. Molecular luminescence techniques include spectrofluorimetry.

iii. Scattering spectroscopy measures the amount of light that a substance scatters at certain wavelengths, incident angles, and polarisation angles. The scattering process is much faster than the absorption/emission process. One of the most useful applications of light scattering spectroscopy is Raman spectroscopy.

Ultraviolet-visible spectroscopy

- Ultraviolet-visible spectroscopy or ultraviolet-visible spectrophotometry (UV/VIS) involves the spectroscopy of photons and spectrophotometry. It uses light in the visible and adjacent to ultra violet (UV) and near infrared (NIR) ranges. In this region of energy space, molecules undergo electronic transitions.
- All atoms absorb in the UV region because photons are energetic enough to excite outer electrons. If the frequency is high enough, photo-ionisation takes place. UV spectroscopy is also used in quantifying protein and DNA concentration as well as the ratio of protein to DNA concentration in a solution.
- Several amino acids usually found in protein, such as tryptophan, absorb light in the 280 nm range and DNA absorbs light in the 260 nm range. For this reason, the ratio of 260/280 nm absorbance is a good general indicator of the relative purity of a solution in terms of these two macromolecules. Reasonable estimates of protein or DNA concentration can also be made this wav using Beer’s law.

Beer-Lambert Law

Beer-Lambert Law:

The Beer-Lambert law (also called the Beer-Lambert-Bouguer law or simply Beer's law) is the linear relationship between absorbance and concentration of an absorber of electromagnetic radiation.

The general Beer-Lambert law is usually written as:

$A = a_\lambda \times b \times c$,

where A is the measured absorbance, a_λ is a wavelength-dependent absorptivity coefficient, b is the path length, and c is the analyte concentration. When working in concentration units of molarity, the Beer-Lambert law is written as;

$A = \varepsilon_\lambda \times b \times c$,

where is the wavelength-dependent molar absorptivity coefficient with units of M^{-1} cm^{-1}. The λ subscript is often dropped with the understanding that a value for ε is for a specific wavelength. If multiple species that absorb light at a given wavelength are present in a sample, the total absorbance at that wavelength is the sum due to all absorbers:

$A = (\varepsilon_1 \times b \times c_1) + (\varepsilon_2 \times b \times c_2) + \ldots \ldots$,

where the subscripts refer to the molar absorptivity and concentration of the different absorbing species that are present.

- **Theory:**
- Experimental measurements are usually made in terms of transmittance (T), which is defined as:
- $T = P/P_0$,
- where P is the power of light after it passes through the sample and P_0 is the initial light power. The relation between A and T is:
- $A = -\log(T) = -\log(P/P_0)$.
- The figure shows the case of absorption of light through an optical filter and includes other processes that decrease the transmittance such as surface reflectance and scattering.

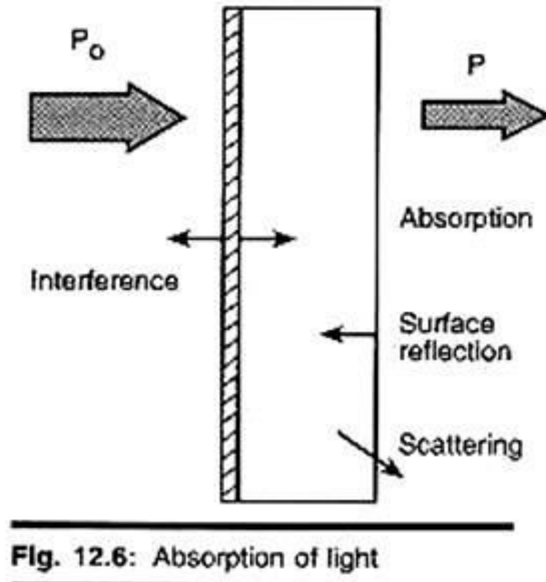


Fig. 12.6: Absorption of light

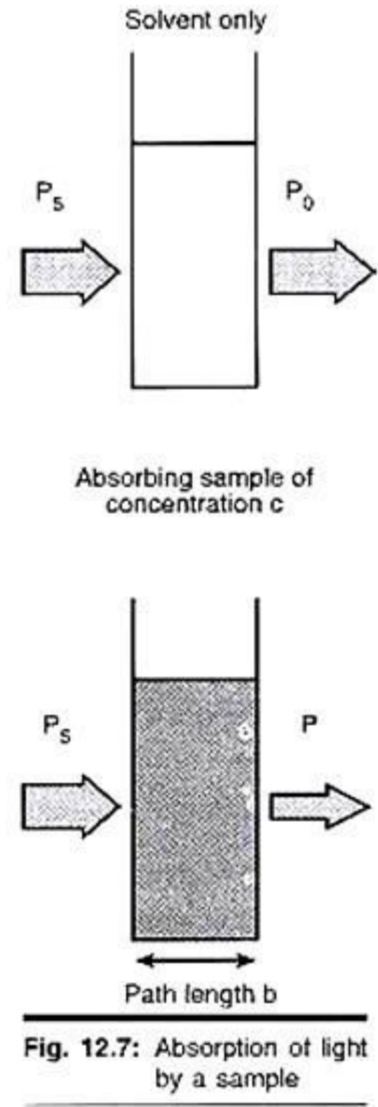


Fig. 12.7: Absorption of light by a sample

- P_s is the source light power that is incident on a sample, P is the measured light power after passing through the analyte, solvent, and sample holder, and P_0 is the measured light power after passing through only the solvent and sample holder. The measured transmittance in this case is attributed to only the analyte.
- Depending on the type of instrument, the reference measurement (top diagram) might be made simultaneously with the sample measurement (bottom diagram) or a reference measurement might be saved on I computer to generate the full spectrum.

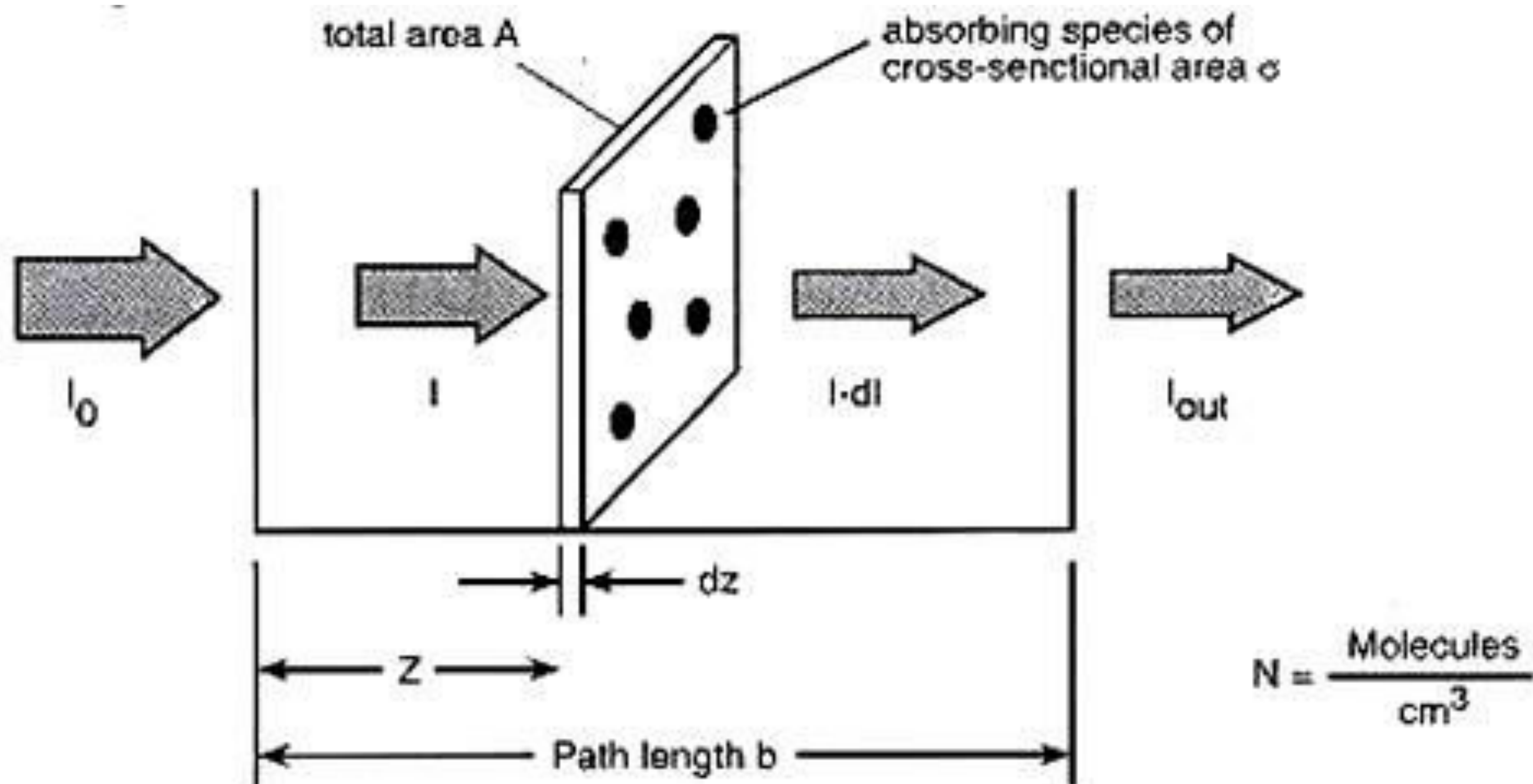


Fig. 12.8: Details of absorption

Spectroscopy – Types

The eight types of spectroscopy techniques are as follows:

1. Infrared (IR) Spectrophotometry
2. Circular Dichromism (CD) Spectroscopy
3. Spectrofluorimetry
4. Luminometry
5. Atomic/Flame Spectrophotometry
6. Electron Spin Resonance (ESR) Spectrometry
7. Nuclear Magnetic Resonance (NMR) Spectrometry
8. Mass Spectrometry.

1. Infrared (IR) Spectrophotometry

IR-light was used in this spectrophotometric analysis, Infra-red spectrophotometry with Gas-Liquid Chromatography or gas analysis techniques often used as a powerful technique for drug metabolism study and also provides a convenient and sensitive means of detecting and measuring differences in the concentration of gases such as carbon monoxide, carbon dioxide and acetylene in biological samples.

- **The light sources in different spectrophotometric techniques are:**

Light Source	Range
1. Tungsten lamp	Visible range (400 to 700 nm)
2. Hydrogen or Deuterium lamp	UV range (200 to 400 nm)

2. Circular Dichromism (CD) Spectroscopy

Information on the three-dimensional structure (conformation) of macromolecules in solution can be obtained by studying their absorption of polarized light, using Circular dichromism (CD) spectroscopy. CD spectroscopy measures this differential absorption of right (R) and left (L) circularly polarized light as a function of wavelength.

The main components of a CD spectrometer are illustrated in Fig. 1.2. Usually L and R circularly polarized radiation is produced from a single monochromator by passing plane-polarized light through an electro-optic modulator.

This is a crystal subject to alternating currents that transits either the R or L component of light dependent on the polarity of the electric field to which it is exposed. The photomultiplier detector produced a voltage in proportion to the ellipticity of polarization of the combined beam falling on the photomultiplier. CD-spectroscopy is often done to get information about the protein conformation (L, B and random coil) and also to study the conformation of nucleic acids.

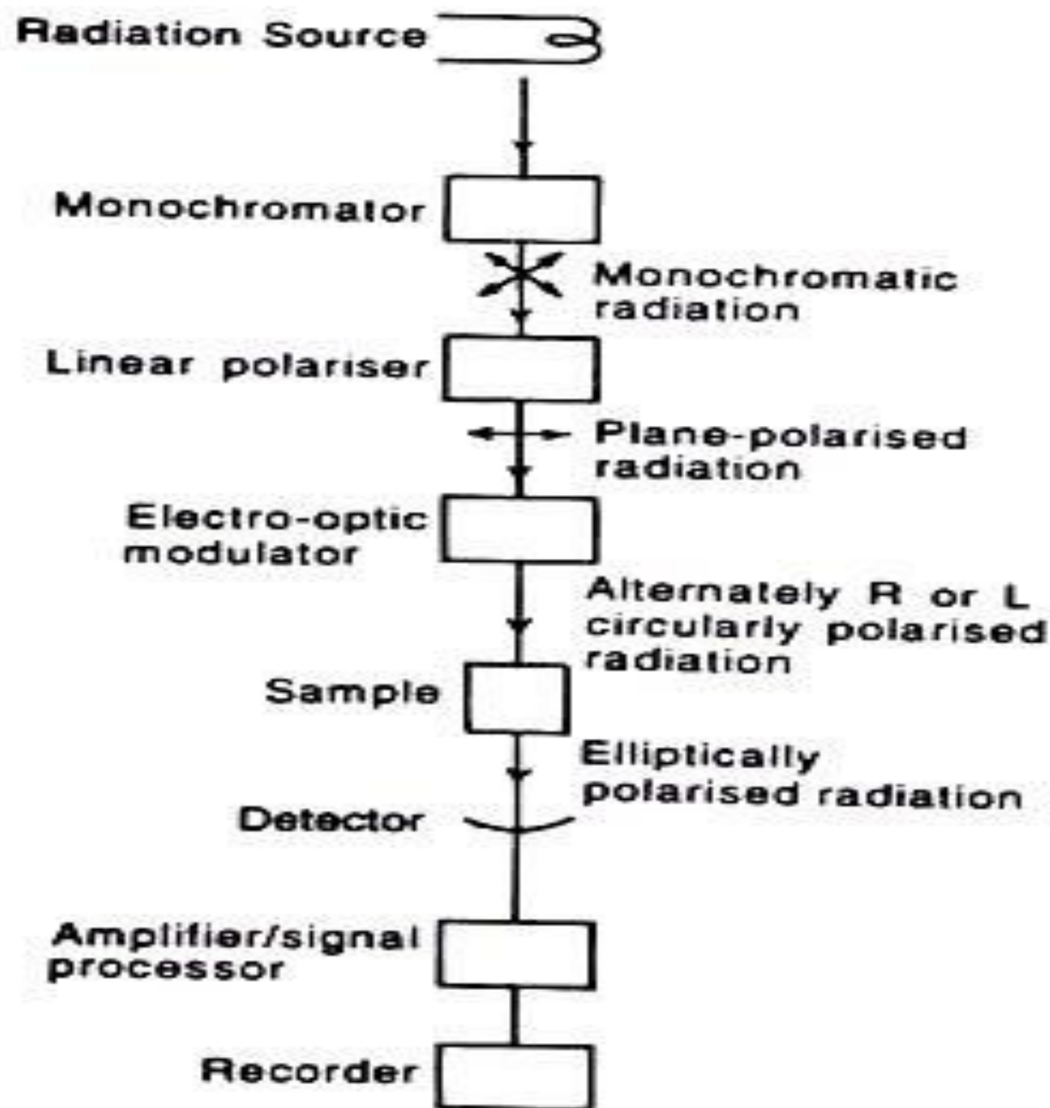


Fig. 1.2 : Optical path in CD-spectroscopy

3. Spectrofluorimetry

Spectrofluorimetry is a specialized spectroscopic method where two monochromators may be used, one selecting the activating wavelength and the other the phorescent wavelength.

No reference cuvette is required, but a calibration curve must be constructed. This method is most accurate at very low concentrations when absorption spectrophotometry is least accurate.

The sensitivity of the instrument is usually easily adjusted over a large range by amplification of the current produced in the photocell circuit.

The basic components of a complete spectra-fluorimeter are mentioned in Fig. 1.3.

This includes a continuous spectrum source (mercury lamp or Xenon arc) a monochromator (M) for irradiating the specimen with any chosen wavelength, a second monochromator (M_2) which, under conditions of constant irradiating wavelengths, enables the determination of I .v fluorescent spectrum of the specimen; and a detector which is usually a sensitive photocell.

This technique is often used for the quantitative study of Vitamin B,, NADH Cork set and other. Group-specific hydrolases may be readily assayed by measuring the rate of appearance of fluorescence of 450 nm of the amino of 4-methylumbellifore one when the enzyme acts upon an ether of ester derivative of 4-methyl umbelliferone.

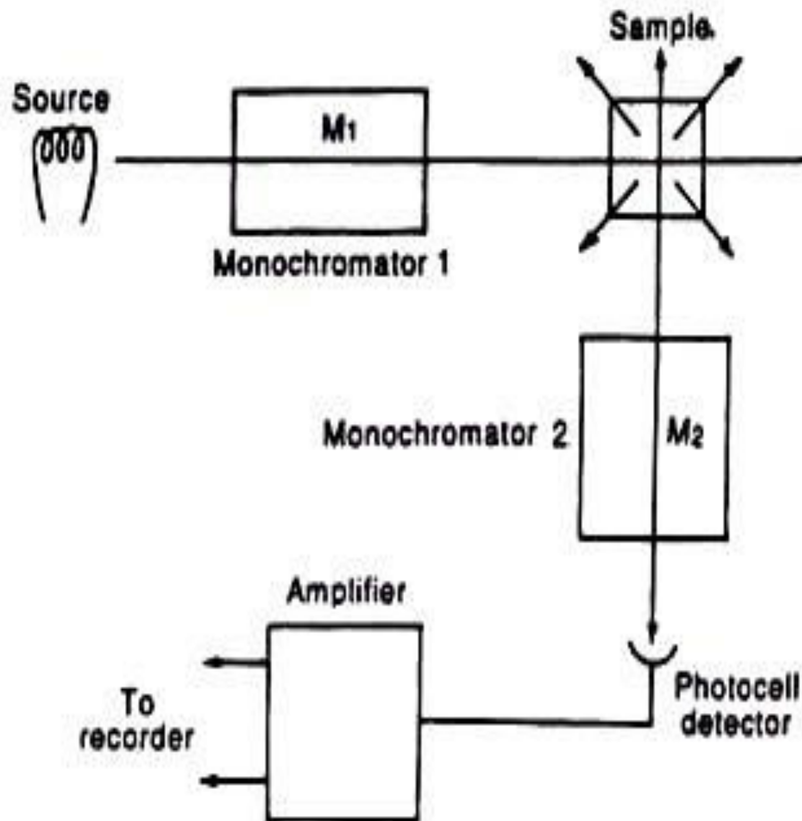


Fig. 1.3 : The basic components of as Spectrofluorimeter

4. Luminometry

- This is a photometric technique in which light — enlisted as a result of a chemical reaction (luminescence) in contrast to the result of a physical reaction (fluorescence or atomic emission) — is measured in a luminometer.
- Luminometers are relatively simple photometers, complicated only slightly by the need to amplify and record the signal from the photocell.
- It has a photomultiplier tube with a well-stabilized high voltage power supply to ensure sensitive, reproducible measurement of light emission, a direct current amplifier with a wide range of sensitivity and linear response and a reaction chamber which allows temperature control, adequate mixing of reactants and protection from extraneous light (Fig. 1.4).

This technique is often used for determining ATP concentration, study of bacterial luciferance system, and chemo luminescence.

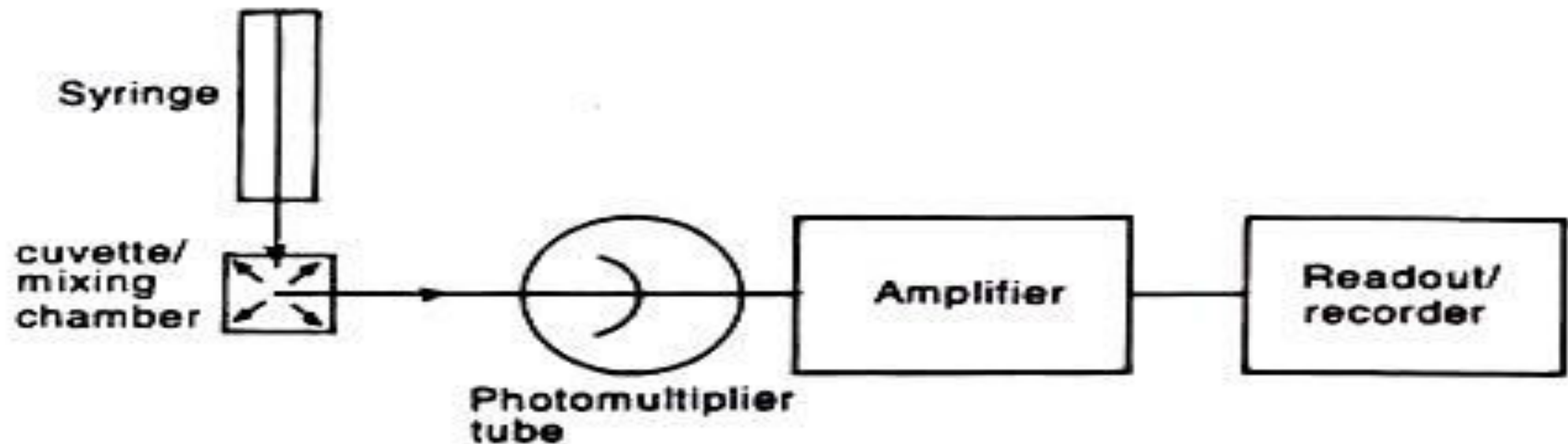


Fig. 1.4 : Light path in Luminometer

5. Atomic/Flame Spectrophotometry

Volatilisation of atoms — either in a flame or electro thermally — causes them to emit and absorb light of specific wavelengths. Atomic/flame spectrophotometry takes advantage of the specificity of line spectra to determine the amounts of a specific elements present. Emission flame spectrophotometry measures the emission of light of a specific wavelength by atoms in a flame.

Atomic absorption spectrophotometry measures the absorption of a beam of monochromatic light by atoms in a flame or alternatively by atoms heated electro thermally in a graphite furnace. The energy absorbed is proportional to the number of atoms present in the official path.

The amount of radiation emitted is proportional to the number of excited atoms present, which depends on the temperature and compositions of the flame. Standard additions must always be used to calibrate the system.

The basic components of an atomic (flame) emission spectrophotometer are: nebulizer, burner, monochromator, detector and read-out unit. Nebulizers are usually of the scent spray type, in which a forced stream of air passes over a capillary tube dipping into the test solution.

Large and small drops of sample solution then passes along with air-stream into the burner. Then light thus emitted is passed through the monochromator or filter. Finally, emitted light was detected and read out.

In order to produce a beam of radiation with a very narrow band, either a source of white light plus a double monochromator, or a hollow cathode discharge lamp is used. The discharge lamps are specific to the element being assayed.

These techniques are often used to detect the metallic elements present in the inorganic and organic compounds — more than 20 elements in biological samples, particularly Ca^{8}, Mg^{++}, Mn^{++}, Zn^{++}, Ca^{++}, Pb^{++}, Cr^{++}, Ni^{++} etc. When assaying metal in biological samples it is usual to degrade organic molecules by ashing.

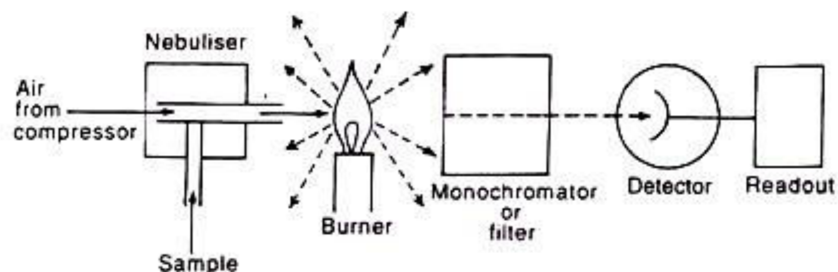


Fig. 1.5 : The major components of atomic emission Spectrophotometer

The detection limits for various elements in emission and absorption flame spectrophotometry are depicted below:

	Emission		Absorption	
Element	Detection Limit (PPM)	Wavelength (nm)	Detection (PPM) Limit	Wavelength (nm)
Calcium	0.005	442.7	0.1	442.7
Copper	0.1	324.8	0.1	324.8
Iron	0.5	372.0	0.2	248.3
Lead	—	—	0.5	283.3
Magnesium	0.1	285.2	0.01	285.2
Manganese	0.02	403.3	0.05	279.5
Sodium	0.0001	589.0	0.03	589.0
Potassium	0.001	766.5	0.03	766.5

6. Electron Spin Resonance (ESR) Spectrometry

This is a technique used for detection of Paramagnetism, i.e. the magnetic movement associated with an unpaired Electron Paramagnetic Resonance (EPR). The technique may be used for detecting transition metal ions and their complexes, free radicals and excited states.

The basic components of an ESR spectrometer are Klystron source, Metal waveguide tube, Field magnet, Sweep coils, Detector, Amplifier, Recorder and Oscillator. Electromagnets generating fields of 50 to 100 millitesla with a uniformity of 1 in 10^6 are required for accurate work.

Most experiments are conducted at around 330 millitesla, in conjunction with an auxiliary sweep of 10 to 100 millitesla. Klystron oscillator produces the monochromatic miss-wave radiation usually with a wavelength of about 3×10^2 m (9,000 MH_2). Samples must be in the solid state, so biological samples are usually frozen in liquid.

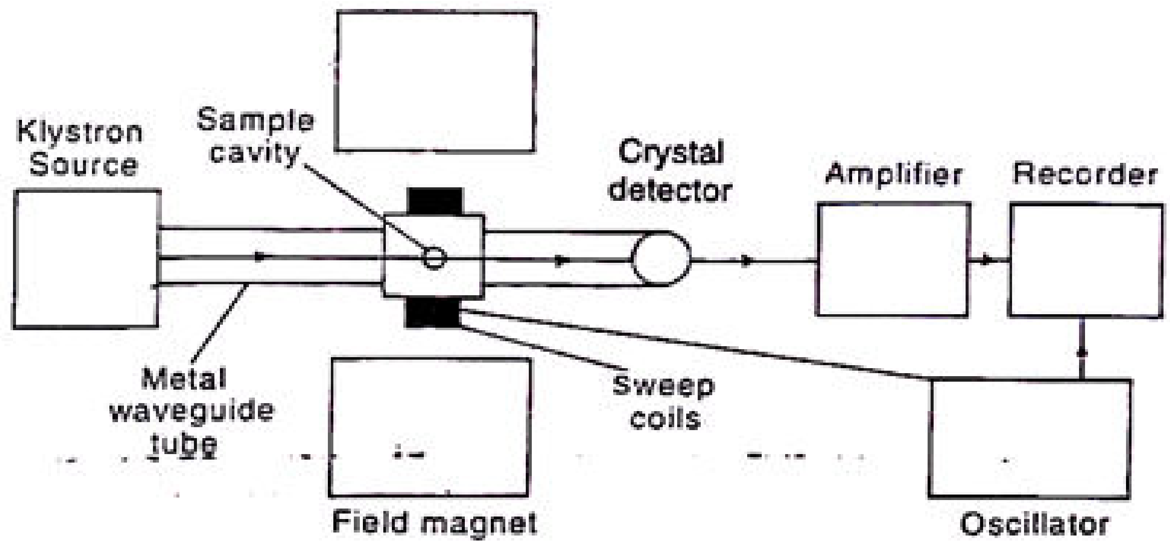


Fig. 1.6 : The main components of an electron spin resonance spectrometer. ESR Spectrometry is one of the main methods used to study metals, particularly those containing mobybdenum, copper and iron

7. Nuclear Magnetic Resonance (NMR) Spectrometry

- NMR-technique is used for detecting atoms which have nuclei that possess a magnetic movement. These are usually atoms containing an odd number of protons in their nuclei. In the same way that pairs of electrons in the same atomic orbital have opposite spin and no resultant magnetic movement, pairs of protons in a nuclei do not have a magnetic moment.
- However, an odd portion in a nudens imports a magnetic moment to the molecule which can interest with an applied magnetic field. This interaction is major concern of nuclear magnetic resonance Spectrometry.
- In a magnetic field of several hundred millitesla (several thousand gauss) such nuclei absorb radiation in the resonance spectrum, giving rise to the phenomenon known as nuclear magnetic resonance (NMR). Most studies are conducted using Hydrogen (H).

- The basic components of an NMR Spectrometer are similar to those illustrated for an ESR-Spectro- meter. A radio frequency transmitter in place of a Klystron source used to irradiate the sample. For DMR the sample must be dissolved to a relatively high concentration in a solvent which lacks protons, such as D_20 or $CDCl_2$.
- To minimize variations in the magnetic field, the sample is contained in a tube of high precision diameter and is usually rotated at high speed by an air turbine. The absorption signal, detected by a radio receiver, is amplified and recorded.
- The major use of NMR is for the study of the molecular structure of relatively simple organic molecules. This structural information relevant to the biological action of the antibiotics has also been obtained from NMR studies. NMR has also been particularly useful in the study of phosphate compounds viz., AMP, ADP, ATP, and phosphocreatine.

8. Mass Spectrometry

It is based upon the principle that moving ion may be deflected by a magnetic field to an extent that is dependent upon its mass and velocity.

Thus ions of a larger momentum are deflected less than ones of lower momentum, whilst a mixture of ions of different mass but constant velocity will be deflected in proportion to their mass.

So, in a Mass Spectrometer, molecules of a compound are ionised, either by ejection of an electron or capture of a proton, to give the parent molecular ion the energy which is such that some fragmentation occurs to give a series of fragment ions.

Knowledge of the mass of the molecular ion and its major fragment ions is frequently sufficient to enable the structure of the parent compound to be uniquely deduced.

- This method is very sensitive and often used for as little as 10^{-6} to 10^{-9} g of material.
- Mass spectra show a series of peaks or lines corresponding to the m/c values of the positive ions produced from the compound.
- The height of the peaks corresponds to the relative abundance of the ions.
- A reference ion of similar m/c value to that of the parent ion is used to calibrate the mass axis (abscissa) of the intensity spectrum.
- The parent ion is the peak with the greatest mass, although it is not necessarily the most abundant (base peak).
- Ion intensities in a mass spectrum are usually recorded as percentage of the intensity of the base peak.

- The basic components of a Mass Spectrometer are
  - Ionization chamber vacuum pump unit,
  - Electrostatic field,
  - Ion trajectory,
  - Magnetic field,
  - Detector,
  - Amplifier and
  - Recorder.

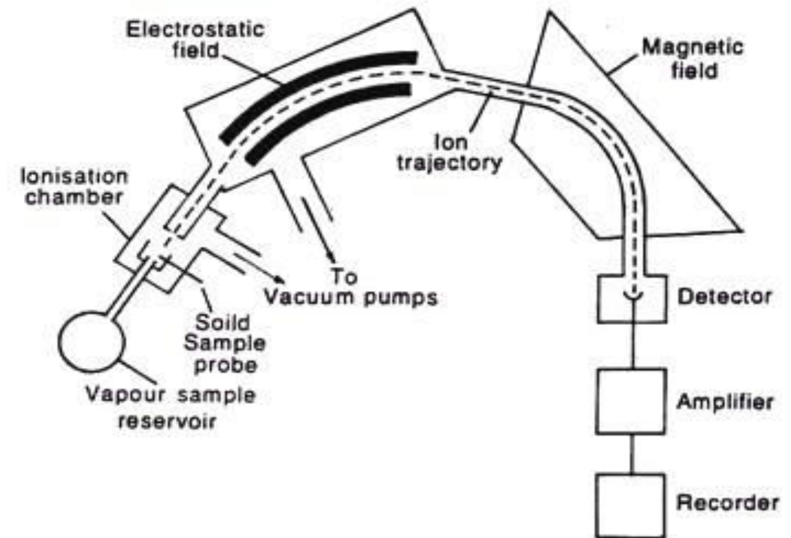
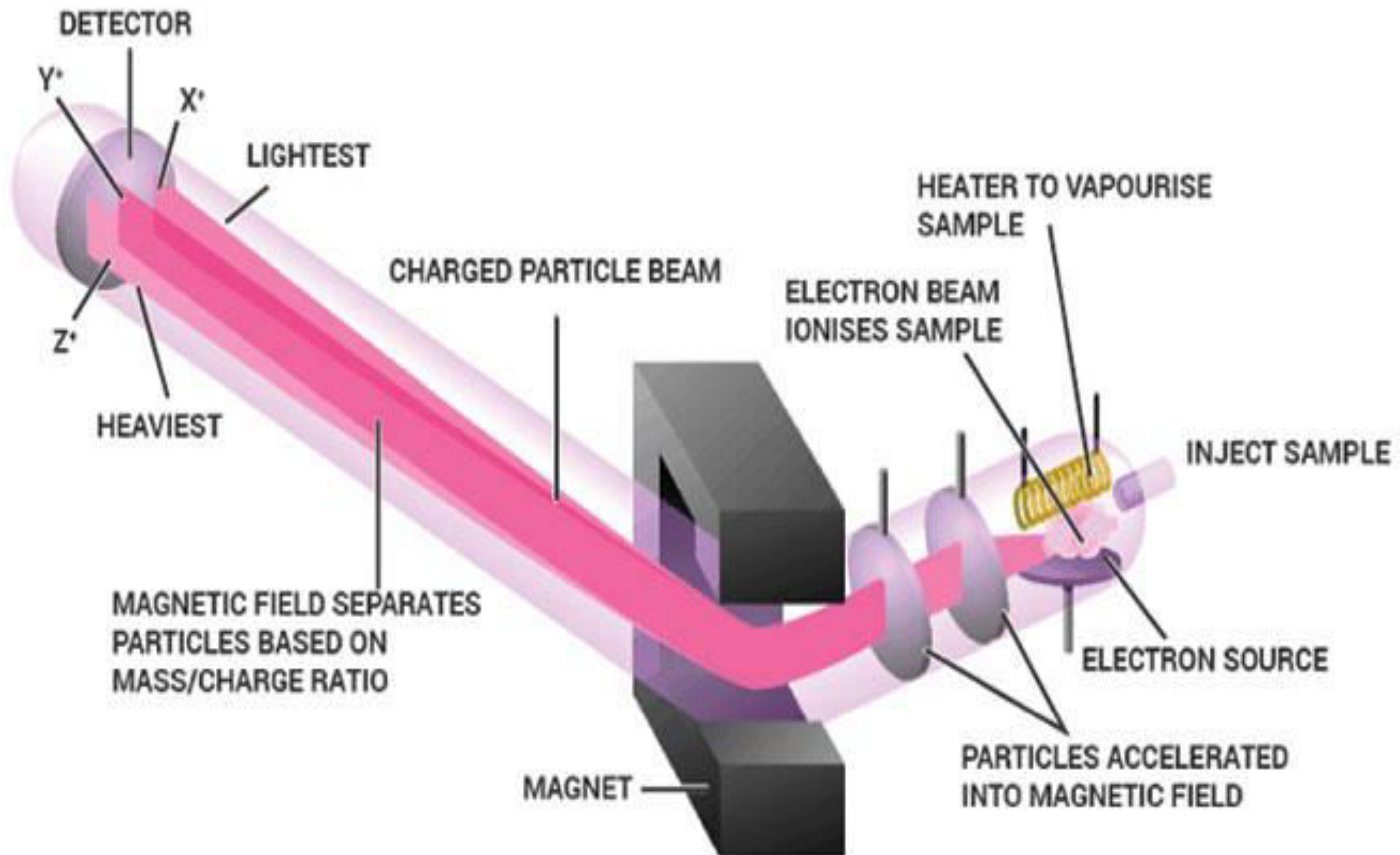


Fig. 1.7 : Basic components of a Mass Spectrometer

- The earliest biochemical uses of mass spectrometry were in the study of metabolic pathways —
- particularly the determination of chemical structure and, hence, the identification of compounds.
- This technique has also been used to determine the amino acid sequence of oligopeptides derived from protein hydrolysate and other sources.

Mass Spectrometry (MS)
Principle, Working, Instrumentation, Steps, Applications



- Mass Spectrometry (MS) is an analytical chemistry technique that helps identify the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions.
- In this instrumental technique, sample is converted to rapidly moving positive ions by electron bombardment and charged particles are separated according to their masses.
- Mass spectrum is a plot of relative abundance against the ratio of mass/charge (m/e).
- These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules and other chemical compounds.

Principle of Mass Spectrometry (MS)

In this technique, molecules are bombarded with a beam of energetic electrons.

The molecules are ionized and broken up into many fragments, some of which are positive ions. Each kind of ion has a particular ratio of mass to charge, i.e. m/e ratio (value).

For most ions, the charge is one and thus, m/e ratio is simply the molecular mass of the ion.

The ions pass through magnetic and electric fields to reach detector where they are detected and signals are recorded to give a mass spectra.

Working of Mass Spectrometry (MS)

In a typical procedure, a sample, which may be solid, liquid, or gas, is ionized, for example by bombarding it with electrons.

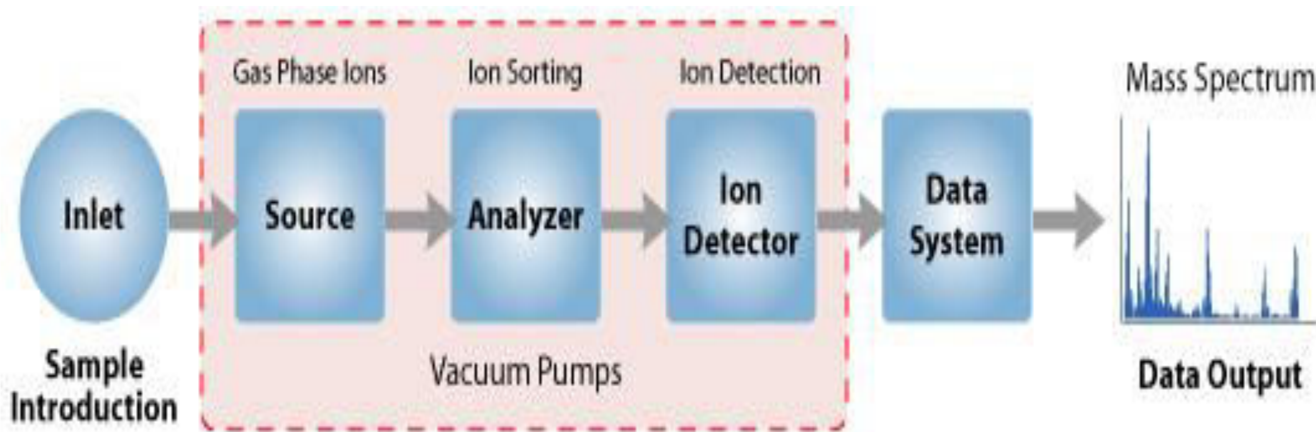
This may cause some of the sample's molecules to break into charged fragments. These ions are then separated according to their mass-to-charge ratio, typically by accelerating them and subjecting them to an electric or magnetic field:

Ions of the same mass-to-charge ratio will undergo the same amount of deflection.

The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio.

The atoms or molecules in the sample can be identified by correlating known masses (e.g. an entire molecule) to the identified masses or through a characteristic fragmentation pattern.

Instrumentation and Steps of Mass Spectrometry



A. Sample Inlet

Sample stored in large reservoir from which molecules reaches ionization chamber at low pressure in steady stream by a pinhole called "Molecular leak".

B. Ionization

Atoms are ionized by knocking one or more electrons off to give positive ions by bombardment with a stream of electrons. Most of the positive ions formed will carry charge of +1.

Ionization can be achieved by :

- Electron Ionization (EI-MS)
- Chemical Ionization (CI-MS)
- Desorption Technique (FAB)

C. Acceleration

Ions are accelerated so that they all have same kinetic energy.

Positive ions pass through 3 slits with voltage in decreasing order.

Middle slit carries intermediate and finals at zero volts.

D. Deflection

Ions are deflected by a magnetic field due to difference in their masses.

The lighter the mass, more they are deflected.

It also depends upon the no. of +ve charge an ion is carrying; the more +ve charge, more it will be deflected.

E. Detection

The beam of ions passing through the mass analyzer is detected by detector on the basis of m/e ratio.

When an ion hit the metal box, charge is neutralized by an electron jumping from metal on to the ion.

Types of analyzers:

- Magnetic sector mass analysers
- Double focussing analysers
- Quadrupole mass analysers
- Time of Flight analysers (TOF)
- Ion trap analyser
- Ion cyclotron analyser

Applications of Mass Spectrometry (MS)

- Environmental monitoring and analysis (soil, water and air pollutants, water quality, etc.)
- Geochemistry – age determination, soil and rock composition, oil and gas surveying
- Chemical and Petrochemical industry – Quality control
- Identify structures of biomolecules, such as carbohydrates, nucleic acids
- Sequence biopolymers such as proteins and oligosaccharides
- Determination of molecular mass of peptides, proteins, and oligonucleotides.
- Monitoring gases in patients breath during surgery.
- Identification of drugs abuse and metabolites of drugs of abuse in blood, urine, and saliva.
- Analyses of aerosol particles.
- Determination of pesticides residues in food.

THANK YOU