Uttar Pradesh Rajarshi Tandon
Open University

PGBCH-102

Analytical Biochemistry

COURSE INTRODUCTION

The objective of this course is to provide knowledge of Analytical Biochemistry. This course cover the brief discussion of different instrumentation tools and techniques required in biochemistry. The course is organized into following blocks :

Block-1 covers the spectroscopy and chromatography techniques

Block-2 deals the centrifugation and electrophoresis techniques

Block-3 describes in brief techniques of microscopy, X-ray diffraction and NMR.

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Analytical Biochemistry

BLOCK

1

SPECTROSCOPY AND CHROMATOGRAPHY

UNIT-1

Spectroscopy

UNIT-2

Chromatography

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BLOCK INTRODUCTION

This is the first block of Analytical biochemistry. It consists of following two units :

Unit-1 : This unit covers the general introduction of absorption, spectroscopy. The details discussed here of atomic adsorption spectroscopy and uv-visible spectroscopy viz. definition, principal, types, instrumentation and application. The spectroscopy, considered as one of the most valuable techniques for the detection of biological sample and useful in qualitative and quantitative detection of chemical compounds.

Unit-2 : This unit covers the history and types of chromatographic in brief. The working principle, instrumentation and application of thin layer, affinity, ion exchange, high performance chromatography are discussed briefly. The chromatography is powerful techniques that used to separate mixture of substance into their individual components. All types of chromatography work on the same principle. There are two phases like stationary and mobile phase exists in all types of chromatography.

UNIT-1 SPECTROSCOPY

1.1 INTRODUCTION

The word spectroscopy comes from the Latin word *specere*, means "to look at" and the Greek word *skopia*, means "to see". So that in spectroscopy refers the techniques that employ light to produce properties of object. However, the light interacts with matter and probe certain features of sample to learn about it consistency or structure. Light is electromagnetic radiation that comprises several regions of wavelength. The electromagnetic radiations have different energy level radiations which gives phenomenon to probe different molecular features'. The spectroscopy methods are the most useful in biochemistry. The visible spectrum is the portion of the electromagnetic radiation that is visible to the human eye. Unlike X-ray, Gamma ray, the UV ray spectroscopy also play important role in analytical techniques. The absorption wavelength is associated with transition that requires a minimum of energy change

Objectives :

- to understand the properties of electromagnetic radiation in spectroscopy .
- to details study of atomic spectroscopy
- sturdy of uv visible spectroscopy
- to understand the principle of Lambert-Beers law

1.2 SPECTROSCOPY OVERVIEW

Spectroscopy helps us to know how the incident radiation affects the sample of specimen. However, before going to details study of spectroscopy the understanding of properties of electromagnetic radiation and it interaction with matter is necessary to every student. The results of spectroscopic data depend on properties of electromagnetic radiation and interacting matter. Spectroscopic data are often represented by an emission spectrum or absorption spectrum. Spectroscopy is a fundamental exploratory tools in the fields of physical, chemical and biological science at atomic and macro scale level. However, the spectroscopy is also helpful in better understanding of tissue and medical image analysis.

1.3 PROPERTIES OF ELECTROMAGNETIC RADIATION

To better understanding of the spectroscopy we should know about the electromagnetic radiation, the interaction of electromagnetic radiations with matter is a quantum phenomenon. Electromagnetic radiation consists of radio waves, microwaves, infrared waves, visible light, ultraviolet radiation, X-rays, and gamma rays. Every radiation has different wavelength, frequency and energy. The wavelength refers for special distance between two consecutive peaks in sinusoidal waveform and is measured in nanometer (nm). The frequency (υ) of electromagnetic radiation defines the number of oscillator made by the wave within the timeframe of 1 second. The energy in electromagnetic radiation exists in the form of photons. The quantum phenomenon of electromagnetic radiation depends upon both; properties of the radiation and the appropriate structural part of the samples involves. The electromagnetic radiation is propagated through free space or through a medium and composed of both electric and magnetic waves. The electric and magnetic waves have oscillations that are perpendicular to each other and also to the direction of travel of the wave.

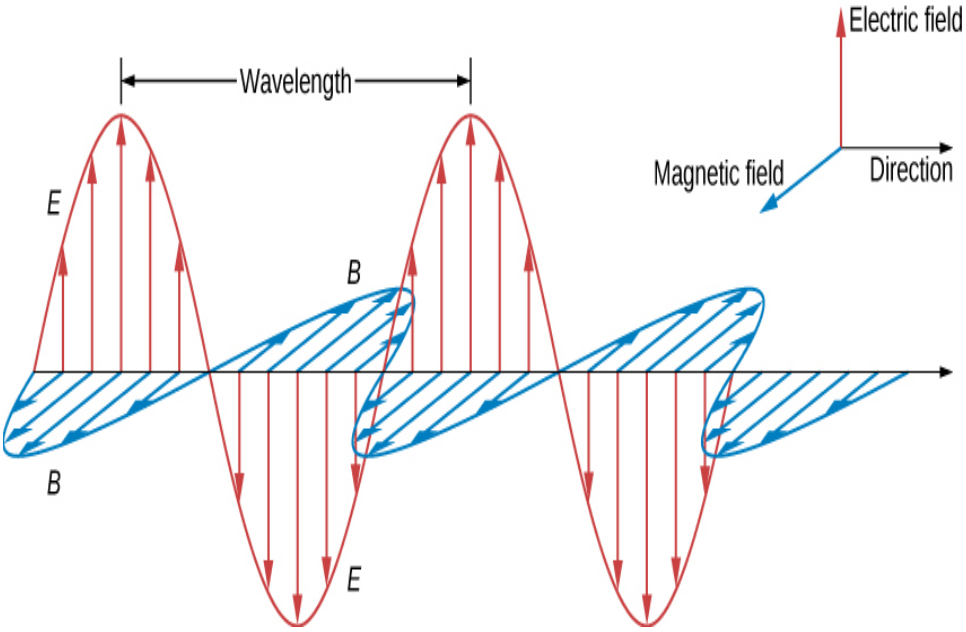

Fig. 1.1 : Nature of electromagnetic waves

There are many sources of electromagnetic radiation, both natural and man-made. Different electromagnetic radiations are given below in Table (1.1) along with their wavelength and frequency. As the wavelength of waves increases frequency get decreases.

Table 1.1 : Region of electromagnetic spectrum

Electromagnetic Rays	**Approximate wavelength range (in meters)**	**Frequency in Hz**
Radiowaves	>1	$3x\ 10^{9}$
Microwaves	$1\times10^{-3}-1$	$3x\ 10^{9}$-$3x\ 10^{11}$
Infrared	$7.0\times10^{-7}-1\times10^{-3}$	$3x\ 10^{11}$-$4x\ 10^{14}$
Visible	$4\times10^{-7}-7\times10^{-7}$	$4x\ 10^{9}$-$7.5\ x\ 10^{14}$
Ultraviolet	$1\times10^{-8}-4\times10^{-7}$	$7.5\ x\ 10^{14}$-$3\ x\ 10^{16}$
X-Rays	$1\times10^{-11}-1\times10^{-8}$	$3\ x\ 10^{16}$-$3\ x\ 10^{19}$
Gamma Rays	$<1\times10^{-11}$	$3\ x\ 10^{19}$

Wavelength units :

A° $=10^{-8}$ cm $= 10^{-10}$m also 1 nm $= 10^{-9}$m, similarly 1 Hz = 1 cycle/sec and 1MHz $= 10^{6}$ Hz

The nanometer has been earlier also designated as millmicron (mμ)

1 nm $= 10^{-6}$ μm $= 10^{-7} = 10^{-9}$m $= 10$A°

Frequency units :

$$1\ \text{Hz} = 1\ \text{cycle/ s}^{-1}$$

$$1\text{MHz (megacycle/sec)} = 10^3\ \text{kHz} = 10^6\ \text{Hz}$$

$$1\ \text{frequency} = 10^{12}\ \text{Hz}$$

Electromagnetic radiation interaction with matter :

We know the EMR annoted types of radiations and various interactions with matter and produce result. The electronic radiations have both properties of wave and particles. Albeit having a mass of zero. As particle, when EMR interact with matter transferring its energy E.

$$E = \frac{hc}{\lambda} = h \quad \text{as} \quad c = \upsilon\lambda \tag{1.1}$$

where h is plank's constant = 6.62 x 10-^{34} joule-second.

In order for a transition the energy must be absorbed. The energy change ΔE needed is defined in quantum terms by the difference in absolute energies between the final and the starting state. The energy change (ΔE) is measured in KJ/mole by following equation.

$$\Delta E = E_{final} - E_{intial} \tag{1.2}$$

From eq. 1.1 & 1.2 the energy will be

$$\Delta E = hv \tag{1.3}$$

Electrons in either atoms or molecules may be distributed between several energy levels but principally reside in the lowest energy level (ground state). In order for an electron to be promoted to a higher energy level (excited state), energy must be put into the system. If this energy E¼h_ is derived from electromagnetic radiation, this gives rise to an absorption spectrum, and an electron is transferred from the electronic ground state to into the first electronic excited state.

If we consider the matter in diatomic molecule, then rotational and vibration level possess discrete energies that only manage into a c continuum at very high energy. Each electronic state of a molecule possesses its own set of rotational and vibrational levels.

Electron either in atom or molecule may be distributed between several energy levels. Knowing $\Delta E = hv$ is derived from electromagnetic radiation this gives rise, to a spectrum and electron is transferred from lower energy level to higher excited state (S_1). The molecule will also be in an excited vibration and rotation state. Subsequent relaxation of the molecule into the vibrational ground state of the first electronic excited state will occur. The electron can then revert back to the electronic ground state. The plot of absorption probability against wavelength is called absorption spectrum. Single atom gives the line spectra while molecules produce band spectra.

Relation between wavelength, Frequency and wave number:

Wavelength (λ) and frequency υ are related as follows

$$c = \upsilon.\lambda \qquad (1.4.)$$

Since c is constant (c = 3 x 10^{10} cm/sec in vaccum. The above relation may be express as

$$\upsilon \propto \frac{1}{\lambda} \qquad (1.5)$$

Reciprocal of wavelength is called wave number

$$\frac{1}{\lambda} = \upsilon^{-}$$

The unit of wave number is cm^{-1}

Table: 1.2 : Difference between atomic and molecular spectroscopy

Atomic spectroscopy concerns only the properties of atoms. Atomic spectroscopy is the study of absorption of light by atom of molecule.	Molecular spectroscopy concerns the molecules which are infinitely more numerous. Molecular spectroscopy is the study of absorption of light by molecules.
Atomic spectra are the transitions of electrons between electronic energy levels in isolated atoms.	Molecular spectra involve transitions in molecules with two or more atoms either the same or different.
Atomic spectra are the transitions of electrons between electronic energy levels in isolated atoms. They are affected by interactions of the transitioning electrons with the nuclei spins and with the other electrons in the atom.	Molecular spectra involve transitions in molecules with two or more atoms (either the same or different). Since the valence electrons are now in different orbital from the atomic orbitals and the orbital structure has changed the electronic transitions are different.
Atomic spectroscopy we can find the nature and the amounts of a	Molecular spectroscopy we can find the nature and the amounts of

given element in the sample. Atoms excited by a high-temperature energy source this light emission is commonly called atomic or optical emission and obtained spectra is called atoms spectrum.	a given molecule in the sample. Molecule is excited by ultraviolet/visible radiation, this light emission is commonly called Molecular emission and obtained spectra is called molecular spectrum.
Atomic spectroscopy has three variations that are most commonly used in spectrochemical analysis, atomic absorption, atomic emission and atomic fluorescence.	molecules exhibit absorption in narrow lines which are very characteristic of the molecule as well as the temperature and pressure of its environment

1.4 ATOMIC SPECTROSCOPY

We know that theory of electromagnetic radiation that says the molecules give rise to band spectra whereas atom gives line spectra. Thus, the study of atomic level, the atomic spectroscopy is very useful to characterization of elements. The atomic spectroscopy leads to both atomic absorption and atomic emission spectra of an atom. The atomic emission occurs due to light of particular wavelength (color) converse. But in atomic absorption spectra occurs when the black line can be observed against a bright light. In general, atomic spectroscopy is not carried out in solution. In order for atoms to emit or absorb monochromatic radiation, they need to be volatilised by exposing them to high thermal energy. Usually, nebulisers are used to spray the sample solution into a flame or an oven. Alternatively, the gaseous form can be generated by using inductively coupled plasma (ICP).

The Absorption spectroscopy, as per name is based on the absorption of electromagnetic radiations by matter. Electron presents in an atom, absorb energy from electromagnetic radiations which fall on it and jump from ground state to excited state. This phenomenon is called absorption and spectroscopy related to it is termed as absorption spectroscopy. An absorption spectrum is measured as a function of wavelength. The absorption spectrum of an atom or molecule depends on its energy level structure and useful for identification of compounds.

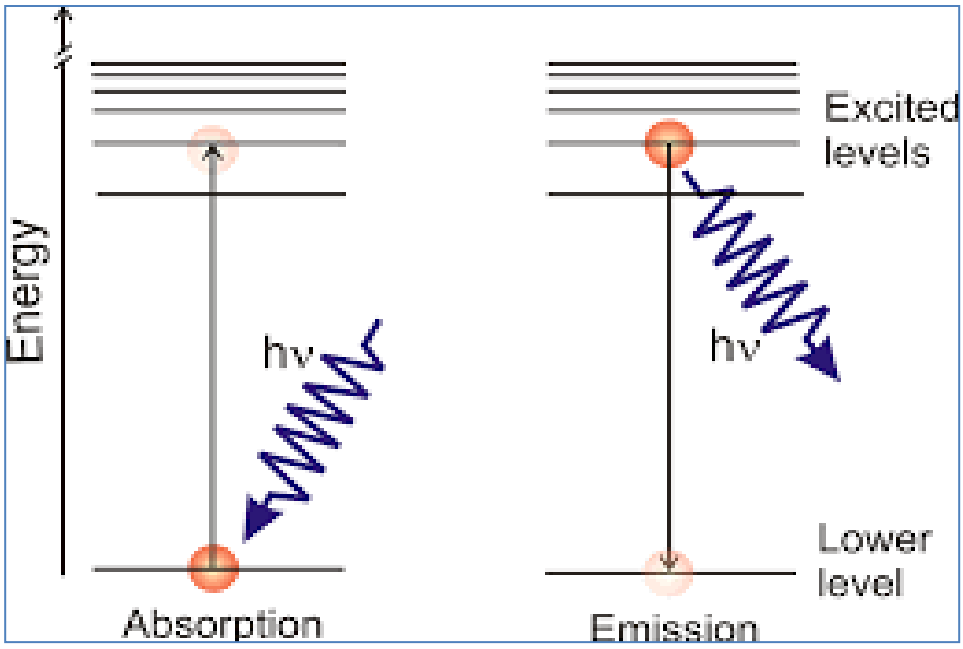

Fig.1.2 : Absorption and Emission of electromagnetic radiation

1.4.1 ATOMIC ABSORPTION SPECTROSCOPY (AAS)

As it is clear from the name, atomic absorption spectroscopy (AAS) that this spectroscopy deals with the absorption of electromagnetic radiation of specific wavelength by atom. The first AA Spectrometer was built by scientist *Alan Walsh* at the Commonwealth Scientific and Industrial Research Organization (CSIRO) in 1954, Division of Chemical Physics, in Melbourne, Australia.

Atomic absorption spectroscopy is a spectro-analytical technique. The absorption wavelength is associated with transition that requires a minimum of energy change. The electronic transition in atom are limited by the availability of empty orbital, because one orbital can be occupied with maximum of two electrons and spin of electron need to be paired in anti parallel fashion. The analyte molar concentration is determined from the amount of absorption. The sample used in atomic absorption spectroscopy, needs to volatile by using the higher energy. Thus, the tested sample either liquid should be converted into vapor form. Atomic absorption is very technique for detecting specific metals and its concentration present in the sample at ppm.

Concentration analysis by atomic absorption spectroscopy is carried out by comparison with calibration standard. It finds extensive applications in the analysis for trace metals in biological serum and drinking water. Some examples of elements detected by AAS are as given below.

Table.1.3 : Some elements detected by AAS.

S. No.	Element	Wavelength (nm)	Limit of Detection (meu g/ml
1	Au	243	0.009
2	Hg	254	0.160
3	Cu	325	0.002
4	Ag	328	0.002
5	U	358	3900
6	Ca	423	0.002
7	Na	589	0.0002
8	K	767	0.002

Principle :

In AAS the free atoms (gas) generated in an atomizer can absorb radiation at specific frequency. Atomized element absorbs energy of a wavelength that is peculiar to that element. In the process of atomization the hallow cathode lamp is used as a light source which emits light of wavelength that is peculiar to that element. When a beam of electromagnetic radiation of a particular wavelength is passed through the vaporized atom present in the flame, the atoms absorb the radiation and extent of radiation will be directly proportional to the number of ground state atoms presented in the flame. Atomic-absorption spectroscopy quantifies the absorption of ground state atoms in the gaseous state. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. The analyte concentration is determined from the amount of absorption of electromagnetic radiation. The atomic absorption is a very common technique for detecting metals and metalloids in environmental samples.

Instrumentation :

The Atomic absorption spectroscopy has simple instrumentation. Every absorption spectrometer must have the three basic component requirements (1) a light source; (2) a sample cell; and (3) a means of specific light measurement. But, unlike other spectroscopy methods, it has two additional requirements. These include a specially designed lamp to produce light of a desired wavelength and a burner to prepare the sample for absorption of light radiation. Additionally, the instrument also sprays the sample in the solution state over an atomizer (burner). This leads to evaporation of the solvent and leaves a fine dry residue. This residue has neutral atoms in the ground state. The sample of interest is aspirated and atomized into the flame. If that metal is present in the sample, its atoms will absorb some of the light, thus reducing its intensity. This decrease in intensity of the light is the process of atomic

absorption. The instrument measures the change in intensity. A computer data system converts this change into an absorbance.

i. Only liquid samples can be analyzed

ii. Non metal cont be analyzed

iii. It may be used for quantitative analysis under special circumstances

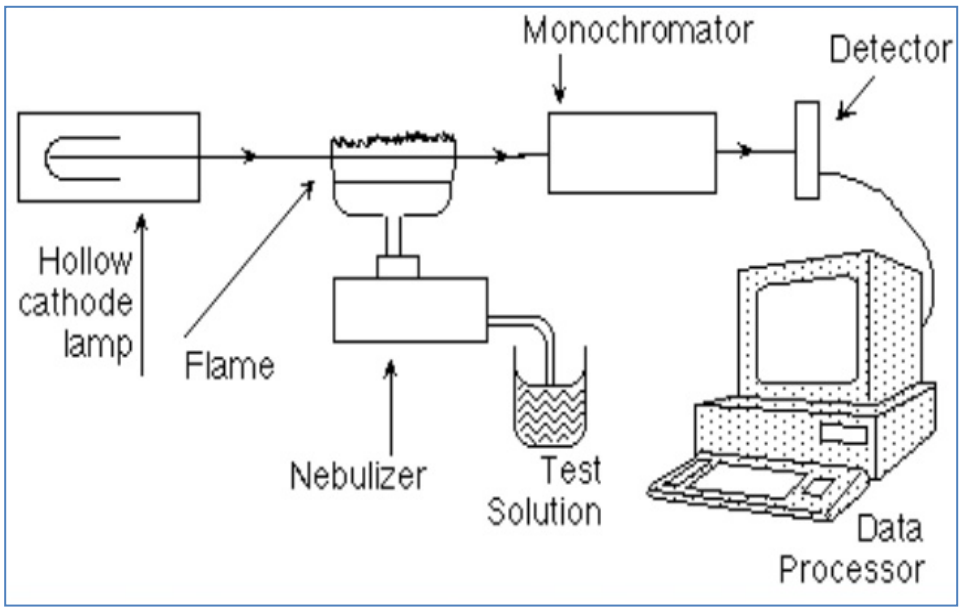

Fig. 1.3 : Diamagnetic representation of AA spectrophotometer

An atom absorbs light at discrete wavelengths. So, it is necessary to use a light source, which emits the specific wavelengths which can be absorbed by the atom. The two most common light sources used in AA are the ‘‘hollow cathode lamp’’ and the ‘‘electrode less discharge lamp. The light should be stable and have sufficient intensity and should produce a narrow spectrum with little background noise. Hollow cathode lamps (HCL) are the most common radiation source in AAS. It contains a tungsten anode and a hollow cylindrical cathode made up of metal to be determined. For instance, if sodium is to be analyzed from the sample, a cathode coated with sodium is used. These are sealed in a glass tube filled with an inert gas like argon or neon which is ionized by an electric arc. The ions get attracted toward cathodes and strike it leading to excitation of metal ions. This leads to the emission of radiation with a characteristic wavelength of analyte metal. The advantage of hollow cathode lamp is that it provides radiation with a bandwidth of 0.001 to 0.01nm. So these lamps give highly specific radiation. The disadvantage of this hollow cathode lamp is that for every metal different cathode lamp has to be employed.

Nebulizer sucks up the liquid sample at controlled rate and creates fine aerosol spray that mixes with fuel and oxidant and where it utilized by exposing them to higher thermal energy and introduce into the flame. The nebulizer uses the combustion flames to atomize and introduce the sample into the light path. More small the size of the droplets produced, more high will be the sensitivity of the element tested. Alternating, the gases form can be generated by using induced coupled plasma (ICP).

Atomization is a separation of particles into individual molecules and breaking molecules into atoms. This is done by exposing the analyte to high temperatures in a flame or graphite furnace. The (spectroscopic) flames and electrothermal (graphite tube) atomizers are the two most common atomizers used nowadays. Other atomizers, such as glow-discharge atomization, hydride atomization, or cold-vapor atomization might be used for special purposes.

Flame atomizers is the oldest and most commonly used atomizers. In this atomizer air-acetylene flame with a temperature of about 2300 °C or air-nitrous oxide flame with a temperature of about 2700 °C are used. Liquid or dissolved samples are typically used with flame atomizers. In flame AAS; a steady-state signal is generated during the time period when the sample is aspirated. This technique is typically used for determinations in the mg L^{-1} range, and may be extended down to a few $\mu g\,L^{-1}$ for some elements.

Electrothermal atomizer uses graphite coated furnace to vaporize the sample. The graphite tubes are heated using a high current power supply. In ET AAS a transient signal is generated, the area of which is directly proportional to the mass of analyte (not its concentration) introduced into the graphite tube. This technique has the advantage that any kind of sample, solid, liquid or gaseous, can be analyzed directly.

Monochromator is a very important part of an AA spectrometer used to select the specific wavelength of light from the lines emitted by the Hollow cathode lamp and transmit it to the detector. It not only selects the specific analytical line, but excludes also all other interfering lines in that region. The selection of specific light allows the determination of the selected elements in the presence of others.

Detector detects the intensity of radiation absorbed by the elements. The detector consists of a photomultiplier tube or simple photocell. The PMT determines the intensity of photons of the analytical line exiting the monochromator.

The processing of electrical signal is fulfilled by a signal amplifier. The signal from the PMT is converted to digital format by a transducer for read-out, or further fed into data station for printout by the requested format. The unknown concentration of the element is then calculated from the calibration curve. The absorbance of each known solution is measured and after that calibration curve of absorbance is plotted against concentration.

Application :

AAS has both qualitative and quantitative application in different areas. The modern emission spectrophotometers allow determination of about 20 elements in biological samples, the most common being calcium, magnesium and manganese. Absorption spectrophotometers are usually more sensitive than emission instruments and can detect less than 1 p.p.m. of each of the common elements with the exception of alkali metals. The relative precision is about 1% in a working range of 20–200 times the detection limit of an element.

- **Clinical analysis :** This spectroscopy is helpful to analyze metal present in biological fluids such as blood and urine.
- **Environmental analysis :** AAS has numerous applications in monitoring our environment such as finding out the levels of various elements in rivers, seawater, drinking water, air, soil, petrol.
- **Pharmaceuticals :** This technique helpful to detect the impurities in drugs because in some pharmaceutical manufacturing processes, minute quantities of a catalyst used in the process (usually a metal) are sometimes present in the final product. By using AAS the amount of catalyst present can be determined. Now a days, AAS is used to detect the amount of heavy metal present in synthetic drugs.
- **Industry :** Many raw materials are examined and AAS is widely used to check that the major elements are present and that toxic impurities are lower than specified. For example, in concrete, where calcium is a major constituent, the lead level should be low because it is toxic.
- **Mining :** Testing the concentration of valuable substances in potential mining areas can be done by using AAS, such as gold in rocks can be determined to see whether it is worth mining the rock to extract the gold.
- **Food industries :** determination of calcium, iron and many other elements present in drinks such as wine, beer and fruit drinks and quality assurance and contamination testing for food materials can also be performed with the help of AAS.

1.5 UV-VISIBLE SPECTROSCOPY

Ultraviolet–visible spectroscopy refers to absorption spectroscopy or reflectance spectroscopy concern with ultraviolet and the visible spectral regions of electromagnetic radiation. Means this technique utilizes visible and adjacent region for analysis work and research into biological probe. The absorption or reflectance in the visible region affects the observed color of the chemical involved. In this region of

the electromagnetic spectrum, atoms and molecules undergo electronic transitions. There are four possible transition ($n \rightarrow \pi^*, \pi \rightarrow \pi^*, n \rightarrow \sigma^* and\, \sigma \rightarrow \sigma^*$) occurs but only two transition states ($n \rightarrow \pi^* and\, \pi \rightarrow \pi^*$) exicited with light form the UV/Vis spectrum for some biological molecules. The electromagnetic transition into molecules can be classified according to the participating molecular orbital.

UV-Vis spectroscopy is used for both the quantitative and qualitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. In qualitative manner, UV-VIS spectroscopy is used to identify the functional group or confirm the identity of compound by matching the absorbance spectrum.

The UV extends from 100–400 nm and the visible spectrum from 400–800 nm. The 100–200 nm range is called the deep UV. Light source for deep UV range is more difficult so it is not commonly used for UV-Vis measurements. Spectroscopy analysis is commonly carried out in solutions but solids and gases may also be studied. The molecular structures which are responsible for interaction with electromagnetic radiation are called Chromophore. Some molecular structure such as protein has three types of chromophore, related to UV/VIS spectra.

- Peptide bond (amide)
- Certain amino acids (tryptophan, tyrosine)
- Certain prosthetic group (porphyrin group in hem)

Table 1.4 : Common colorimetric and UV absorption assays

Substance	Reagent	Wavelength (nm)
Amino acids	a. Ninhydrin b. Cupric salts	570 (proline : 420) 620
Cysteine residues, thiolates	Ellman reagent (di-sodium-bis-(3-carboxy4-nitrophenyl)-disulphide)	412
Protein	(a) Folin (phosphomolybdate, phosphotungstate, cupric salt)	660
	(b) Biuret (reacts with peptide bonds)	540
	(c) BCA reagent (bicinchoninic	562

	acid)	
	(d) Coomassie Brilliant Blue	595
	(e) Direct	Tyr, Trp: 278, peptide bond : 190
Glucose	Glucose oxidase, peroxidase, o-dianisidine, phosphate buffer	420
Ketohexose	(a) Resorcinol, thiourea, ethanoic acid, HCl	520
DNA	(a) Diphenylamine	595
	(b) Direct	260
RNA	Bial (orcinol, ethanol, $FeCl_3$, HCl)	665

Characterization of abortions ultraviolet :

It is particularly useful for identification of functional groups and the structure of molecules containing unsaturated bond (π- electron) such as aldehyde ketone. It may also we used for the quantities determination of unsaturated compounds. It is not for trance quantitative analysis. It provides no information on saturated bond (σ bond).

Table1.5 : Difference between atomic absorption spectroscopy and atomic emission spectroscopy

Atomic absorption	Atomic emission
Atomic absorption is the adsorption of electromagnetic radiation by atom	Atomic emission is the emission of electromagnetic radiation by atom
Atomic absorption is the adsorption occurs when atom absorb certain wavelength of electromagnetic radiation	Atomic absorption is the adsorption occurs when atom emit certain wavelength of electromagnetic radiation
Electron are excited to higher	Electron move to lower energy

energy level	level
Absorption density does not depend on the temperature	Emission density influence by temperature variation
Beers law is obeyed over wide range of concentration	Beers law does not obeyed
Relation between absorption density vs. concentration is not much linear	Relation between emission density vs concentration is not much linear
Absorbance vs. concentration data is obtained	Intensity vs. concentration data is obtained

Principle :

When a photon hits a molecule, it is absorbed by molecules, given by an extinction with itself depend on the wavelength λ of photon. The photon is promoted into a more excited energetic state. UV-visible light has enough energy to excite the electrons from ground state to a higher electronic state. The energy difference between the lower to higher energy level is called the band gap. The energy of the photon must exactly match the band gap for the photon to be absorbed. When the incident light intensity (I_o) passes through a sample with appropriate transparency through the path length (thickness) l, then the change in intensity occurs this is represented as Observed Intensity (I).

The characteristic absorption parameter for the sample is the extinction coefficient a, yielding the correlation $I = I_o$. The ratio $T = I/I_o$ is called transmission.

Thus, molecules with different chemical structures have different energy band gaps and different absorption spectra. The larger the band gap between the energy levels, the greater the energy required to promote the electron to the higher energy level, ultimately resulting in light of higher frequency, and therefore shorter wavelength, being absorbed.

1.5.1 ELECTRONIC TRANSITIONS

Ultraviolet and visible radiation interacts with matter which causes electronic transitions that is promotion of electrons from the ground state to a high energy state. There are four types of electronic transitions are possible.

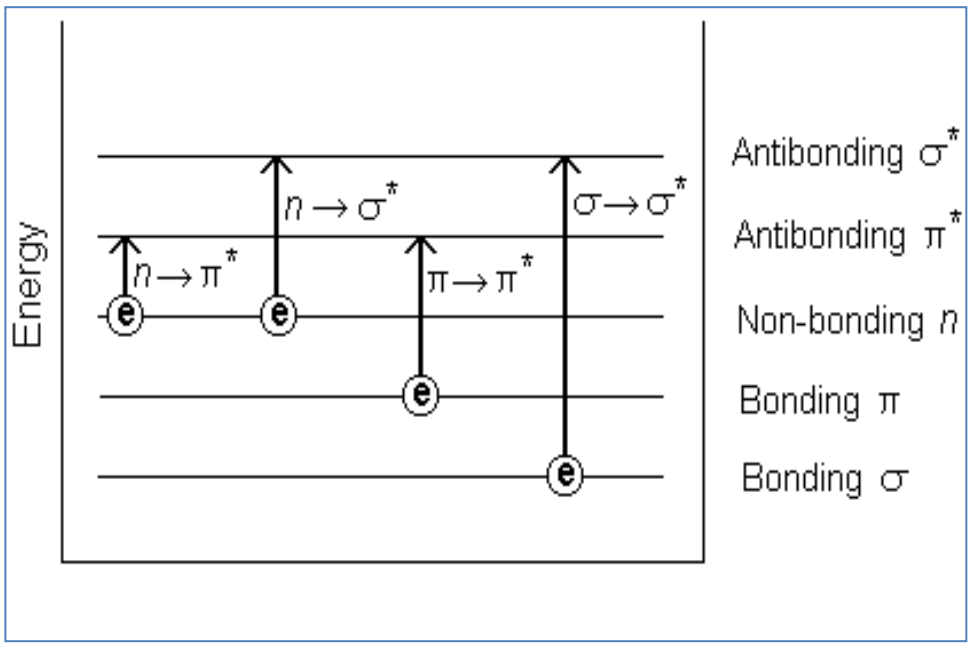

Fig.1.4 : Electronic Transition in UV-Vis Spectroscopy

σ→σ* Transitions :

This type of transition takes place when an electron in a bonding sigma orbital is excited to the corresponding antibonding orbital i.e. $\sigma \rightarrow \sigma^*$ transitions, associated with all saturated hydrocarbons and high energy (150 nm) is required. As the energy of UV region is 200-400 nm, so for this type of transition we have to go in vacuum UV region. For example, methane shows an absorbance maximum at 125 nm which has only C-H bonds, and can only undergo $\sigma \rightarrow \sigma^*$ transitions. Absorption maxima due to $\sigma \rightarrow \sigma$ transitions fall in deep UV, so they are less useful.

n→σ* Transitions :

Transition of electron takes place from non-bonding orbital to antibonding σ* orbital. For this transition energy required is 175 nm. This type of transition occurred when any hetero atom is present in saturated compounds such as ketone, amine, aldehyde, alcohol, etc. For example, methyl chloride shows absorption at 169 nm.

π→π* Transitions :

This is associated with the transition of electron from non-bonding orbital to pi- antibonding orbital and energy required is more than 200 nm. It may be 800 nm depends upon conjugation present in that particular compound. Doubly and triply bonded hydrocarbons and aromatic compounds shows π→π* transition.

n→π* Transitions :

This electronic transition involve transition of electron from non-bonding orbital to pi- antibonding orbital and energy associated with it is more than 200nm and extended up to 800 nm depending upon coupling

conjugation present in that particular compound. Example of compound associated with this n→π* transition is carbonyl compound.

Absorption Law :

We know the biological samples mainly comprise aqueous solutions; the detection of substance present in sample is measured in molar concentration c. The transition phenomenon in solution is governed by Lambert- Beer's law. The absorption of light by any absorbing material is governed by two empirical laws. Lambert's Law states that "when a beam of monochromatic radiation is passed through the absorbing medium, then the decrease in the intensity of radiation will be directly proportional to the thickness (pathlength) of the vessel containing solution. Beer's Law states that "when a beam of monochromatic radiation is passed through the absorbing medium the decrease in the intensity of radiation will be directly proportional to the concentration of solution." Thus the combination of Lambert's law and Beer's law is

$$log\left(\frac{I_0}{I}\right) = \mathcal{E}cl$$

$log_{10}\left(\frac{I_0}{I}\right)$ is the absorption, A (optical density), $\mathcal{E}$ is molecular absorptivity (extinction coefficient), c is concentration of the solution in dm^{-3} and l path length in cm.

Factor affecting UV-Vis absorption :

The Uv- visible spectrum gets affected by the nature of solvent used such as water act as native solvent for some of compounds like protein and peptide; feels comfortable in aqueous solution for detection. But the wavelength, about 700-200nm i.e. spectrum of water does not show band and thus act as silent component of sample.

The chromophore partially determine by it chain structure in absorption spectroscopy because of some factors:

- Protonation/deprotonation (pH, RedOx);
- Solvent polarity (dielectric constant of the solvent); and
- Orientation effects.

Beside those factors, the immediate environment of chromophore can be probed by assessing their absorption that is following:

- Due to bathochromic effect: a wavelength shift to higher values is called red shift or bathochromic effect.
- Due to hypsochromic effect : when a wavelength shift to lower wavelengths is called blue shift or hypsochromic effect
- Due to increase in hyperchromicity (more colour')
- Due to decrease in absorption ('less colour')

In addition, the solvent polarity also affects the difference between the ground and excited states. The orientation effect such as increase in order of nuclei single standard to double standard DNA leads to different absorption behavior.

Instrumentation :

Ultraviolet- visible spectroscopy involves the spectroscopy of photons in the UV-visible region. There is an interaction between UV visible light and sample to be analyzed. As a result of this interaction, some photons (photons of UV-Vis EMR) are absorbed and this absorption of UV visible is measured by an instrument named UV visible spectrophotometer. It measures the intensity of light passing out through a sample (I), and compares it to the intensity of light before it passes through the sample (I_0). The ratio (I/I_0) is called the transmittance, and is usually expressed as a percentage (%T). From the transmittance (T), the absorbance can be calculated as, $A = -logT$.

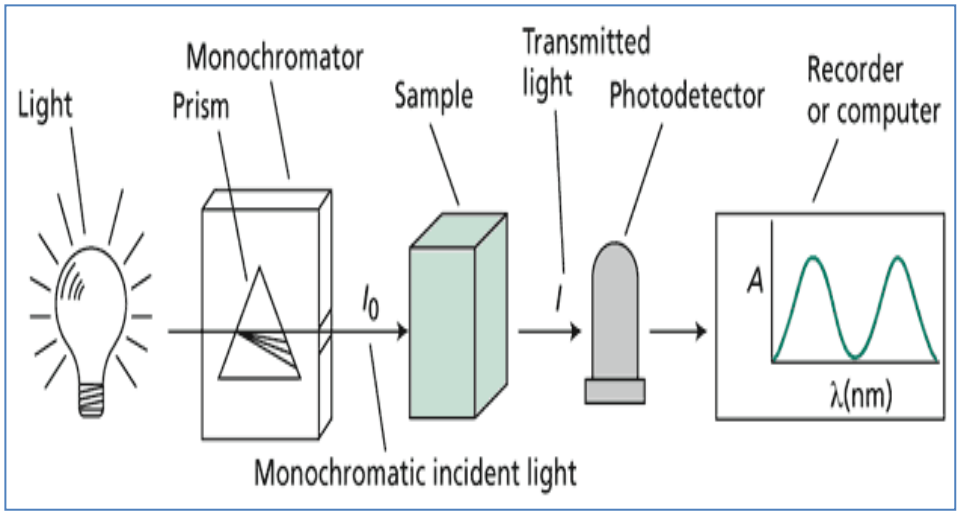

Fig. 1.5 Scheme the representation of working spectrophotometer

The components of spectrophotometer are as follows.

Light source : Light source is the basic part of spectrophotometer. It must be stable and provide continuous radiation. Light source must be of the sufficient intensity for the transmitted energy to be detected at the end of the optical path. Most commonly radiation source used in UV-Vis spectroscopy are as follows.

- **Deuterium lamp :** Deuterium arc lamp, which is continuous over the ultraviolet region (190–400 nm). The intensity of radiation of deuterium lamp is 3-5 times than the hydrogen lamp.
- **Hydrogen discharge lamp :** Hydrogen discharge lamp consists of two electrodes contain in deuterium filled silica envelope. This lamp covers a range from 160-375 nm. These lamps are stable, robust and widely used.

- **Tungsten lamps :** It is similar to house hold lamp in construction and provide a supply of radiation in wave length ranging from 320-2500 nm.
- **Xenon discharge lamp :** These are enclosed in a glass tube with quartz or fused silica and xenon gas is filled under pressure, contains two tungsten electrodes separated by a distance. An intense arc is formed between electrodes by applying high voltage.
- **Mercury arc lamps :** In mercury arc lamp, mercury vapor is stored under high pressure and excitation of mercury atom is done by electric discharge.

Monochromator : It is used to remove the radiation of desired wavelength from the wavelength of continuous spectra. Following types of monochromator are used.

- Filters
- Prisms
- Gratings

Sample compartment : Cells or cuvettes are used for holding liquid sample. Sample holder should be transparent to the wavelength region to be recorded. Quartz or fused silica cuvettes are required for spectroscopy in the UV region. Cells may be rectangular in shape or cylindrical with flat ends generally having 1 cm thickness.

Detector : Detectors convert light energy into electrical signals that are display on read out device. It measures the absorption of an analyte via the intensity of transmitted light. Mainly three types of photosensitive devices are used.

- Barrier layer cell / Photovoltaic cell
- Phototubes / Photo emissive tube
- Photomultiplier tube

Recorder: Signals from the detector are finally received by the recording device.

Terminology used in UV-Visible spectroscopy :

- **Chromophore and auxochromophore**

  It is covalently unsaturated group responsible for absorption in the UV-Vis region. Some examples are C=C, C=O, N=N, C≡N, C≡C etc. Chromophores can be divided into two groups.1) this type of Chromophore contain sigma and pi electrons and undergo $\pi \rightarrow \pi^*$ transitions. Ethylenes and acetylenes are the example of

such chromophores.2) second type of Chromophore contains sigma, pi and nonbonding electrons. They undergo two types of transitions; $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$. Carbonyl, nitriles, azo compounds, nitro compounds etc. are the example of such chromophores.

- Auxochromes are covalently saturated group with one or more lone pair of electrons. These groups themselves do not show any characteristics absorption above 200 nm but when attached to a given chromophore usually cause a shift of the absorption band to longer wavelength with a simultaneous increase in the intensity of the absorption band. Auxochrome generally increase the value of wavelength as well absorbance by extending the conjugation through resonance or hyper conjugation. Some common examples are halogens, –OH, -SH, $-NH_2$ and their derivatives such as –OR, -NHR, $-NR_2$ etc.

- **Bathochromic shift or Red shift**: Shift of absorption maxima to a longer wavelength is called bath chromic shift. $n \rightarrow \pi^*$ transition of carbonyl compounds observes this type of shift.

- **Hypsochromic shift or Blue shift:** A shift of absorption maxima to shorter wavelength is called hypsochromic shift or blue shift. Generally it is caused due to the removal of conjugation or by changing the polarity of the solvents.

- **Hyper chromic shift:** Due to hyperchromic shift intensity of absorption maxima increases. For example, benzene shows B-band at 256 nm, ε_{max} at 200 whereas aniline shows B-band at 280 nm, ε_{max} 1430. The increase of 1230 in the value of aniline compared to that of benzene is due to the hyperchromic effect of the auxochrome NH_2.

- **Hypochromic shift:** It is defined as effect due to which the intensity of absorption maxima decreases. This is caused by reduction in conjugation. For example, aniline shows λ_{max} 280 and ε_{max} 1430 whereas the anilinium ion shows λ_{max} 254 nm and ε_{max} 160.

Application of UV-Vis Spectroscopy :

UV-Vis is used in many chemical analyses. It is used to quantitative the amount of protein in a solution, as most proteins absorb strongly at 280 nm such an example spectrum of cytochrome C, which has a high absorbance at 280 and also at 450 nm because of a heme group. UV-Vis is also used as a standard technique to quantify the amount of DNA in a sample, as all the bases absorb strongly at 260 nm. RNA and proteins also absorb at 260 nm, so absorbance at other wavelengths can be measured to check for interferences. Specifically, proteins absorb strongly at 280 nm, so the ratio of absorbance at 280/260 can give a measure of the ratio of protein to DNA in a sample. In addition, other applications of UV-Visible spectroscopy are as follows.

Impurities Analysis :

This spectroscopy helps to determine the impurities in organic molecules. Additional peak in UV-Vis spectra is due to the presence of impurities in the sample and can be compared with that of standard raw material. By measuring the absorbance at specific wavelength, impurities can be detected. For example, benzene appears as common impurity in cyclohexane which can easily detected as benzene shows absorption at 255nm. UV-Vis spectroscopy is useful in elucidation of structure of organic compounds, presence and absence of unsaturation and hetero atoms. Common applications for difference UV spectroscopy include the determination of the number of aromatic amino acids exposed to solvent, detection of conformational changes occurring in proteins, detection of aromatic amino acids in active sites of enzymes, and monitoring of reactions involving 'catalytic' chromophores (prosthetic groups, coenzymes).

Qualitative Analysis :

The qualitative analysis is carried out by the UV-Vis spectroscopy when the atom or molecules absorb UV radiation and identification is done by comparing the absorption spectra with the spectra of known compound. Qualitative analysis is done in UV/Vis regions to identify certain classes of compounds both in the pure state and in biological mixtures (e.g. protein-bound). The application of UV/Vis spectroscopy to further analytical purposes is rather limited, but possible for systems where appropriate features and parameters are known. Most commonly, this type of spectroscopy is used for quantification of biological samples either directly or via colorimetric assays. In many cases, proteins can be quantified directly using their intrinsic chromophores, tyrosine and tryptophan. The characteristic picks in protein spectrum are a band at 278/280 nm and another at 190 nm . The region from 500 to 300nm provides valuable information about the presence of any prosthetic groups or coenzymes. Protein quantification by single wavelength measurements at 280 and 260nm only should be avoided, as the presence of larger aggregates (contaminations or protein aggregates) gives rise to considerable Rayleigh scattering.

Difference spectra :

Difference spectrum is obtained by subtracting one absorption spectrum from another. Difference spectra can be obtained in two ways: either by subtraction of one absolute absorption spectrum from another, or by placing one sample in the reference cuvette and another in the test cuvette. Difference spectra have three distinct features as compared to absolute spectra shown in Fig 1.6.

- difference spectra may contain negative absorbance values;

- absorption maxima and minima may be displaced and the extinction coefficients are different from those in peaks of absolute spectra;
- there are points of zero absorbance, usually accompanied by a change of sign of the absorbance values. These points are observed at wavelengths where both species of related molecules exhibit identical absorbance's (isosbestic points), and which may be used for checking for the presence of interfering substances.

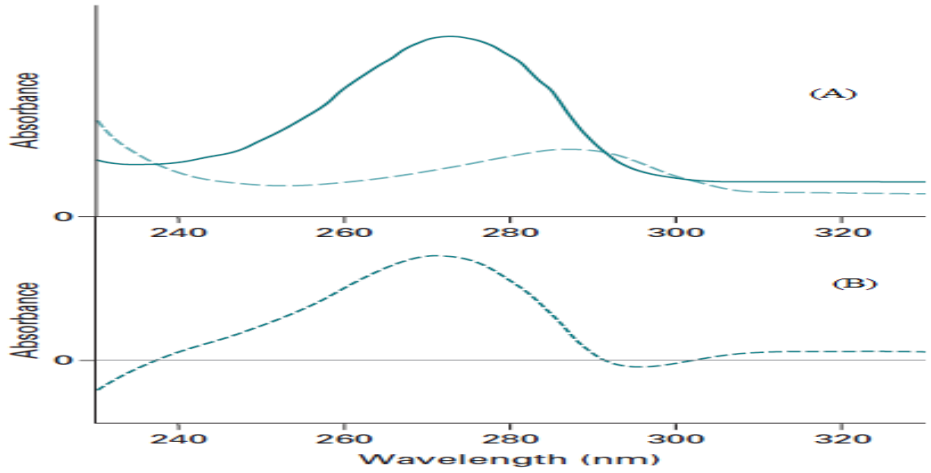

Fig. 1.6 : a) Absolute spectra of ubiquinone (solid curve) and ubiquinol (dotted curve).b) Difference spectrum.

1.6 SUMMARY

After reading this block student will learn about various aspects of spectroscopy. Students will explore his knowledge about the instrumental techniques that would be beneficial for diagnosis and characterization of various biological samples. We lean the spectroscopy is a fundamental tool in field of physical, chemical and biological sciences which is used in determination of electronic structure, composition of matter from atomic scale to macro scale level. The atomic adsorption and UV-Visible spectroscopy used the visible light for detection of specimen. The atomic microscopy is very useful in detection of heavy metal form the biological samples. There is an interaction between UV visible light and sample to be analyzed. By measuring the absorbance at specific wavelength impurities can be detected by UV visible spectroscopy.

1.7 TERMINAL QUESTIONS

Q.1. What do you understand for spectroscopy? Write the principle and application of uv-visible spectroscopy.

Answer:----------

Q.2. Distinguish clearly between emission and absorption spectrum.

Answer:----------

Q.3. UV-Visible work on which law. Discuss the Limbert Beer's Law.

Answer:----------

Q.4. Write the principles of Atomic adsorption spectroscopy and it applications.

Answer:----------

Q.5. Briefly discuss chromophore and auxochrome in spectroscopy.

Answer:----------

Q.6. What is the difference between atomic and molecular spectroscopy?

Answer:----------

1.8 FURTHER READINGS

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UNIT-2 CHROMATOGRAPHY

Structure

2.1 INTRODUCTION

All chromatography consists of stationary and mobile phase, which may be liquid or gaseous, useful for the separation of biological sample. Mobile phase passed over or through the stationary phase after the mixture of analytes to be separated applied to stationary phase. During chromatography, separation of analysts carried out by the phase back and forth occurs between the two phase. So that difference in their distribution coefficient result of their separation. All types of chromatography work on the same principle. There must be exist two phase. Mobile phase should be separated and stationary phase should be gel/ liquid or solid mixture that is immobilized. Chromatography may be preparative or analytical.

Objective :

- To understand the principle of chromatography
- To learn about the process and instrumentation of different chromatography
- To understand the role of different chromatography in biochemistry

- To understand the role of different phase of chromatography

2.2 CHROMATOGRAPHY OVERVIEW

Chromatography is derived from the Greek word *chroma*, which means “color” and *graphein*, which means "to write". So the word chromatography means "color writing". A Russian botanist M.S. Tswett invented chromatography in 1903, while studying the coloring materials in plant life. The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of fatty acid mixtures. Chromatography is a powerful technique used to separate mixture of substance into their individual components, so that the individual components can be thoroughly analyzed. All types of chromatography work on the same principle. There must be exist two phase.

Stationary phase : The stationary means doesn't move. It either a solid or a liquid supported on a thin film on the surface of inert solid, through which the sample contained in the mobile phase percolates.

Mobile phase : This phase flowing over the surface of the stationary phase. The mobile phase may be either a liquid or a gas.

The mobile phase flows through the stationary phase and carries the components of the mixture with it. The various components of the mixture travel at different rates, causing them to separate.

Chromatography is such an outstanding technique that two Nobel prizes have been awarded to chromatographers. Over 60% of chemical analysis worldwide is currently done with chromatography. Chromatography may be preparative or analytical. In preparative chromatography the components of a mixture are separated for later use, so that it a form of purification. Whereas analytical chromatography is perform for establishing the presence or measuring the relative proportions of analytes in a mixture. Liquid chromatography is used for the identification of liquid mixture as well as quantitative determination of components of high molecular weight.

2.3 PRINCIPLE OF CHROMATOGRAPHY

In the chromatography substance are separated due to their relative affinities for the stationary and mobile phase. The distribution coefficient ‘K’ governs the distribution. The fraction with a greater affinity to stationary phase travels slower and at a shorter distance, while that with a lesser affinity travels faster and longer.

$$K = \frac{\text{concentration of a component in moving phase}}{\text{concentration of that component in the stationary phase}}$$

2.4 TYPES OF CHROMATOGRAPHY

There are several types of chromatography, each differing in the kind of stationary and mobile phase they use. The commonly used chromatographic techniques are tabulated below.

Technique	**Stationary phase**	**Mobile phase**	**Basis of separation**	**Notes**
Paper chromatography (PC)	Solid (cellulose)	Liquid	Polarity of molecules	Compound spotted directly on a cellulose paper
Thin layer chromatography (TLC)	Solid (silica or alumina)	Liquid	Polarity of molecules	Glass is coated with thin layer of silica on which is spotted the compound
Liquid column chromatography (LCC)	Solid (silica or alumina)	Liquid	Polarity of molecules	Glass column is packed with slurry of silica
Size exclusion chromatography Or Gel permeation chromatography (GPC)	Solid (micro porous beads of silica)	Liquid	Size of molecules	Small molecules get trapped in the pores of the stationary phase, while large molecules flow through the gaps between the beads and have very small retention times. So larger molecules come out first. In this type of chromatography there isn't any interaction, physical or chemical, between the analyte and the stationary phase.
Ion-	Solid	Liquid	Ionic	Molecules

exchange chromatography	(cationic or anionic resin)		charge of the molecules	possessing the opposite charge as the resin will bind tightly to the resin, and molecules having the same charge as the resin will flow through the column and elute out first.
Bio-affinity chromatography	Solid (agarose or porous glass beads on to which are immobilized molecules like enzymes and antibodies)	Liquid	Binding affinity of the analyte molecule to the molecule immobilized on the stationary phase	If the molecule is a substrate for the enzyme, it will bind tightly to the enzyme and the unbound analytes will pass through in the mobile phase, and elute out of the column, leaving the substrate bound to the enzyme, which can then be detached from the stationary phase and eluted out of the column with an appropriate solvent.
Gas chromatography	Liquid or solid support	Gas (inert gas like argon or helium)	Boiling point of the molecules	Samples are volatilized and the molecule with lowest boiling point comes out of the column first. The molecule with the highest boiling point comes out of the column last.

2.4.1 THIN LAYER CHROMATOGRAPHY

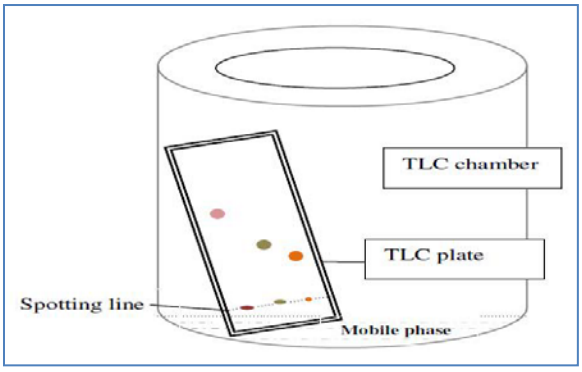
Thin layer chromatography is type of planar chromatography. The important difference between TLC and other chromatography is of particular technique, in place of physical phenomenon like adsorption, partition etc. in TLC the stationary phase consists of a thin layer of sorbent (e.g. silica gel, cellulose powder, alumina) coated on a inert, rigid backing material such as glass plate, aluminum foil or silver foil. As a result, the separation process takes place on a flat 2D surface. TLC is an analytical tool widely used because of its simplicity, relative low cost, high sensitivity, and speed of separation. This analytical technique can be performed as a means of monitoring the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Characteristics of stationary and mobile phase in TLC are as follows. The stationary phase present in TLC is finally divide powder. Stationary plate spread over supporting material which is made up of glass or aluminium. The thickness of stationary phase should be 250 micrometer and the particle size in stationary phase will be 1-40 micrometer. While the mobile phase is always liquid that is used in TLC. Both polar and non polar liquid can also be used in TLC.

Principle :

It is based on the principle of adsorption chromatography or partition chromatography or combination of both, depending on adsorbent, its treatment and nature of solvent employed. In this technique finally divide stationary phase is used and it is spread over a supporting plate and a mobile phase is also used. This mobile phase migrate over these supporting material or stationary phase against the gravitational force. The mechanism behind this migration of mobile phase is the capillary action. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is achieved. Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or characters are identified by means of suitable detection techniques.

Experimental :

TLC system consists of following components.



- **TLC plate :** TLC plates can be prepared in lab but usually commercially available, with standard particle size ranges to improve reproducibility. These plates are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like gypsum and water. This mixture spreads as thick slurry on a non-reactive carrier sheet, generally made up of glass, thick aluminum foil or plastic.

- **TLC chamber :** TLC chamber helps to maintain a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.

- **Mobile phase :** This comprises of a solvent or solvent mixture which is suitable to separate the chemical mixture. The solvents used should be chemically inert with the sample, a stationary phase.

Methodology :

To run a thin layer chromatography plate, three steps - spotting, development and visualization are carried out.

Firstly draw a line using a ruler and pencil approximately 1 cm from the edge of the TLC plate. Put a small spot of solution containing sample with the help of TLC pipettes. Make sure enough sample is spotted on the plate. After the first spot is applied, leave it to dry. The sample should be applied to the same spot three to four time depending on how dilute the sample is never touch the TLC plate on the surface, but carefully held only by the edges. This will avoid possible contamination due to perspiration. Plate is developed in TLC chamber which contain solvent or mobile phase. A small amount of suitable solvent is poured into the chamber to a depth less than 1cm. A moistened filter paper in the mobile phase is placed on the inner wall of the chamber. Close the chamber with a glass cover for a few minute to let the solvent vapors ascend the filter paper and maintain equal humidity. Now placed the TLC plate in the chamber, in such a way that the sample does not touch the surface of eluent in the camber and closed it with a lid. The solvent travel up the stationary phase by capillary action meets the sample mixture and carries it up the plate. Once the mobile phase has reached approximately two-third up the plate, remove it from the chamber and let it dry. Mark the solvent front immediately. In Visualization process the simple is visualized colored compounds, as the spots can be directly observed after development. The silica gel on the TLC plate is impregnated with a fluorescent material that glows under ultraviolet (UV) light and dark spots will appear on a glowing background. These dark spots should be circled with a pencil to mark their locations. Sometimes it is not possible to see the spots on the plate as these are UV inactive compounds. For these types of compounds, a number of chemical stains can be used. Iodine is among the most common stains. Most organic compounds will form a dark-

colored complex with iodine and dark brown spots will appear on the plate.

Any individual solute (steroids, drugs, dyes etc) will move by a constant ratio with respect to the solvent front, under constant condition of temperature, solvent system and adsorbent. This is known as retardation factor or R_f value.

$$\mathbf{R_f} = \frac{\boldsymbol{distance\ travel\ by\ solute\ molecule\ from\ origin}}{\boldsymbol{distance\ travel\ by\ solvent\ front\ from\ origin}}$$

Applications :

Purity of any sample : TLC can be used to check the purity of the sample by comparing it with standard or authentic sample. If any impurity is present, then it shows extra spots and this can be detected easily.

Identification of compounds : Thin layer chromatography can be used in purification, isolation and identification of natural products like volatile oil or essential oil, fixed oil, waxes, alkaloids, glycosides, steroids etc. In spite of these TLC is employed to identify the compound of interest in mixture, extracted from plant, animal or microbial sources by running the TLC of both standard and extracted sample together and matching their Rf value.

Biochemical analysis : TLC is extremely useful in isolation, separation and characterization of biochemical metabolites or constituent from its body fluids, blood, plasma, serum, urine etc.

In food and cosmetic industry : TLC method is used for separation and identification and characterization of colors, various cosmetic products, preservatives and sweetening agent. It will perform easily as it does not require any sophisticated equipment and is also time-efficient.

Examination of reactions **:** TLC can perform to monitor the progress and rate of reaction at particular intervals. To determine this it is observed that at the beginning of a reaction the entire spot is occupied by the starting chemicals or materials on the plate. As the reaction starts taking place the spot formed by the initial chemicals starts reducing and eventually replaces the whole spot of starting chemicals with a new product present on the plate. The formation of an entirely new spot determines the completion of a reaction.

In Pharmaceutical Industry : Quantitatively, TLC is used to monitor the purity of several drugs, including sedatives, antihistamines, analgesics, tranquilizers, and steroids and separate different metabolites present in a drug. TLC can be used to identify the presence of drug residues and antibiotics in food, such as poultry, beef, pork, milk, and fish among others.

In forensic Science : TLC can be effectively used in forensic studies where body fluids, such as urine and blood can be tested for the presence of drugs and it nature. For example acidic and neutral drugs are identified

by using octadecyl silica in the stationary phase, while plain silica and octadecyl silica are used to identify basic drugs.

A special application of TLC is in the characterization of radio labeled compounds, where it is used to determine radiochemical purity.

2.4.2 ION EXCHANGE CHROMATOGRAPHY

As it name suggest ion exchange chromatography, means ion exchanger resin will be there and due to presence of that resin ion exchange will take place i.e. sample is ionized and that sample will exchange with counter ion present in stationary phase and ultimately separation will take place. This technique separates charged or polar molecule in a mixture. Only hydrophilic molecules can be separated out from this technology.

In this type of chromatography separation occurs as a result of formation of ionic or electrostatic bond between the charged group of biomolecules and an ion exchange resin bearing opposite charge. Ions exist in a state of equilibrium between the mobile phase and stationary phases which give rise two possible formats, anion and cation exchange are referred to as counter ion (Fig. 2.1). These exchangeable matrix counter ions may include protons (H^+), hydroxide groups (OH^-), single charged mono atomic ions (Na^+, K^+, Cl^-), double charged mono atomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}) as well as organic bases (NR_2H^+) and acids (COO^-). Cations are separated on cation-exchange resin column and anions on an anion exchange resin column.

It is one of the most important adsorption chromatography, performed for separation of peptides, proteins, nucleic acids and related biopolymers which have different molecular sizes and molecular nature with electronic charge. Advantage of using ion chromatography is that only one interaction involved during the separation as compared to other separation techniques; therefore, ion chromatography may have higher matrix tolerance.

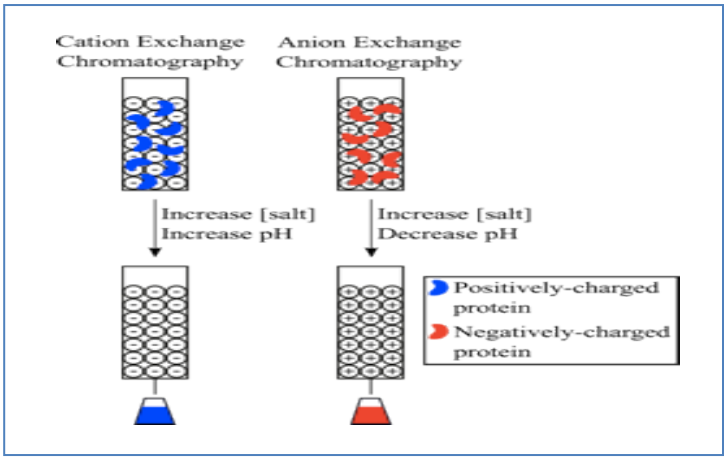


Fig. 2.1 : Diagrammatical representation of ion exchange chromatography

Stationary phase is solid and have resin polymers forming network. Most commonly used cellulose, agarose and polymethacrylate. These stationary phases consist of an immobile matrix which covalently bound to charged molecules. Mobile phase is liquid containing sample that is to be separated.

Principle :

Ion exchange chromatography is based on the reversible electrostatic interaction of charged species with the ion exchange matrix and ultimately separation takes place. On the basis of ions separated, the ion exchange chromatography can be divided into two categories.

a. Anion Exchange Chromatography:

$$\text{Regin-OH}^- + \text{A} \longrightarrow \text{Regin-A} + \text{OH}$$

(in solution) (in eluting solution)

Anion exchanges regin

When molecule of interest is negatively charged then anion exchange chromatography is used. In this process anion in a mobile (liquid) phase exchanges with another anion that is previously bound to a positively charged solid support or matrix. Commonly used anion exchange resins are Q-resin, a Quaternary amine; and DEAE resin, Diethylaminoethane. Anion exchange chromatography is used both for preparative and analytical purposes and may also be usedchromatographically, to separate

anions and medicinally to remove an anion from gastric contents or bile acids in the intestine.

b. Cation Exchange Chromatography:

$$\text{Regin-}\ \text{H}^+ + \text{M}^+ \longrightarrow \text{Regin-}\ \text{M}^+ + \text{H}^+$$

(in solution) (in eluting solution)

Using cation exchange regin

Cation exchange chromatography is used when the desired molecules to separate are cations in mobile phase. Positively charged molecules are attracted to a negatively charged solid support. S-resin, sulfate derivatives; and CM resins, carboxylate derived ions are commonly used as cation exchange resins.This type of chromatography is used both for preparative and analytical purposes and can separate a large range of molecules from amino acids and nucleotides to large proteins.

Instrumentation :

Typical Ion exchange chromatography instrumentation includes the following components.

1. **Pump :** It is one of the most important components in the ion exchange chromatography system which has to provide a continuous constant flow of the eluent through the IC injector, column, and detector. The constant-flow pump is the most widely used.

2. **Injector :** Sample is injected in column by using injection valve. Liquid samples may be injected directly and solid samples need only to be dissolved in an appropriate solvent. Injectors should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 ml of volume with high reproducibility and under high pressure (up to the 4000 psi).

3. **Columns :** Column is made up of stainless steel, titanium, glass or an inert plastic according to its ultimate use. The column can vary in diameter from about 2mm to 5 cm and in length from 3 cm to 50 cm depending on whether it is to be used for normal analytical purposes, microanalysis, high speed analyze or preparative work.

4. **Guard Column :** It is placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove particles that clog the separation column

5. **Suppressor :** This device reduces the background conductivity of the chemicals used to elute samples from the ion-exchange column which improves the conductivity measurement of the ions being tested. It is membrane-based devices which are designed to convert the ionic eluent to water as a means of enhancing the sensitivity.

6. **Detectors :** Detector used in ion exchange chromatography has wide range to run instrument both on analytical and preparative scale. Generally electrical conductivity detector is used.

7. **Data system :** In routine analysis a pre-programmed computing integrator may be sufficient but for higher control levels, a more intelligent device is necessary, such as a data station or minicomputer.

Methodology :

Separation through ion exchange chromatography carried out in a column packed with ion exchange. Generally these ion exchangers are commercially available, made up of styrene and divinyl benzene. Selection of ion exchanger depends upon charge of particle to be separated. To separate anion “Anionic exchanger” for example DEAD-cellulose is used, whereas to separate cations “Cationic exchanger” such as CM-cellulose is used. The following four steps are involved in the separation process.

1. **Equilibration :** The first step is the equilibration of the stationary phase to the desired start condition. When equilibrium is reached all stationary phase charged group is associated with exchangeable counter ions such as chloride or sodium.

2. **Sample application and wash :** The second step is sample application and wash. The column is filled with ion exchanger then the sample is applied followed by the buffer. Generally tris-buffer, pyridine buffer, acetate buffer, citrate and phosphate buffers are used. The sample buffer should have the same pH and ionic strength as the starting buffer in order to bind all appropriated charged molecule. The goal in this step is to bind the target molecule and wash out all the unbounded materials.

3. **Elution :** In the third step elution, biomolecules are released from the bioexchanger by a change in buffer composition. A common way is to increase the ionic strength with sodium chloride, or another simple salt, in order to desorb the bound molecule. The particles which have high affinity for ion exchanger will come down the column along with buffers.

4. **Regeneration :** The final step is regeneration, removes all molecules still bound. This ensures that the full capacity of the stationary phase is available for next run.

Application :

- Ion exchange chromatography is most effective method for water purification. As hard water is one of the common problems in most parts of the world and it is necessary to make hard water soft for drinking purpose. The calcium and other salts present in water are removed by this method.

- Complete deionization of water or a non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ion.

- An important use of ion-exchange chromatography is the routine analysis of amino acid mixtures. The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated in a few hours, and their concentrations are determined automatically as the amino acid “Autoanalyzer” is based on in exchange principle.

- Separation of many vitamins, other biological amines, carbohydrates, nucleotides, proteins and organic acids and bases etc are performed with the help of ion exchange chromatography.

- In biochemistry, ion exchange chromatography used for separation of drugs and metabolites from blood, urine, etc which find application in clinical diagnosis. This separation technique

effectively used in purification of enzymes after extracting from the tissues.

2.4.3 AFFINITY CHROMATOGRAPHY

Affinity chromatography is also called bio-affinity chromatography. It is one type of liquid chromatography that depends upon the reversible adsorption of biomolecules in a biochemical mixture through biospecific interaction on the ligands. This type of interaction may take place between antigen and antibody, enzyme and substrate, receptor

and ligand, or protein and nucleic acid. These interactions which are used for purification by placing one of the interacting molecules, referred to as affinity ligand, onto a solid matrix to create a stationary phase while the target molecule is in the mobile phase. The high selectivity of affinity chromatography is take place by allowing the desired molecule to interact with the stationary phase and be bound within the column in order to be separated from the undesired material which will not interact with ligand and elute first.

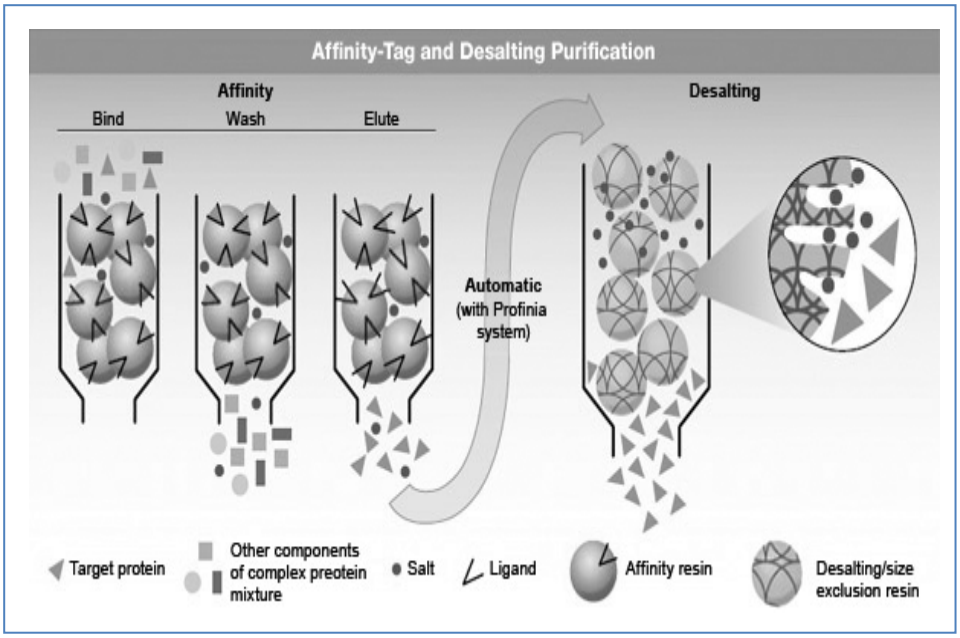
This technique was first developed by a Swedish biochemist A. Wilhelm Tiselius in 1930 while studying enzyme and other proteins. He won the Nobel Prize in 1948.

Affinity chromatography is one of the most powerful and unique technique used for the purification and isolation of biological molecules on the basis of its biological function or individual chemical structure. As a result, purifications that would otherwise be time consuming and complicated, can often be easily achieved with affinity chromatography.

This technique offers high selectivity, resolution, and capacity in most protein purification schemes. This chromatography can be used to purify and concentrate a substance from a chemical mixture into a buffering solution, reduce the amount of unwanted substances in a mixture, identify the biological compounds binding to a particular substance, purify and concentrate an enzyme solution. The molecule of interest can be immobilized through covalent bonds. In bio-affinity chromatography the stationary phase is solid modified resin and the mobile phase is liquid containing sample mixture or buffer is used.

Principle :

The principle of affinity chromatography involves highly specific biological or chemical interaction between an immobilized ligand and a desired target molecule, which can bind selectively and interact with the target even in complex solutions containing many other components. So due to this specific interaction others molecules or contamination present in mixture are eluted while target molecules are separated out from all other molecules that cannot bind the ligand.



Instrumentation :

The affinity chromatography must have the following three components.

1. **Matrix :** The matrix is an inert support; simply provide a structure to increase surface area to which ligand can coupled directly or indirectly. It should be chemically and physically inert and stable. The matrix must be insoluble in solvents and buffers employed in the process. It must be easily coupled to a ligand or spacer arm onto which the ligand can be attached. Matrix is made up of agarose and other polyacrylamide materials.

2. **Spacer arm :** To prevent satiric interference or overlap during the binding process of target molecule to the ligand an inhibitor is used. This inhibitor contains a hydrocarbon chain firstly attached to agarose bead and referred as spacer arm.

3. **Ligand :** Ligand is refers to the molecule that reversibly binds to the target molecule, enabling purification by affinity chromatography. The ligand can be selected only after the nature of the macromolecule to be isolated is known.

Methodology :

Usually affinity chromatography has been completed in following steps.

Preparation of column :

The column is loaded with solid resin such as agarose, sepharose, cellulose etc and ligand is selected according to desired component. Spacer arm is attached between ligand and matrix.

Sample injection :

Before the injection of the components, the column should pre-equilibrate with starting or binding buffer. The sample must also be equilibrated with this starting binding buffer. After that solution containing a mixture of components is loaded into the elution column and allowed to run at a controlled rate.

Adsorption of components of interest :

The interested molecule can be adsorbed to the ligands when they pass through the column whereas other components, which have no affinity to the ligands, will be pushed to the end of the column by the solvent. If there are several components in the mixture have affinity to the stationary phase, there will be a competitive adsorption on the ligands. The component, which has the strongest affinity toward the ligands, will replace the components of weaker affinity and take the adsorption sites. Ultimately, the components which have weaker affinity to the ligands will also be pushed to the end of the column by the solvent.

Washing (Removal of impurities) :

Column is washed with wash buffer solution which is generally having low salt concentration. When we load the wash buffer on the column, the unwanted molecules washed out from the mixture.

Elution of desired components :

Elution buffer is prepared on the basis of pH or salt. Generally high salt concentration solution is used. Due to high salt concentration interaction between desired molecule and stationary phase is adsorbed component of interest is removing from the column. These are collected in collecting tube for further testing.

Applications :

- Affinity chromatography is one of the most useful methods for the separation, purification and concentration of specific products.
- It is a powerful protein separation method, based on the specific interaction between immobilized ligands and target proteins.
- Peptides can also be separated effectively by affinity chromatography through the use of peptide-specific ligands.
- This chromatography is the basis for immuno chromatographic test (ICT) strips, provides a rapid means of diagnosis in patient care. ICT detection is highly specific to the microbe causing an infection.
- It is also used in enzyme assays and to identify the binding sites of enzymes.

- It is used in vitro antigen-antibody reactions.
- Detection of single nuceotide polymorphisms and mutations in nucleic acids can also be done by using Affinity chromatography.

2.4.4 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

In recent years hydrophobic interaction chromatography (HIC) has become a powerful technique for purification of biological compounds on a laboratory scale as well as at the industrial scale.

As the name suggest hydrophobic interaction, means this type of chromatography separates molecules based on their hydrophobicity. It is a powerful separation technique for separating and purifying proteins in their native state which are based on the reversible interaction between the protein surface and a hydrophobic chromatographic sorbent. HIC is also useful in isolating protein complexes and in studying protein folding and unfolding. In this separation technique, hydrophobic groups such as phenyl, octal or butyl, are attached to the stationary column. Proteins that pass through the column bearing hydrophobic amino acid side chains on their surfaces are able to interact with and bind to the hydrophobic groups on the column.

This technique is based on the adsorption of biomolecules to a weakly hydrophobic surface at high salt concentrations, followed by elution with a descending salt gradient. The more hydrophobic the molecule, the less salt needed to promote binding.

Hydrophobic interaction chromatography is a reversed-phase separation technique that often designed using the opposite conditions of those used in ion exchange chromatography and size-exclusion chromatography for the separation and purification of proteins. HIC is carried out with an aqueous buffer of higher salt concentration at neutral conditions and uses a weak hydrophobic stationary phase for the separation of proteins. The mobile phase in HIC is neutral and nonorganic substance which protects enzymes from denaturation. In HIC polar stationary phase such as silica is used.

Principle :

Separation in hydrophobic interaction chromatography depends upon the reversible adsorption of biomolecules according to their hydrophobicity.

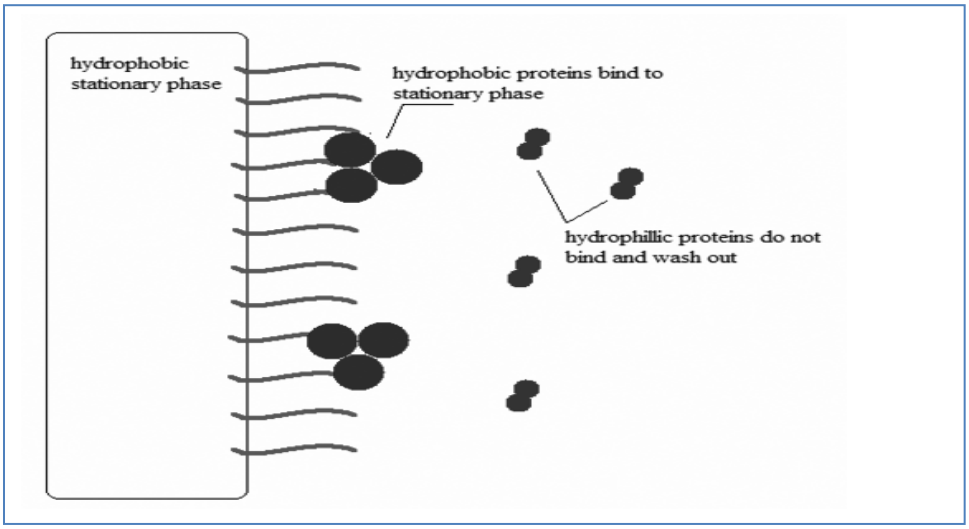


Fig. 2.2 : Diagrammatical representation of Hydrophobic Interaction Chromatography

Methodology :

Equilibration : The first step of separation in HIC is equilibration of the stationary phase which is performed by adding salt to the mobile phase. Generally used equilibration buffer in this step is 50 milimolar sodium phosphate with 1 to 1.5 molar ammonium sulphates.

Sample application and wash : This step includes the binding of target molecule and wash out all undesired molecules. The binding is promoted by moderately high concentration of salt. The concentration of salt that give optimum purification is chosen.

Elution : In the elution step biomolecules leaves from the hydrophobic surface by a change in the buffer composition. A common way is to decrease the salt concentration of the buffer. Gradient solution can be used to the target molecule from the other bound molecules in the sample. Normally a gradient of 10-15 column volume is sufficient.

Regeneration : Regeneration is the final step to remove all molecules that are still bound. These have to show the full capacity of stationary phase for the next run.

Applications :

- Hydrophobic interaction chromatography can be used in analysis of highly polar substances including biologically active compounds, such as pharmaceutical drugs, peptides and proteins.
- Hydrophilic interaction chromatography is a useful analytical tool that rivals RP-HPLC in many applications dealing with polar or ionized compounds in complex matrices.
- HIC is used in purification of monoclonal antibody for clinical studies of passive immunotherapy of HIV-1

- HIC is used in purification of human autotoxin.
- HIC, in combination with ion exchange chromatography used to purify mammalian transcription factor.

2.4.5 GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography is a type of size exclusion chromatography, which separate molecule according to their size and shape. With some exceptions, the separation of the components in the sample mixture correlates with their molecular weight. In these cases gel filtration chromatography can be worked as analytical tool to find out the molecular weight of an uncharacterized molecule.

It is also an important preparative technique which can be used to separate protein, peptides and oligonucleotides on the basis of size. In gel filtration chromatography separation is achieved by physical means, which make it differ from other types of chromatography. As gel filtration chromatography does not involve any interaction of the sample or the solvent with the matrix in a column. Aqueous solution or buffer solution used as mobile phase in gel filtration chromatography. Gel consisting of porous beads or carbohydrates cross linking agents is used as stationary phase. Most commonly used gels are dextron (sephadex), agarose (sepharose) and polyacrylamide (Bio Gel).

Principle :

Separation in gel filtration chromatography is based on the differences in sizes from biomolecules as they pass through a column packed with a chromatographic medium or stationary phase, which is a gel. So the larger size molecules separate out first from the sample solution, after which smaller size molecules separated.

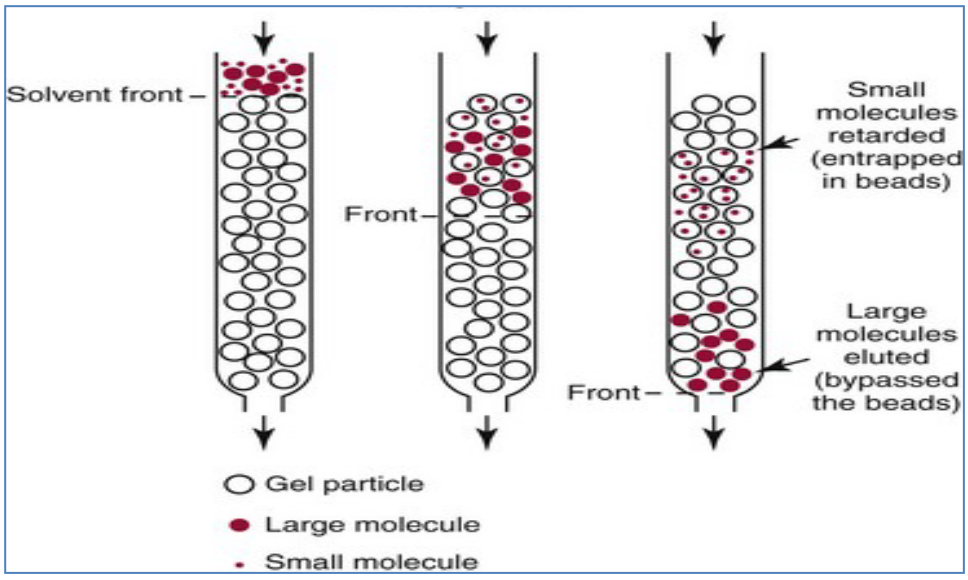


Fig. 2.3 : Separation process in gel filtration chromatography

Instrumentation :

The basic components of the gel filtration chromatography are as follows.

The matrix : The matrix is the material in the column that is actually the separation medium or known as stationary phase. The matrix should be chemically and physically stable and inert so that it has lack of reactivity and absorptivity.(fully describe)

Chromatography column : It is tube filled with matrix and had a frit and elution spout fitted at the bottom. The frit is a membrane or porous disk that supports and retains the matrix in the column but allows water and dissolved solutes to pass.

The elution buffer : It is the mobile phase of the chromatography and flows through the matrix and out of the column. The column, with the matrix and applied sample, is "developed" by the elution buffer. The molecules in the sample are carried by the flow of buffer into the matrix where they are gradually separated.

Methodology :

- To perform separation, porous matrix is packed into a column to form packed bed. Before packing, the matrix are soaked into selected mobile for overnight, to prevent breaking or bursting of column due to swelling of the stationary phase.
- Sample is applied to the column.
- Buffer and sample move through the column. Molecules diffuse in and out of the pores of the matrix.
- It is difficult for larger molecules to pass through the pores and penetrate the beads. So that larger molecules trends to flow around and in between the beads whereas smaller molecules diffuse into the beads and are delayed in their passage down the column.
- Consequently larger molecules are eluted from the column before smaller molecules. Larger molecules take the faster more direct path that involves less time in the beads.

Application :

Gel filtration chromatography helps to determine the molecular weight distribution of polymers.

As this technique separate the substance on the basis of different size and mass. So that organic compounds like sugars, polypeptides, polystyrenes, etc can be separated as they have different size.

- Fractionation of molecules and complexes within a predetermined size range

- It helps in size analysis and in removal of large proteins and complexes.
- This chromatography can be used in desalting process.
- It can remove small molecules such as nucleotides, primers, dyes, and contaminants from mixture.
- It is effectively used in separation of bound from unbound radioisotopes

Give some exercise related to gel filtration chromatography

2.4.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC stands for high-performance liquid chromatography. As its name suggest, high performance means that this analytical technique used for proper separation, identification and for quantification of components from the mixture**.** HPLC is an instrumental form of liquid chromatography and gives high performance due to the small particle size of the stationary phase. The particle size of stationary phase is 3.5 to 10 micrometer. Due to smaller size, surface area of the particle is high and ultimately HETP (height equivalent to theoretical pressure) increased and thereby help in achieving more efficient separation of the components of the mixture than those used in conventional liquid chromatography.

In this chromatography particle size of stationary phase is small and due to small size, packing of stationary phase will be high. Due to tight packing flow rate of mobile phase is reduced. So that to increase the flow rate or to increase efficiency or to increase the separation of the mixture high pressure is applied. The applied pressure is about 1000-4000 psi. Because of the use of high pressure in this technique, it is sometimes also known as high pressure liquid chromatography. So we can say HPLC is modern application of liquid chromatography.

HPLC has the ability to separate, and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion. Because of this versatility, HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals.

Principle :

HPLC is highly automated and extremely sensitive technique as compared to column chromatography because the components of a mixture are separated from each other due to their different degrees of interaction with the absorbent particles. This causes different elution rates for the different components and leads to the separation of the components as they flow out from the column. Solvents are used as mobile phase. In general organic compounds are analyzed using HPLC and these organic compounds are soluble in polar solvents. Some commonly used solvents

as mobile phase are methanol, acetonitrile and water. Acidifiers or basifies or buffer solution are added to the solvent to achieve better separation. As these neutralize the ionized analytes or compounds present in the column. If analyte or compounds present in column will be ionized so affinity with stationary phase will be lost and proper separation does not take place. Means every analyte immediately come with the mobile phase. Particle size of stationary phase ranges 3.5 to 10 micrometer. There are two types of stationary phase. i) Normal stationary phase for example silica gel is used and ii) Reversed stationary phase for example octa decyl silane silica gel etc. There are generally two types of columns are used. Normal phase column and reversed phase column.

Instrumentation :

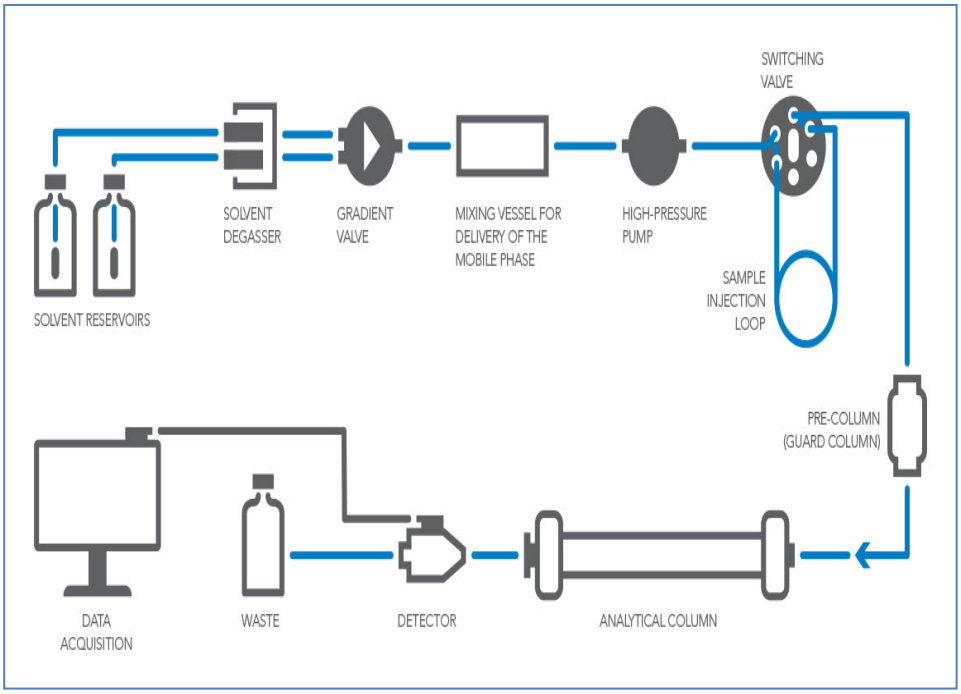


Fig. 2.4 : Diagrammatical representation of HPLC

The main components of HPLC are as follows:

1. **Solvent Reservoir :** The solvent reservoir holds the solvent, which is referred to as the mobile phase. Two types of reservoir are used.

   a. **Binary system :** In this type two reservoir are present.

   b. **Quaternary system :** In this type four reservoir are present.

   In HPLC we use specific type of solvent that are filtered known as HPLC grade solvent. This solvent is highly pure.

2. **Degasser :** It is used to remove the gases which are dissolved in mobile phase or in solvent. Degassing is done by using vacuum pump. So vacuum pump remove the any type of gas or air present

in solvent and make it suitable for better separation. If these gases interact with mobile phase and go to the column, proper functioning of the column does not take place i.e. proper separation may hindered.

3. **Solvent Mixing Valve:** This valve used to mix the solvent. If we use binary system two solvents will get mix together and if we use quaternary system, four solvents mix together.

4. **HPLC Pump:** Two types of pumps are used.

   a. **Constant pressure pump :** Pressure of the pump is constant so that flow rate may vary and this will not do proper separation.

   b. **Constant flow rate pump:** Flow rate is constant and pressure may be changed. So according to our column resistance pressure will be changed and due to that changed pressure flow rate of the column does not affected. Generally 1 ml/min flow rate is used in HPLC.

5. **Precolumn or Guard Column:** HPLC used precolumn or guard column to remove impurity of the solvent. As the name suggested precolumn, means it is used before the analytical column. As stationary phase is same in guard column. Solvent first go through the guard column after that it will go to the analytical column. So any type of contamination present in the solvent is removed by this column, before it go to analytical column. Size of the guard column is low as compared the analytical column. Internal diameter is 4-5 mm and length is 2-10 cm.

6. **Sample Injector : Three types of sample injectors are used.**

   a. **Septum Injector** : In this type one auto selling in present and solvent is injected using micro siring.

   b. **Stop flow septum less injector :** In this type of injector firstly we have to stop the mobile phase then we open the upper part of the column and after that we put the sample here and again start the flow of mobile phase.

   c. **Microvolume sampling injector :** This is the modern and sophisticated injector. By using this Injector highest accuracy will be achieved and auto sampling technique is applied by using this method.

7. **Analytical Column :** Length of analytical column is 10-30 cm and internal diameter is 4-5 mm. this column is made up of stainless steel which can resist the high pressure used in HPLC. During packing of analytical column 14000-15000 psi pressure are applied.

8. **Detector :** It is used to separate compound bands as they elute from the high pressure column. There are several types of detector used in HPLC. Some of them are as follows.

   a. **Bulk property detector :** Like refractive index detector.

   b. **Solute property detector :** Like florescence detector, UV detector.

   c. **Multipurpose detector :** It is combination of 2 or 3 types of detector.

   d. **Electrochemical detector :** Like colometric, emperometric detector.

   e. **Mass detector :** It is also known as LCMS. It is highly sensitive and most commonly used detector in HPLC.

The information is sent from the detector to a computer which generates the chromatogram. The mobile phase exits the detector and is either sent to a waste, or collected as desired.

Methodology :

To run HPLC following process goes on.

- The sampler brings the sample mixture to be evaluated into a stream of mobile phase which is flowing at a defined pressure.
- Now the injected mixture does flow over the stationary phase inside the column under the influence of pressure along with the mobile phase.
- During this flows based on the affinity of individual compounds in the mixture towards stationary and mobile phase, some compounds get eluted out of the column first before others.
- Outside the column they are sent into a detector where individual compounds are detected and recorded in computer installed chromatography software.
- The recordings (preferably in the form of quantitative peaks) are compared with those of standard compound's HPLC values, and the individual compounds are identified. So the overall theory of HPLC is relative separation and detection of compounds.

Application :

- ***In Environment :*** HPLC can be use in detection of phenolic compounds and other contaminants present in drinking water. It also behaves as bio-monitor of pollutants.
- ***In Forensics Science :*** This separation technique is used to determine quantify of steroids, cocaine and other drugs in blood,

urine etc. Forensic analysis of textile dyes can also be performed by using this technique.

- ***In Food and Flavor* :** It helps in measurement of quality of soft drinks and water, sugar analysis in fruit juices, polycyclic compounds analysis in vegetables. Preservative analysis can also be done by this technique.
- ***In* clinical diagnosis and health industry :** HPLC is used in Urine analysis, antibiotics analysis in blood. Many disorders related to body metabolism, those related to endocrine and exocrine gland secretion, alteration in body fluids are diagnosed by HPLC analysis of concerned fluids. For example, detection of bilirubin, biliverdin in hepatic disorders and endogenous Neuropeptides in extracellular fluid of brain can be performed by using HPLC.
- **In scientific research :** HPLC system is a mandatory tool in most of the scientific research such as medical, biological, chemical, biochemical, and phytochemical research. This technique finds its major application to analyze and quantify the molecules and can easily distinguished the components with similar chemistry and properties.
- **In pharmaceutical industry :** In the pharmaceutical industry, the qualitative type of HPLC analysis is widely used. It can be used to control drug stability and pharmaceutical quality. HPLC also analyzed any new molecule under development or in a preclinical trial to see their concentration in the blood after certain intervals of administration. This helps to evaluate the metabolic profile, plasma concentration, bioavailability, etc. of the formulation or chemical moieties under development.

2.5 SUMMARY

All types of chromatography work on the same principle and used mobile phase and stationary phase separation of biological samples. The chromatography basically classified into thin layer, ion exchange, affinity, gel filtration, high performance liquid, hydrophobic interaction chromatography etc. In thin layer chromatography the stationary phase is used and it is spread over a supporting plate and a mobile phase is also used. This mobile phase migrate over these supporting material or stationary phase against the gravitational force. In ion exchange chromatography, separation occurs as a result of formation of ionic or electrostatic bond between the charged group of biomolecules and an ion exchange resin bearing opposite charge. HPLC has the ability to separate, and identify the compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion HPLC also used for identification and for quantification of components in a biological mixture.

2.6 TERMINAL QUESTION

Q.1. What is chromatography? Define the role of various chromatography in biochemistry.

Answer: ----------

Q.2. Write the principle and instrumentation of HPLC.

Answer: ----------

Q.3. Write the principle, instrumentation and application of affinity Chromatography.

Answer: ----------

Q.4. Discuss about gel chromatography in brief.

Answer: ----------

Q.5. Discuss ion exchange chromatography in brief.

Answer: ----------

Q.6. Write the principle of thin layer chromatography.

Answer: ----------

2.7 FURTHER READING

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Analytical Biochemistry

BLOCK

2

CENTRIFUGATION AND ELECTROPHORESIS

UNIT-3

Centrifugation

UNIT-4

Electrophoretic techniques

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BLOCK INTRODUCTION

This is the second block of analytical biochemistry. It consists of following two units.

Unit-3 : This unit covers the type, principle and application of centrifugation. However, the differential centrifugation, density gradient, and ultracentrifugation are briefly discussed. These techniques are very useful in separation and purification of organic molecules.

Unit-4 : This unit overs the basic understanding of electrophoresis. The various types of electrophoresis techniques is discussed briefly. The electrophoresis of proteins, native-PAGE, SDS-PAGE and agarose gel electrophoresis for DNA are discussed. The isoelectric- focusing and 2D gel electrophoresis also discusses in briefly. The electrophoresis is most valuable techniques over the process of separation of macromolecules.

UNIT-3 CENTRIFUGATION

Structure

3.1 INTRODUCTION

This unit discusses the principle, instrumentation and applications of centrifugation. The centrifugation is one of technique that has important role in the field of biochemistry, because it implies to separate and purify the biological fluids from samples by applying centrifugal forces (Fig. 3.1). Here discuss the various centrifugation techniques such as differential centrifugation, density gradient and ultracentrifugation. However, the centrifugation is based on the density gradient of molecules. The sedimentations rate of particles can be increasing by using centrifugal forces. The rapid spinning imposes high centrifugal forces on suspended particles or even molecules in solution through ultracentrifugation that cause separation of particles on the basis of difference in weight. The biological centrifugation is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium. It is a key technique for isolating and analyzing cells, sub cellular fractions,

supramolecular complexes and isolated macromolecules such as proteins or nucleic acids.

Fig. 3.1 : Image of centrifuge

Objectives :

The basic objectives of this unit is

- To understand basic nature and types of centrifuge.
- To briefly know the basic principle and instrumentation of different types of centrifuge.
- To understand the role and application of different types of centrifuge.

3.2 CENTRIFUGATION OVERVIEW

The centrifugation is the method of separating two things with different density, by rapid rotating it in a circular motion which eventually, separates the less dense and denser particles. It forces less dense particles to come out on the surface of the mixture. The centrifugation force is play significance role to separate different density

particles over the time. In practice, centrifugal force is necessary to separate most of the particles. The rate of separation in a suspension of particles by means of gravitational force mainly depends on the particles, size and density. Particles of higher density or large size typically travel at a faster rate and at some point will be separated from particles less dense or smaller. This sedimentation rate of particles, which describes the movement of a sphere in a gravitational field, is shown in equation 1, which calculates the velocity of sedimentation.

$$\upsilon = \frac{d^2(p-L)g}{18\,n} \qquad (3.1)$$

υ = sedimentation rate or velocity of the sphere

d = diameter of the sphere

p = particle density

L = medium density

n = velocity of medium

g = gravitational force

3.3 BASIC PRINCIPAL OF CENTRIFUGATION

A centrifuge is a device for separating particles for the solution according to their size, shape, density, velocity of medium and rotor speed. In a solution, particles whose density is higher than that of the solvent sink and particles that are lighter than it float to the top. However, those particles which have size more than 5 μm are considered in sedimentation at the bottom due to gravitational force. If the size of particles is less than 5 μm they undergo Brownian motion. The centrifugation involves principle of sedimentation, where the acceleration at centrifugal force causes denser substance to separate out along the radial direction at the bottom of tube. Considering a body of mass m rotating in a circular path of radius r at a velocity υ. The force acting on the body in a radial direction is given by

$$F = \frac{m\upsilon^2}{r} \qquad (3.2)$$

where

F = centrifugal force

m = mass of body

υ = velocity of the body

r = radius of circle of rotation

The gravitational force acting upon the body; G=mg, where G= gravitational force, g = acceleration due to gravity.

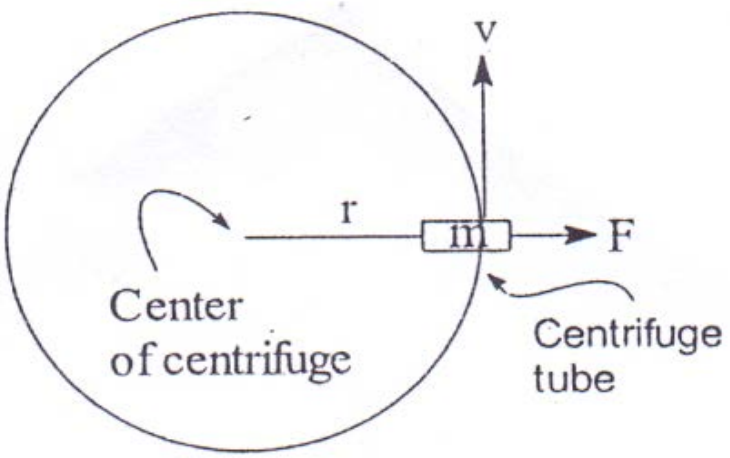

Fig. 3.2 : Illustation of the principal of centrifugation

However, the rate of sedimentation is dependent upon the applied centrifugal field (cm s^{-2}), G, that is determined by the radial distance, r, of the particle from the axis of rotation (in cm) and the square of the angular velocity, ω of the rotor.

$$G = \omega^2 r \qquad (3.3)$$

In centrifugatio proces, the five important behaviors of particles are cosidered.

i. The rate of particles sedimentation is proportional to the particle size.

ii. The sedimentation rate is propertional to the difference in density b/w the particle and the medium.

iii. The sedimentation rate is zero when the particle density is the same as the medium density.

iv. The sedimentation rate decreases as the medium viscosity increases.

v. The sedimentatation rate increase as the gravitational force increases.

vi. The denser the biological buffer system is, the slower the particle will move in a centrifugal field;

vii. The greater the frictional coefficient is, the slower a particle will move

viii. The greater the centrifugal force is, the faster the particle sediments

ix. The sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal

RCF (relative centrifugal force), which is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity. RCF is measured in force x gravity or g-force. This is the force exerted on the contents of the rotor, resulting from the revolutions of the rotor. RCF is dependent on the speed of rotation in rpm and the distance of the particles from the centre of rotation. Where the speed of rotation is given in rpm (θ) and the distance (r) is expressed in centimeters, RCF can be calculated by using the formula in equaction (3.4).

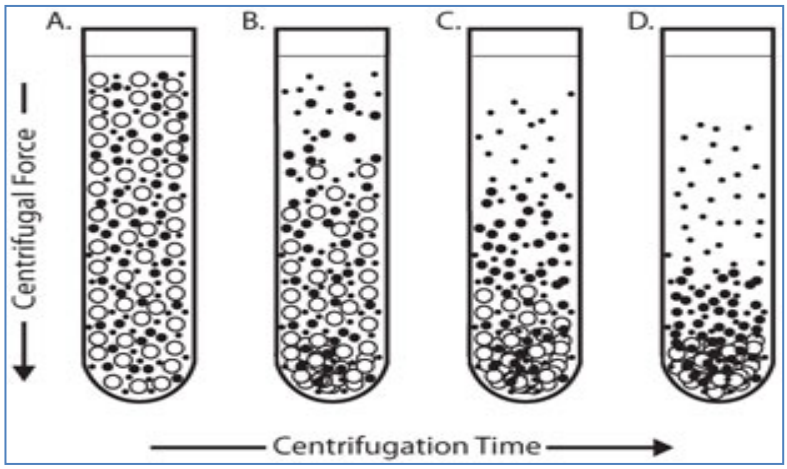

Fig.3.3 : Schemtic representation of different centrifugation

$$RCF = \frac{4\pi^2(\text{rev min}^{-1})^2 r}{360 \text{ X } 981} = \frac{G}{g} \qquad (3.4)$$

RCF units are therefore dimensionless (denoting multiples of g) and revolutions per minute are usually abbreviated as r.p.m.: RCF =1.12X10^{-5} r.p.m.

When designing a centrifugation protocol, it is important to keep in mind that

- The more dense a biological structure is, the faster it sediment in a centrifugal field.
- The more massive a biological particle is, the faster it moves in a centrifugal field.
- The denser the biological buffer system is, the slower the particle will move in a centrifugal field.
- The greater the frictional coefficient is, the slower particle will move.
- The greater the centrifugal force is, the faster the particle sediments.
- The sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.

3.4 TYPES OF ROTOR CENTRIFUGES

A centrifuge rotor is the rotating unit of the centrifuge, which has fixed holes drilled at an angle. Test tubes are placed inside these holes and the rotor spins to aid in the separation of the materials. There are three types of centrifuge rotors: swing-bucket, fixed-angle and vertical rotors. Companies usually name rotors according to their type of design, the maximum allowable speed and sometimes the material composition. Depending on the use in a simple low-speed centrifuge, a high-speed centrifuge or an ultracentrifuge, different centrifugal forces are encountered by a spinning rotor.

Swing-Bucket Rotors : It is usually supports samples ranging in volume from 36 mL to 2.2 mL. Swing-buckets can support two types of separations: rate-zonal and isopycnic. Swing-buckets are preferred for rate-zonal separations.

Fixed-Angle Rotors : It has cavities range from 0.2 mL to 1 mL and used for pelleting applications to either pellet particles from a suspension or remove the excess debris. The most important aspect in deciding to use a fixed-angle rotor is the K factor.

Vertical Rotors : Vertical rotors are highly specialized and has very short to run. It is typically used to band DNA in cesium chloride. Vertical rotors have very low K factors, which is useful if the particle must only move a short distance until it pellets.

3.5 APPLICATION OF CENTRIFUGATION

- A centrifuge is used to separate two miscible substances:

- Cells
- Sub-cellular components
- Proteins -Nucleic acids

- Centrifugation is basis the of size, shape and density of particles
- It utilizes density difference between the particles/macromolecules and the medium in which these are dispersed
- To analyze the hydrodynamic properties of macromolecules and purification of mammalian cells
- To Separation of urine components and blood components in forensic and research laboratories.
- It use to separate fraction of sub-cellular organelles and fractionation of membrane vesicles
- A tube of anti-coagulated whole blood left standing on a bench top will eventually separate into plasma, red blood cell and white blood cell fractions.
- It utilizes density difference between the particles/macromolecules and the medium in which these are dispersed. Dispersed systems are subjected to artificially induced gravitational fields

3.6 TYPES OF CENTRIFUGE

Centrifugation techniques take a central position in modern biochemical, cellular and molecular biological studies. Depending on the particular application, centrifuges differ in their overall design and size. However, a common feature in all centrifuges is the central motor that spins a rotor containing the samples to be separated. Particles of biochemical interest are usually suspended in a liquid buffer system contained in specific tubes or separation chambers that are located in specialised rotors.

Many different types of centrifuges are commercially available including:

- large-capacity low-speed preparative centrifuges
- refrigerated high-speed preparative centrifuges
- analytical ultracentrifuges
- preparative ultracentrifuges
- large-scale clinical centrifuges
- small-scale laboratory microfuges

The low speed centrifuge has a maximum speed of 4000-5000 rpm. This intrument usually operate at the room tempreature with no means of temperature control. It is manily used in the sedimentation of red blood cells unit. In this techniques the particles are titly packed into a pettet and the suspention is separated by decantation. The low speed centrifuge have following types of rotor such as fixed angle and swinging bucket.

The hish speed centrifugation has a moximum speed of 15,000-20,000 rpm. The operator of this instrumet can be carefully contral speed and temparature which is requre for sensitive biological sample. The high speed centrifuge have following types of rotor such as fixed angle, swinging bucket and vertical rotors.

Ultracentrifuge has maximum speed of 65,000 rpm. Intense heat is generated due to high speed thus the spining chambers must be refrigerated and kept a high vaccum. It is used both in preparative and analytical work.

3.7 DIFFERENTIAL CENTRIFUGATION

Differential centrifugation is simplest form of separation deferential palliating. It separates components of a cell on the basis of their densities and mass. The cell membrane is first ruptured to release the cell's components by using a homogenizer. The resulting mixture is referred to as the homogenate. The homogenate is centrifuged to obtain a pellet containing the most dense organelles. Compounds that are the densest will form a pellet at lower centrifuge speeds while the less dense compounds will likely remain in the liquid supernatant above the pellet. Different pelleting is commonly used for harvesting or producing crude sub cellar fractions for tissue homogenizing, for example a rat liver hematogenate containing nuclei, mitochondria, lysosomes and membrane vesicles centrifuge at low speed for a short time will pellet mainly the larger and more dense nuclei.

Equilibrium sedimentation uses a gradient of a solution to separate particles based on their individual densities (mass/volume). The sedimentation rate can be increased by using centrifugal forces. Different densities or size of particles will sediment at different rates with largest and most dense particles sedimenting the fastest followed by less dense and smaller particles. Subsequent centrifugation at a higher centrifugal force will pellet particles of the next lower order of size e.g. Mitochondria and so on. It is usually for more than four differential centrifugation cycles. For a normal tissues damage due to the heterogeneity in biological particles, differential centrifugation suffers from contamination and poor recoveries. The contamination by different particles types can be addressed by resuspension and repeating the centrifugation steps.

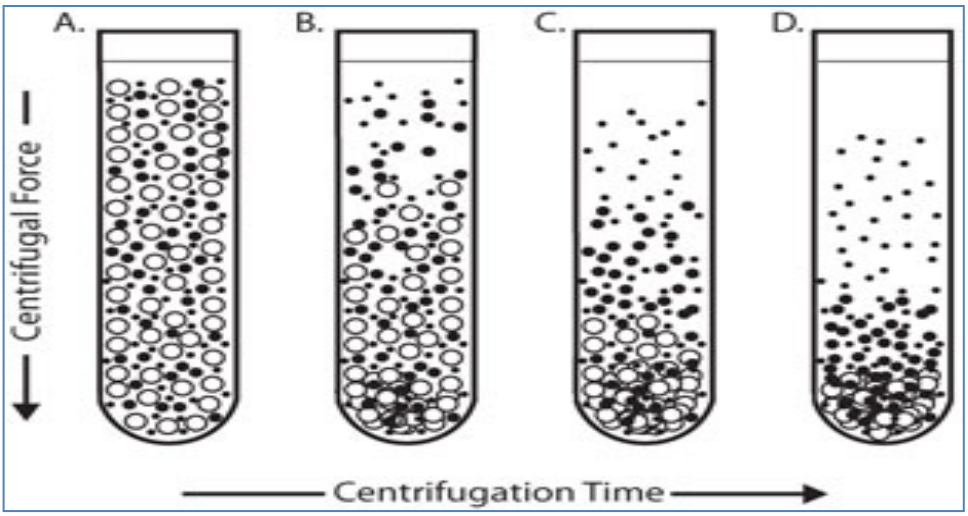

Fig. 3.4 : Diagram of different centrifugation

The differential sedimentation of a particulate suspension in a centrifugal field is diagrammatically shown in Fig. 3.4. In this process the homogenate mixtures are evenly distributed throughout the centrifuge tube and then move down the tube at their respective sedimentation rate during centrifugation. The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant. However, during the initial centrifugation step smaller particles also become entrapped in the pellet causing a certain degree of contamination. At the end of each differential centrifugation step, the pellet and supernatant fraction are carefully separated from each other.

3.7.1 DENSITY GRADIENT CENTRIFUGATION

It is the preferred method to for purification of sub cellular organelles and macromolecules. Density gradient can be generated for placing layer after layer of gradient media such as sucrose in a tube with the heaviest layer at the bottom and the lightest at the top in either a discontinuous mode. The cell function to be separate is placed on top of the layer and centrifuged. Density gradient separation can be classified into two categories such as Rate-zonal centrifugation and Isopycnic centrifugation.

3.7.2 RATE-ZONAL CENTRIFUGATION

The problem of cross contamination of particles of different sedimentation rates may be avoided by layering the sample as a narrow zone on top of density gradient in rate-zonal centrifugation. In this way the faster sediment particles are not contaminated by the slower particles as occurs in differential centrifugation. However, the narrow load zone limits the volume of sample that can be accumulated on the density gradient. The gradient stabilizes the bands and provides a medium of increasing density and viscosity. In the Fig. 3.5 shows diagram of rate-zonal centrifugation.

Sample is layer (a) the top of a density gradient, (b) under centrifugation force, particles move at different rates depending on this mass, (c) the speed at which particles sediment, primarily depends on their size and mass instead of density. As the particles in the bond more down through the density medium, zone containing particles of similar size the faster sedimenting particles move a head of the slower ones. Because the density of the particles is greater than the density of the gradient, all the particles will eventually form a pellet if centrifuged is long enough.

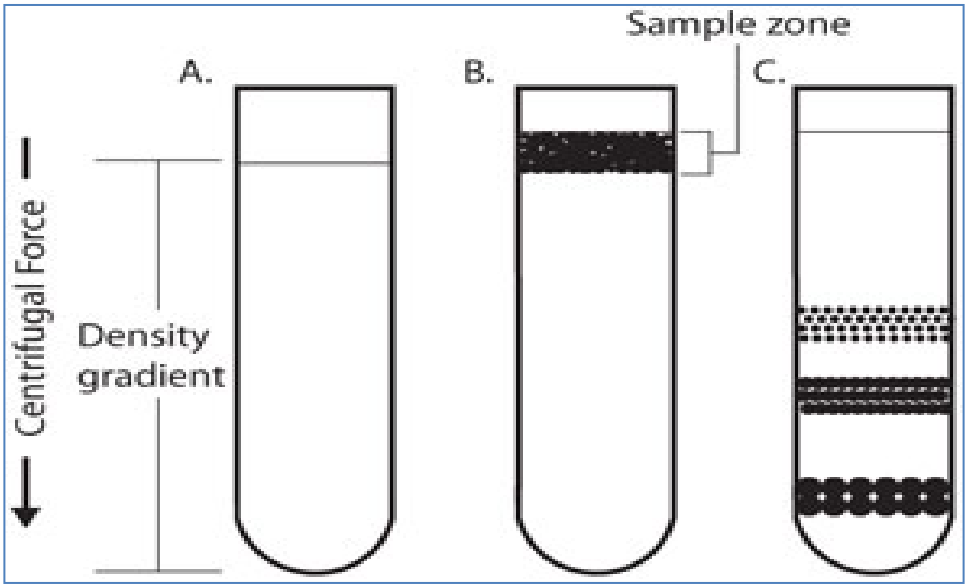

Fig 3.5 : Diagram of Rate-zonal centrifugation

Rate-zonal separations cannot be carried out in single head rotors as the sample mixes with the gradient during acceleration of the rotor and until recently, the capacity of swing-out rotors was severely limited. As the sedimenting zones are as broad as, or broader than, the starting zone, the volume of material which can be loaded onto a rotor is limited if any degree of separation is to be achieved. Rate-zonal centrifugation was initially used mainly for analytical separations such as the analysis of the size distribution of samples of polysomes or of RNA. Although very soon after the introduction of the technique Thomson and his colleagues used rate sedimentation to separate mitochondria and lysosomes.

3.8 ISOPYCNIC CENTRIFUGATION

Isopycnic centrifugation designates density gradient centrifugation methods, where the macromolecules end up in a position where their apparent density or buoyant density is equal to the local density of the gradient. However, in isopycnic separation, the particles are also separated solely on the basis of their density. Particles size only affects the rate at which particles move until then density is the same as the surrounding the particles to be separated. In this method the particles will

never sediment to the bottom of the tube no matter how long the centrifugation time. In isopycnic centrifugation molecules separated on equilibrium position, not by rates of sedimentation.

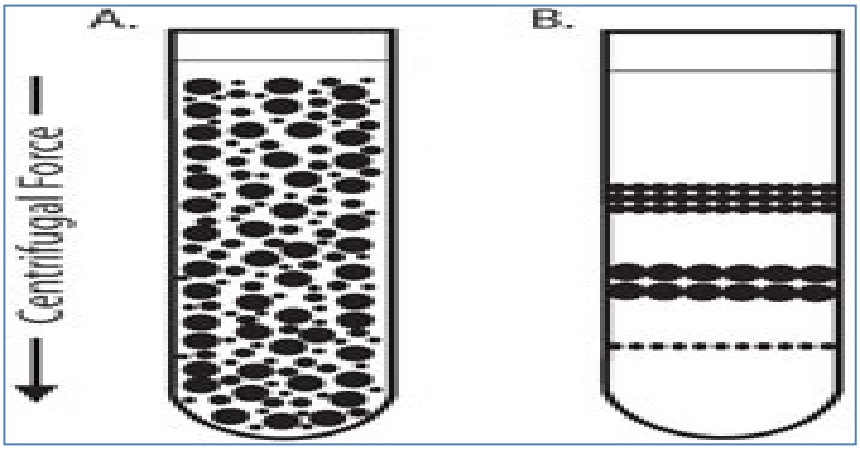

Fig. 3.6 : Diagram of Isopycnic centrifugation

The sample (Fig.3.6) of density gradient (A) is under centrifugal force, particles move until their density is the same as the surrounding medium (B). Upon centrifugation, particles of specific density sediment until they reach the point where their density is the same as the gradient media (i.e. the equilibrium position). The gradient is then said to be isopycnic and the particles are separated according to their buoyancy. Since the density of biological properties is sensitive to the osmotic pressure of the gradient, isopycnic separation may vary significantly depending on the gradient medium used. Although a continuous gradient may be more suited for analytical purpose and the preparative techniques commonly used for discontinuous gradient in which the particles bond at the interaction between the density gradient layers. This makes harvesting certain biological particles (e.g. lymphocytes) series.

3.9 ULTRACENTRIFUGATION

An important tool in biochemical research is the centrifuge, which through rapid spinning imposes high centrifugal forces on suspended particles or even molecules in solution and cause separations of such matter on the basis of difference in weight. For example, red blood cells may be separated from plasma of blood, nuclei from mitochondria in cell homogenates and one protein from another in complex mixture.

Proteins are separated by ultra centrifugation at very high speed spinning with appropriate of the protein layers as they form in the centrifugal field. It is possible to determine the molecular weight of the proteins. The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speed, capable of generating acceleration as high as 19600 Km/ S^2 (around 5000 rpm).

Biological centrifugation is a process that uses centrifugal force to separate and purify mixture of biological particles in a liquid medium. The smaller the particle higher the g- force required for the separation. It is a key technique for isolating and analyzing cells, subcellular fractions, supramolecular complexes and with high g-forces instruments or ultra-centrifuges (up to 60000 revolutions per minute corresponding to 200000 g) isolated macromolecules such as proteins or nucleic acids. Such high speed devices require a vacuum to avoid overheating.

The development of the first analytical centrifuge with a specially designed optical system for monitoring and recording the sedimentation process by ***Svedberg*** in the late 1920 and the technical refinement of the preparative centrifugation technology at the centre of biological and biomedical research for many decades. Today, centrifugation techniques represent a critical tool for modern biochemistry and are employed in almost all invasive subcellular studies. Analytical ultracentrifugation is mainly concern with the study of purified macromolecules or isolated supramolecular assemblies; preparative centrifugation methodology is devoted to actual separation of tissues cells, sub cellular structures, membrane vesicles and other particles of biochemical interest. The basic principles of centrifugation, the general design of various rotors and separation processes are diagrammatically represented. The learning process of undergraduate students is hampering by the lack of a proper linkage between theoretical knowledge of practical applications.

The isolation of fractions from skeletal muscle homogenates as example of ultra centrifugation. Since affinity isolation methods not only represent an extremely powerful tool in purifying biomolecules.

3.10 TYPES OF ULTRACENTRIFUGATION

- Analytical ultracentrifugation
- Preparative ultracentrifugation

3.10.1 ANALYTICAL ULTRACENTRIFUGATION

Analytical ultracentrifugation (AUC) refers to the analysis of a macromolecular solution by its subjection to gravitational forces up to 300000-fold greater than gravity. From its inception by Svedberg in the mid-1920s, analytical ultracentrifugation has played a leading role in studies of macromolecular systems. AUC is also involves the measurement of the radial concentration gradients of molecules created by centrifugal force. Three optical systems are available for the analytical ultracentrifuge (absorbance, interference and fluorescence), that permit precise and selective observation of sedimentation in real time. Some of the most attractive features of AUC are:

Versatility : a wide variety of samples can be examined by AUC, including molecules ranging in size from sucrose to virus particles.

Rigor : AUC experiments are directly interpreted in the context of thermodynamic and hydrodynamic theory, so it is not necessary to run standards to calibrate each experiment.

Convenience : The data analysis methods have made AUC are much more convenient and accessible to the general biochemistry and polymer science communities. In contrast to earlier instruments, experiments are easy to set up and centrifugation parameters and data acquisition are all under computer control.

Since sedimentation relies on the principal property of mass and centrifugal force, it is a valuable technique for a wide variety of solution conditions.

1. What can be done with AUC to characterize a sample:

a. Determine number of components and number of species detection of impurities.

b. Molar mass of each species.

c. Kind and stiochiometry of chemical reactions present in solution, including association with ligands and self-association.

d. Shape and charge of the molecules, as inferred from their sedimentation frictional behavior.

2. Examples of molecules that can be analyzed:

a. Protein

b. Polysaccharides

c. Nucleic acids

d. Small molecules, drugs, ligands gases

e. Large aggregates, organ cells.

3. Kinds of buffers and additional solutes:

a. BME, DTT

b. Triton X100, Twean – 80

c. Nucleotides

d. Salt and neutral molecules that significantly affect density

e. PEG, glycerol affect viscosity and density strongly

f. 6 M Gdn and 8 M Urea

In an analytical ultracentrifuge a sample being spun can be monitored in real time through an optical detection system using ultraviolet light absorption and optical refractive index sensitive system.

Working : As a rotor turns, the images of the cell (proteins) are projected by an optical system on to film or a computer. The concentration of the solution at various points in the cell is determined by absorption of a light of appropriate wavelength. This can be accomplished either by measuring the degree of blackening of a photographic film or by the deflection of the recorder of the scanning system, fed into a computer.

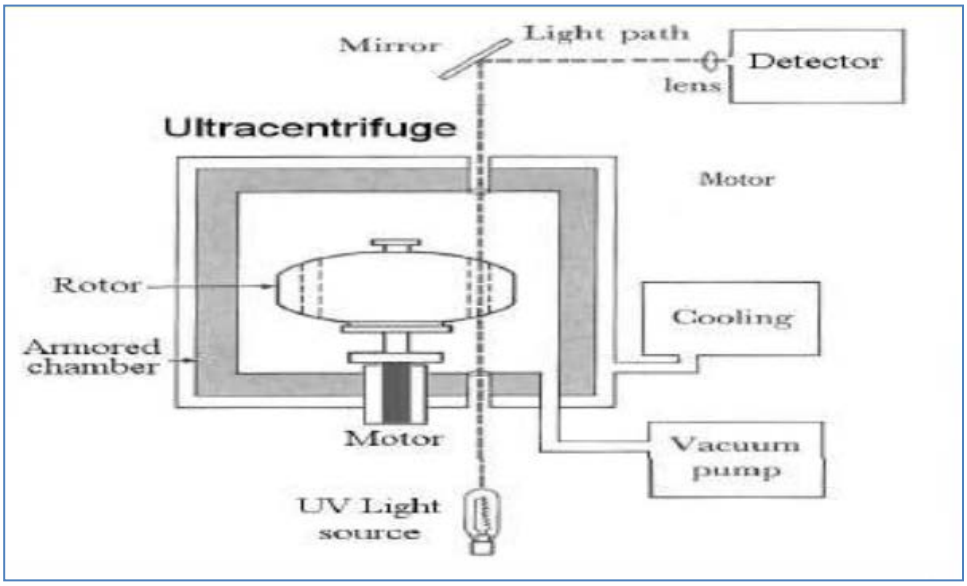

Fig. 3.7 : Image of ultracentrifugation

This allows the operator to observe the separation of the sample concentration verses the axis of rotation. As a result of the applied centrifugal field two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation equilibrium experiments.

Sedimentation velocity experiments aim to interrupt the entire time course of sedimentation and report on the surface and molar mass of the dissolved macromolecules as well as their size- distribution. Sedimentation velocity experiments can also be used to study reversible chemical equilibria between macromolecular species by monitoring the number and molar mass of macromolecular complexes.

Sedimentation equilibrium experiments are concerned only with the final steady state of the experiments where sedimentation is balanced by diffusion opposing the concentration gradients, resulting in a time independent concentration profile. Analytical centrifugation involves measuring the physical properties of the sedimenting particles such as sedimentation coefficient or molecular weight.

3.10.2 PREPARATIVE ULTRACENTRIFUGATION

Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. Swinging bucket rotors allow the

tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates. Fixed angle rotors are made of a single block of metal and hold the tubes in cavities bored at a predetermined angle. Zonal rotors are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high. They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution. Sucrose gradients are typically used for separation of cellular organ cells. Gradients of casesium salts are used for separation of nucleic acids. After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube to isolate the separated components.

The Preparative ultracentrifuges are also used in biology for pelleting of fine particulate fractions, such as cellular organelles such as mitochondria, microsomes, ribosomes and viruses.

- Used for gradient separations. Gradients of sucrose for separation of cellular organelles.
- Gradients of calcium salts for separation of nucleic acids.
- After the spun at high speed for sufficient time, allow the rotor to come to a smooth stop
- Gradient is gently pumped out of each tube to isolate the separated components.

3.11 SUMMARY

Centrifugation is very useful techniques for the separation and purification of biological particles in liquid medium. It is also useful because it use centrifugal force to separate the samples. The Centrifugation techniques are critical tools for modern biochemistry. Centrifugal force is necessary to separate the most of particles. In addition, the potential degradation of biological compounds during prolonged storage means faster separation techniques are needed. The rate of separation is a suspension of particles by means of gravitational force; it mainly depends on the particles size and density. Particles having a size above 5 um sedimentation at the bottom due to gravitational force. Such a suspension can be separated by simple filtration technique the most common application is separation of solid substance for high concentrated suspensions. Proteins are separated by ultra centrifugation very high speed spinning with positively of appropriate photography of the protein layers as they from in the centrifugal field.

3.12 TERMINAL QUESTIONS

Q.1. Introduce about centrifuge and centrifugation.

Answer: ----------

Q.2. Write the basic principle and application of centrifugation.

Answer: ----------

Q.3. Discus the types of centrifugation and role of it in purification of macromolecules.

Answer: ----------

Q.4. What is rate-zonal centrifugation? Discuss about it.

Answer: ----------

Q.5. What is analytical ultracentrifugation?

Answer: ----------

Q.6. What is Differential centrifugation and write it application in biochemistry?

Answer: ----------

3.13 FURTHER READINGS

1. Y R Sharma, Elementary organic spectroscopy; S Chand publication 2007.
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UNIT-4 ELECTROPHORETIC TECHNIQUES

Structure

4.1 INTRODUCTION

The electrophoretic techniques is very useful in separation of electrically charge species existed as either a cations (+) or anions (-) in solution. Thus, this technique is useful in the separation of biological macromolecules under the influence of an anode, depending on the nature of their net charge. The electrophoresis is available for running either vertical or horizontal gel system. The moving boundary, native PAGE, SDS-Polyacrylamide, gel electrophoresis (SDS-PAGE) and Zone electrophoresis are discussed briefly in this unit. The electrophoresis's also used in separation of nucleic acid is described here briefly. The various application of electrophoretic is also discussed.

Objective :

- To discuss the principle and application of gel electrophoresis.
- To discuss about agarose Gel electrophoresis for DNA
- To understand the SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

4.2 ELECTROPHORETIC OVERVIEW

Electrophoresis is the process of moving charged molecules in solution by applying an electric field across the mixture. Because molecules in an electric field move with a speed dependent on their charge, shape, and size. Electrophoresis has been extensively developed for molecular separations. The electrophoresis is useful in the determination of the number, amount and mobility of components in a given sample, to obtain information about the electrical double layers surrounding the particles. It is also useful into determination of molecular weight of proteins and DNA sequencing. The electrophoresis of different types as mentioned in Fig. (4.1).

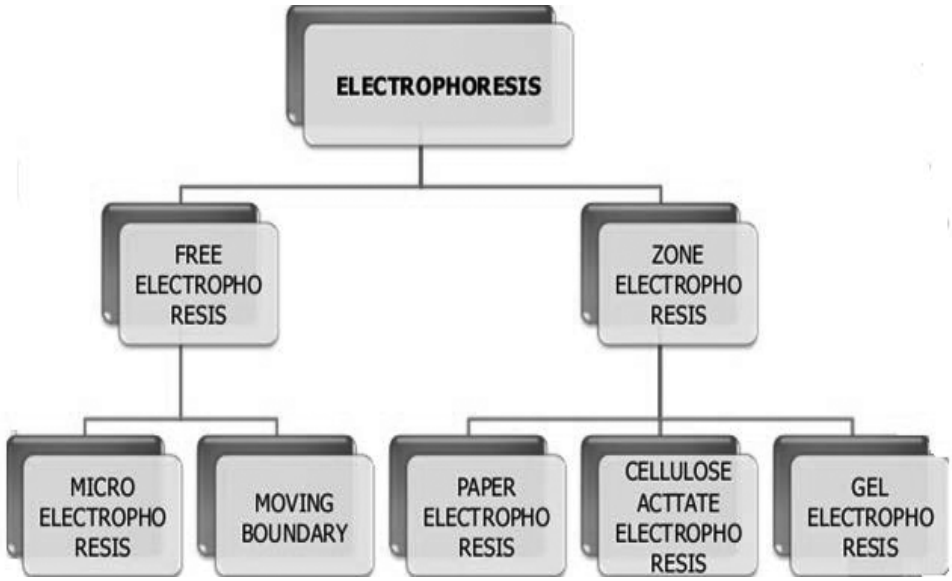

Fig.4.1 : Flow chart of different electrophoresis

4.3 GENERAL PRINCIPLE OF ELECTROPHORESIS

Electrophoretic literally means running in the electric field. The charge molecule moves their counter charge electrode but electric field is removed before it reaches the electrode. Charge species movement in an electric field gives differential mobility to the sample based on the charge and consequently resolves them. The moment of charged particles is retarded with the addition of a polymeric gel so that a different time is available for resolving the sample. Polymeric gel is inert, uncharged and does not cause retardation by different size (depending on the concentration of polymer) and sample pass through the pore and as a result their electrophoretic mobility is reduced.

If migration velocity is υ and E is applied electric field strength them electrophoretic mobility μ, M is positive or negative while neutral species have on mobility.

$$\mu = \frac{\upsilon}{E} \quad (4.1)$$

$$\upsilon = \frac{EQ}{\eta\pi} \quad (4.2)$$

where Q= charge of molecule; E = magnetite of applied potential, η = viscosity of medium; π = shape of molecule in terms of radius. M is +Ve and, –Ve while neutral species have no mobility. Different types of electrophoresis techniques are designated depending upon whether it carried out in the presence or absence of a supporting media.

4.4 ELECTROPHORESIS OF PROTEINS

Protein electrophoresis is a standard laboratory technique by which charged protein molecules are transported through a solvent by an electrical field. Both proteins and nucleic acids may be separated by electrophoresis, which is a simple, rapid, and sensitive analytical tool. The mobility of a molecule through an electric field is depending on field strength, net charge on the molecule, size and shape of the molecule. Apart from that it also depends on ionic strength, and properties of the matrix through which the molecule migrates (e.g., viscosity, pore size). Polyacrylamide and agarose are two support matrices commonly used in electrophoresis. Agarose gel has a large pore size and is suitable for separating nucleic acids and large protein complexes. Polyacrylamide gel has a smaller pore size and is ideal for separating majority of proteins and smaller nucleic acids. Several forms of polyacrylamide gel electrophoresis (PAGE) and (SDS)-PAGE are widely used.

4.5 GEL ELECTROPHORESIS

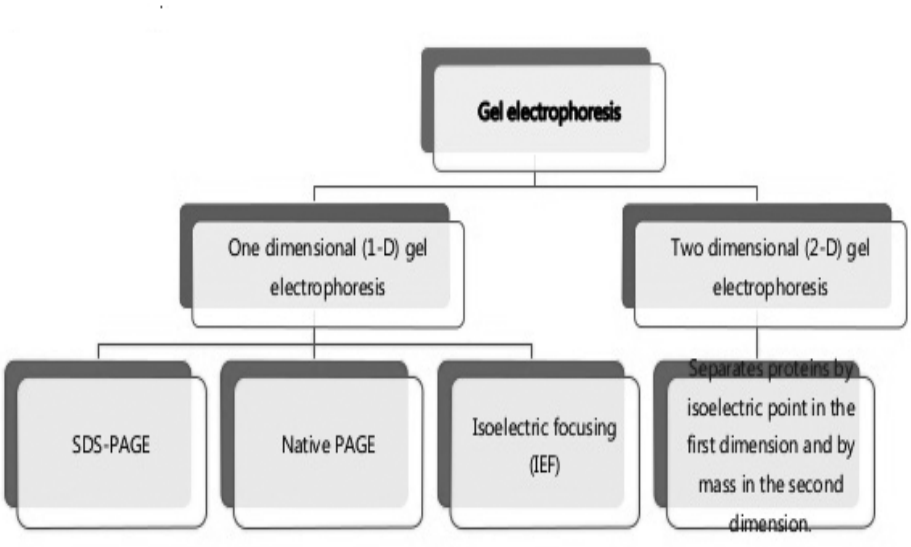

Fig. 4.2 : Flow chart of different gel electrophoresis

Principle of gel electrophoresis :

When we place any charged molecules in an electric field, they move toward the positive or negative pole according to the charge they are having. Proteins do not have any net charge whereas nucleic acids have a negative charge so they move towards the anode when electric field is applied.

Factors affecting gel electrophoresis :

Electrophoretic velocity depends on :

Inherent Factors :

- How much charge the particles have
- What is the molecular weight
- Secondary structures (i.e., its shape).

External Environment :

- pH of solution
- Electric field
- Solution viscosity
- Temperature

Gel electrophoresis is a technique used for the separation of Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA) or protein molecules according to their size and electrical charge using an electric current applied to a gel matrix. Gel is a cross linked polymer whose composition and porosity is chosen based on the specific weight and porosity of the target molecules. Types of gels used are as

i) Polyacrylamide gel :

Polyacrylamide gel electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility.

ii) Agarose gel :

Agarose is a polysaccharide extracted from seaweed (Gelidium) and contains many agarobiose subunits. During solidification, agarose form a network of polymers and its pore sizes can be determined by its concentration. It is usually used at concentrations of 0.5 to 2%. Stiffer gel means the agarose concentration is higher. Heat agarose with buffer to prepare gel and after it cools down it is poured in to the tray called as casting tray. These gels are not toxic unlike acrylamide gels. Range of separation in agarose gels is higher but resolving power is low.

4.5.1 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively. It is particularly useful for monitoring protein purification. The SDS-PAGE method is based on the separation of proteins according to their size; it can also be used to determine the relative molecular mass of proteins. When proteins are separated by electrophoresis through a gel matrix, smaller proteins migrate faster due to less resistance from the gel matrix. Other influences on the rate of migration through the gel matrix include the structure and charge of the proteins. In SDS-PAGE, the use of sodium dodecyl sulfate (SDS, also known as sodium lauryl sulfate) and polyacrylamide gel largely eliminates the influence of the structure and charge. SDS is an anionic detergent. Samples to be run on SDS–PAGE are firstly boiled for 5 min in sample buffer containing b-mercaptoethanol and SDS. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the negatively charged SDS molecules.

By heating the protein sample between 70-100°C in the presence of excess SDS and thiol reagent, disulfide bonds are cleaved, and the protein is fully dissociated into its subunits. In the presence of SDS and a reducing agent that cleaves disulfide bonds critical for proper folding, proteins unfold into linear chains with negative charge proportional to the polypeptide chain length. Polymerized acrylamide (polyacrylamide) forms a mesh-like matrix suitable for the separation of proteins of typical size. Protein is separated based on their polypeptide chain length by electrophoresis in a polyacrylamide gel with an appropriate mesh size.

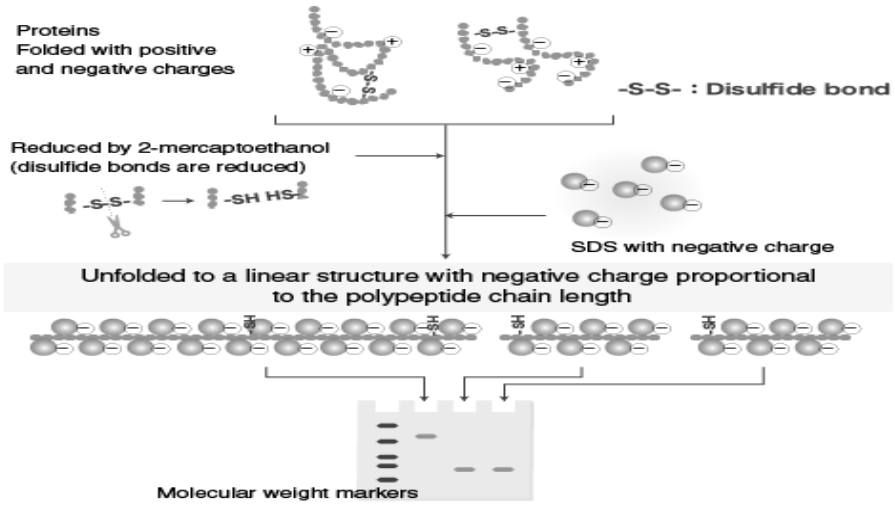

Fig. 4.3 : Process of SDS-PAGE

Source : https://ruo.mbl.co.jp/bio/e/support/method/sds-page.html

The negatively charged protein–SDS complexes move towards the anode because they have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. An application of SDS-PAGE.SDS-PAGE is used mainly for the following purposes:

- Measuring molecular weight.
- Peptide mapping.
- Estimation of protein size.
- Determination of protein subunits or aggregation structures.
- Estimation of protein purity.
- Protein quantization.
- Monitoring protein integrity.
- Comparison of the polypeptide and composition of different samples.
- Analysis of the number and size of polypeptide subunits.
- Post-electrophoresis applications, such as Western blotting.
- Staining of Proteins in Gels with Coomassie G-250 without Organic Solvent and Acetic Acid.
- Pouring and Running a Protein Gel by reusing Commercial Cassettes.

4.5.2 NATIVE PAGE

Native PAGE, separates acidic water-soluble and membrane proteins in a polyacrylamide gradient gel. In native PAGE, proteins are separated according to the net negative charge, size, and shape of their native structure. Due to present of net negative charge in alkaline running buffers the protein molecules are electrophoretic migrate. The electrophoretic migration depends on the negative charge density. If protein has high negative charge density then it will migrate faster than less negative charge density. At the same time, the frictional force of the gel matrix creates a sieving effect, regulating the movement of proteins according to their size and three-dimensional shape. Small proteins face only a small frictional force, while larger proteins face a larger frictional force. Thus native PAGE separates proteins based upon both their charge and mass. However, the no denaturants are used in native PAGE, subunit interactions within a multimeric protein are generally retained and information can be gained about the quaternary structure. In addition, some proteins retain their enzymatic activity (function) following

separation by native PAGE. Thus, this technique may be used for preparation of purified, active proteins.

4.5.3 ISOELECTRIC FOCUSING

Isoelectric focusing is an electrophoretic method in which proteins are separated on bans of Pls. It makes use of the property of proteins that their net charge is determined by the pH of their local environments. Proteins carry positive, negative or zero net electrical charge, depending on the pH of their surroundings. The net charge of any particular protein is the sum of all of its positive and negative charges. These are determined by the ionize able acidic and basic side chains of the constituent amino acids and prosthetic group of the protein. If the number of acidic groups in proteins exceeds the number of basic group, the pl of that protein will be at a low pH value and protein is classified as being acidic. When the basic groups outnumber the acidic group in proteins, the pl will be high with the proteins classified as basic. Proteins show considerable variation isoelectric points, but Pl values usually fall in the range of pH of 3-12 with a great many having pl between pH 4 and pH7.

Proteins are positively charged in solution at pH values bellow their pl and negatively charge above their isoelectric points thus at pH values bellow the pl of particular protein it will migrate toward the anode. Protein at its isoelectric point will not move in an electric field.

When a protein is placed in a medium with a linear pH gradient and subjected to an electric field, it will initially move toward the electrode with the opposite charge in this Fig.4.4.

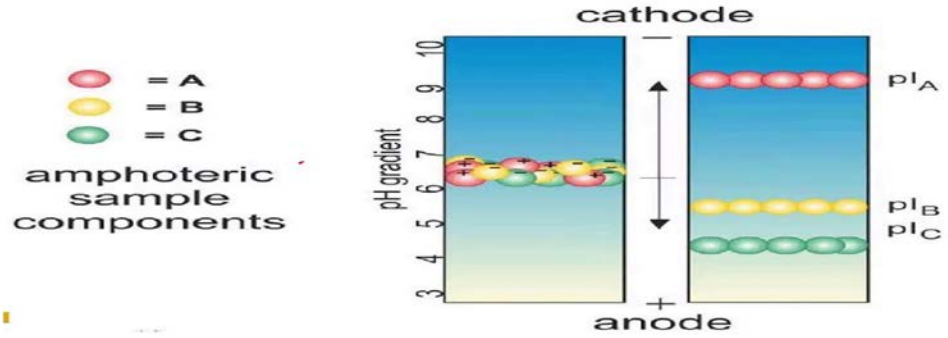

Fig 4.4 : Isoelectric focusing

The motion of a protein undergoing isoelectric focusing is depicted (circles)

Migration through the pH gradient the protein will either pickup or lose protons. As it does its net charge and mobility will decrease and the protein will slow down. Eventually the protein will arrive at the point in pH gradient. There being uncharged, it will stop migrating of a particles at its pl should happen to diffuse to a region of lower pH it will become protonated and be forced towards the cathodes by the electric field. If on the other hand , it diffuses into a pH higher than its pl , the protein will

becomes negatively charged and will be down towards in this way, protons condense or forces, into sharp bands in the pH gradient at this individual characteristic pl values .

Focusing is a steady state mechanism with regard to pH. Proteins approach their respective pl values at differing rates but remain relatively fixed at those pH values for extended periods. These types of motion are in contrast to conversional electrophoresis in which proteins continue to move through the medium until the electric field is removed. Moreover, in IEF, proteins migrate to their steady state position from anywhere in the system. Thus the sample application point. In fact the sample can be initially distributed through the entire separation system like. Establishing pH gradient. Gels for isoelectric focusing, power condition resolution in isoelectric focusing, protein solublization for isoelectric focusing.

4.6 AGAROSE GEL ELECTROPHORESIS FOR DNA

Agarose gel electrophoresis is performed in a continuous fashion with both electrodes and gel cassette submerged within the buffer. The electrophoreses chamber has two platinum electrodes placed on the both ends connected to the external power supply from a power pack which supplies a direct current or DC voltage . The tank filled with the running buffer and the gel casted is submerged inside the buffer. There are additional accessories needed for casting the agarose gel such as comb, spacer, gel, caster etc.

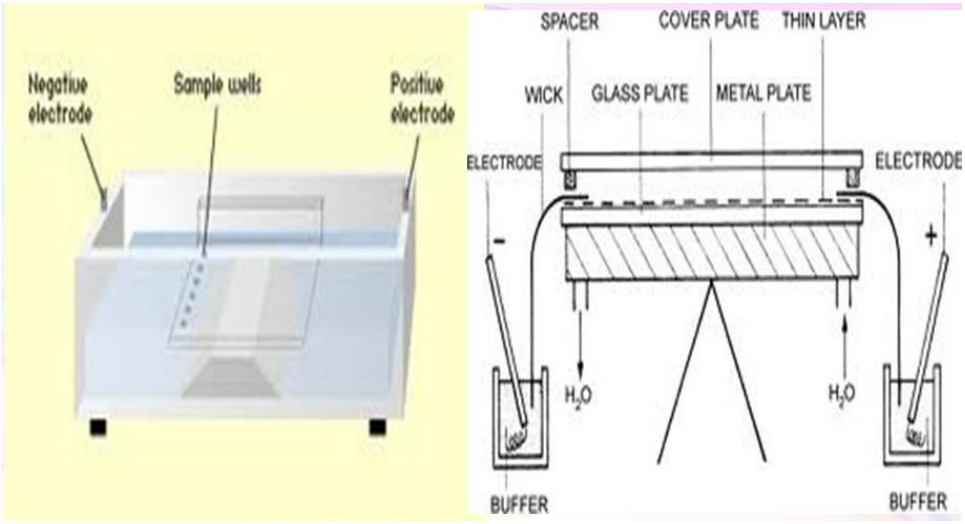

Fig 4.5 : Different components of horizontal gel electrophoresis apparatus

The purpose of each reagents used in horizontal gel electrophoreses are

1. **Agarose –** Polymeric sugar used to prepare horizontal gel for DNA analysis

2. **Ethidium bromide –** for staining of loading dye for horizontal gel to the DNA .

3. **Sucrose**-for preparation of loading dye for horizontal gel

4. **Tris HCl**- The component of the running buffer.

5. **Bromophenol blue –** Tracking dye to monitor the progress of the electrophoresis.

The agarose powder is dissolved in a buffer and heated to melt the agarose .Hot agarose is poured into gel cassette and allowed in to set. A comb can be insert in to the hot agarose to cast the well for loading the sample .In some cases Ethidium bromide is added within the gel so that it stains the DNA while electrophoresis.

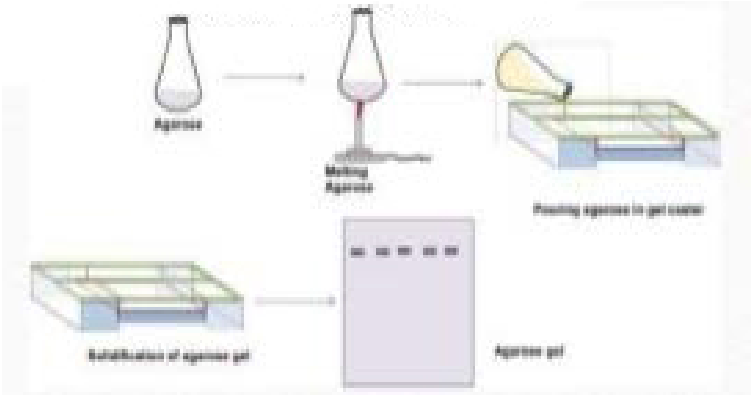

Fig. 4.6 : Different steps in casting of the agarose gel for horizontal electrophoresis

The gel cassette is placed in the electrophoresis tank submerged completely and DNA loaded in to the well with the help of pipetman and run with the constant voltage. DNA runs from negative to positive end and Ethidium bromide present in the gel stain the DNA Observing the agarose gel in a UV chamber shown the DNA stained with ether as organ color fluorescence.

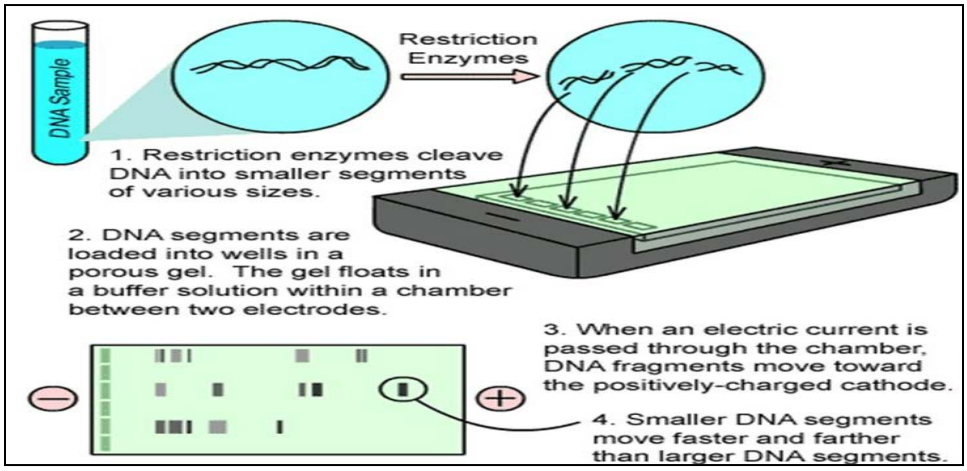

Fig. 4.7 : Observation of DNA stained with etbrina U.V. Chamber

For DNA agarose gel electrophoresis can be determined by comparing the size of the known DNA molecules. The DNA of known size is resolved on 0.8 % agarose along with the unknown sample. The value of the relative migration (R f) of each DNA band is calculated from the agarose gel, the values of relative migration (R f) and size of the DNA is used to draw the calibration curve to calculate the size of the unkwon DNA sample.

DNA protein introduction – DNA is negatively charged molecule and it interact with positively charged protein to form DNA – protein complex.

The size and hydrodynamic volume changes.

DNA + Protein ⟶ DNA-Protein

The DNA protein interaction, a fixed amount of DNA is incubated with the increasing concentration of protein. Due to the formation of DNA protein complex .The hydrodynamic volume of the complex increases and a shift in band is observed. The DNA has an extended structure and it provide docking site for several protein molecules such as single stranded binding protein. Gradual shift in DNA band will be observed until the DNA binding site is not saturated with the protein molecules. The DNA proteins interaction will be able to several aspects-

- Whether protein–X has a affinity for DNA and interaction is specific in nature.
- What will be affinity parameter of the interaction of DNA to protein in making DNA protein complex?

4.7 2-D GEL ELECTROPHORESIS

2D electrophoresis is a form of gel electrophoresis which is used to analyze protein mixture is separated by two dimensions on 2D gels. 2D electrophoresis begins with electrophoresis in the first dimension and then separates the molecules perpendicularly from the first to create an electropherogram in the second dimension. In electrophoresis, in the first dimension, molecules are separated linearly two molecules will be similar in two distinct properties; molecules are then separated at 90 degrees from the first electropherogram according to molecular mass. Since it is unlikely that two molecules will be similar in two distinct properties, molecules are more effectively separated in 2D electrophoresis then in 1D electrophoresis.

The two dimensions properties are separated into using this technique can be isoelectric point, protein complex mass in the native state or protein mass. Separation of proteins by isoelectric point is called isoelectric focusing. There by the gradient of pH is applied to a gel an electric potential is applied across the gel. All pH value other than their isoelectric point proteins will be charged. If they will be pulled to the more positive end of the gel. The proteins applied in the first dimension will

more along the gel and will accumulate at their isoelectric point, that is the point at which the overall charge on the protein is 0(a neutral charge) for the analysis of the functioning of proteins in a cell. Most often proteins act together in complexes to be fully functional. The analysis of this sub organelle organization of the cell requires techniques conserving the native state of the protein complexes. In native polyacrymide gel electrophoresis (native PAGE), protein remains in their native state and are separated in the electric field following their mass and the mass of their complexes respectively. To obtain a separation by size and not by net charge as in IEF, an additional charge is transferred to the proteins by the use of Coomassie brilliant blue or Lithium clodayete sulfate. After completion of the first dimension, the complexes are destroyed by applying the de nurturing SDS PAGE in the second dimension, Where the proteins of which the complex is composed are separated by their mass. Before separating the proteins by mass, they are treated with sodium clodayete sulfate (SDS) along with other reagents (SDS PAGE in ID).

This denatures the proteins and binds a number of SDS molecules roughly proportional to the proteins length b/c a proteins is roughly proportional to its mass this is equivalent to the proteins mass.

The SDS molecules are negatively charged, the result of this is that all of the proteins will have approximately the same mass to charge ratio as each other in addition proteins will not migrate when they have no charge, there for the coating of proteins in SDS allows migration of the protein in the second dimension . In the second dimension, an electric potential is again applied, but at a 90 degree angle from the first field. The protein will be attracted to the more positive side of the gel proportional to their mass to charge ratio. As previously explained this ratio will be nearly the same for all proteins.

The proteins progress will be slowed by frictional forces, the gel there force acts like a molecule sieve when the current is applied, separating the proteins on the basis of their molecular weight with larger proteins being retain higher in the gel and smaller proteins being able to pass through the sieve and reach lower regions of the gel. This result a gel with proteins spread out on its surface. These proteins can then be detected by a variety of means, but the most commonly used stains are silver Coomassie brilliant blue staining. In the former case a silver colloid is applied to the gel. The silver binds to cystein groups within the proteins. The silver is darkened by exposure to ultraviolet light. The amount of silver can be related to the dark and therefore the amount can only give location on the gel. This measurement can only gives approximate amount, but is adequate for most purposes. Silver starting is 100 time more sensitive than coomessie brilliant blue with a 40 fold range of linearity. Molecules other than proteins can be separated by 2D electrophoresis. In super coiling assay, coiled DNA is separated in the first dimension and denature by a DNA intercalate (such as Ethidium bromide or the less carcinogenic chloroquine) in the second, this is comparable to the combination of native PADE/SDS-PAGE in protein separation.

4.8 SUMMARY

Migration of charged particles under the influence of an electric field describes the process of electrophoresis. There are several macromolecules such as amino acids, peptides, proteins, and nucleotides etc have ionizable group therefore, at given pH they can be migrated toward different charge under the influence of eclectic field. The electrophoresis equipment has basically two units such as power pack and Electrophoretic unit. The Electrophoretic unit runs either vertical or horizontal system. Thus, this technique useful in the separation of biological macromolecules under the influence of an anode, depending on the nature of their net charge. Moving boundary electrophoretic is carried out in a U shape tube with platinum electrodes attached to the end of both arms. Agarose gel electrophoresis is performed in a continuous fashion with both electrodes and gel cassette submerged within the buffer. SDS-PAGE is a technique widely used in biochemistry, forensics, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility.

4.9 TERMINAL QUESTIONS

Q.1. What is electrophoresis? How it is useful in biochemistry.

Answer: ----------

Q.2. Write the principle of electrophoresis.

Answer: ----------

Q.3. What is Native PAGE electrophoresis?

Answer: ----------

Q.4. Define the SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).

Answer: ----------

Q.5. Define the role of Agarose Gel electrophoresis for DNA.

Answer: ----------

Q.6. Write the 2-D Gel electrophoresis.

Answer: ----------

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Analytical Biochemistry

BLOCK

3

MICROSCOPY, X-RAY DIFFRACTION AND NMR

UNIT-5

Microscopy

UNIT-6

X-Ray Diffraction and NMR

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BLOCK INTRODUCTION

This is the third block of analytical biochemistry. It consists following two units.

Unit-5 : Unit one comprises the different types of microscopic techniques, Principle, instrumentation and application of light and electron microscope easily reveal how to apply this technique in characterization on biological sample. The microscope is most valuable techniques for the visualization and understanding of morphology of specimen.

Unit-6 : XRD and NMR are discussed in this unit. XRD is a nondestructive techniques applied for determination of crystal size of particles. The NMR is most valuable techniques over the nuclei which has odd number of electron in their nucleus. The principle, phenomenon, instrumentation and application of (^{1}H and ^{p3}CMR) techniques are also described in this unit.

UNIT-5 MICROSCOPY

Structure

5.1 INTRODUCTION

Our nature has lots of diversity in which number of plant and animals are found in different shape and size. Some organisms are very small in size and they cannot see by naked eyes. The microscopy is the special instruments that are useful in biochemical analysis because it produces magnified visual or photographic images of tissues, and cellular organelles to see with the naked eyes. In other words, the microscopy is defined as the optical instrument that consists of lens or combination of lenses to magnify the minute's objects. Microscopy was invented more than 400 years ago, and it is amongst the most useful instruments for all scientific applications. Most microscopys use light or lens which are curved pieces of glass or plastics to magnify the objects. There are two fundamentally different microscopys viz. light and electron microscopy used to study of specimen. The light microscopy used in study of very small organism or objects. The electron microscopy viz. transmission and scanning microcope is very useful in detection of size and morphological

image of specimen. The light microscopy used a series of lens or glass to focus light in order to from an image, while electron microscopy used electronic lens to focus beam of electron. Light microscopy image magnify about 1500 times whereas electron microscopy image magnify 2000000 times. The magnifying power of a microscopy is an expression of the number of times the object being examined appears to be enlarged and is a dimensionless ratio.

Objectives :

- Understand the function and application of microscopy.
- To discuss, working principle of light microscopy.
- To describe the features of electron microscopy and their application in analysis of biological sample
- To understand the role of instrument that used in the light and electron microscopy.

5.2 MICROSCOPY OVERVIEW

This microscopy is the special instruments that produce magnified visual or photographic images of objects to see with the naked eyes. In other words, the microscopy is defined as the optical instrument that consists of lens or combination of lenses for magnifies the minute's objects. The lenses used in the microcopy tend to bend and focus the light rays to produce large image of tiny substances. Thus, we can say that microscopy is the biggest tools for us that reveal the mystery and beauty of unseen world.

In 1660s, the Anton von Leeuwenhoek was first who used simple microscopy or magnifies glass to see the single-celled animals and even some larger bacteria with a simple microscopy. The microcopies invented by British microbiologist Robert Hooke sometime in the 1660s.The microscopy instrument consists generally perform three tasks: i) it magnifies the image of specimen, ii) it separates the details of the image and, iii) it renders the details visible to the human eye or camera. The microscopy which contain only single lens is called simple microscopy whereas the two or more lens occupied microscopy is called compound microscopy. A light compound microscopy that have many lens are combined together has useful magnification of about 1500 times. The limit of any optical instrument is given approximately by abbes relationship

$$\text{Resoluation} = \frac{\text{wavelenth } \lambda}{\text{numerical aperature } (\text{nsin}\alpha)}$$

These relationships explain that high resolution in microscopy can only achieved by manipulating a small number of variables such as in

wavelength of the illuminating radiation, the refractive index and the aperture.

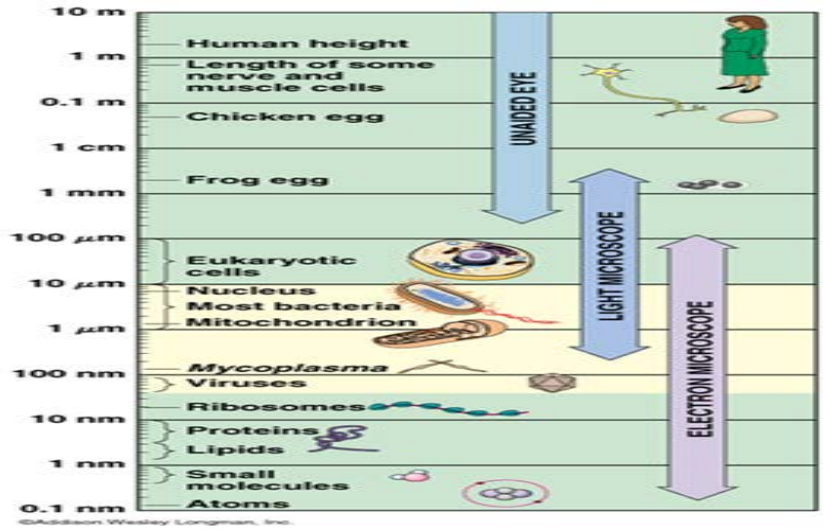

Fig. 5.1 : Use of different microscopy for different objects

5.3 ELECTRON MICROCOPY

Electron microscopy is considered very advanced techniques for resolution of imaging technology for scientist and researcher to image because it produce greatest image of biological organelle and non specimen. Electron microscopys reveal the ultra structure of cell, cell organelles and disease affected cell. There are fundamentally two different types of electron microscopy viz.- Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM). In transmission electron microscopy, the electron pass through the specimen and produce image while in scanning electron microscopy, the electron reflected back from the specimen (secondary electron of same frequency) collected on florescence screen and thus image is produced.

5.3.1 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Transmission electron microscopys use electrons in a way that is analogous to the way light microscopys use visible light. To obtain a TEM image, a thin sample of about 200 nm is subjected with a high energy electron beam which is directed using electromagnetic lenses.

Basic Principle :

Transmission electron microscopy (TEM) works on principle of transmission of electron in the specimen to produce image. During this process, beam of electrons transmitted through an ultra-thin specimen, interacting with the specimen and finally produce image. The electron gun is the source of electron beam that produce at when high voltage about

400V and 100000V is passed between the anode and cathode. Since electrons cannot pass through a glass lens, magnetic lenses are used to focus the beam. The beam of electron illuminates entire specimen. Some of electrons absorb or scattered by atom of metallic strain, because the certain metal are used in specimen for contrast in transmission electron microscopy. The object magnifies the image of specimen and the image received by the third magnetic lens which works as projection lens.

However, in TEM, the transmission of electron beam is highly dependent on the properties of material being examined. Such properties include density, composition, etc. The electrons have limited power to permit at the material thus specimen should be 50-100 nm in size which allows them to pass it. As a result, a specimen with a non-uniform density can be examined by this technique. Whatever part is transmitted is projected onto a phosphor screen for the user to see.

Instrumentation :

TEM has high magnification about 500000 times and it was discovered by Knoll and Rules of Germany in 1932. The transmission microscopy consists of following parts shown in Fig. 5.2.

- **Electron Gun and anode :** Electron gun is a source of light in the TEM. Electron gun consists of electrically heated tungsten or cathode, when high voltage about 400 and 100000 passes between the anode and cathode and the tungsten filament emit electron. This electron must have high velocity with wavelength of 0.005 to 0.003 nm.
- **Microscopy columns :** Electron can travel in straight line in vacuum only because in the air collides with oxygen or nitrogen.
- **Condense lens :** It is magnetic coil, which focus or condense the electron beam in the path of object.

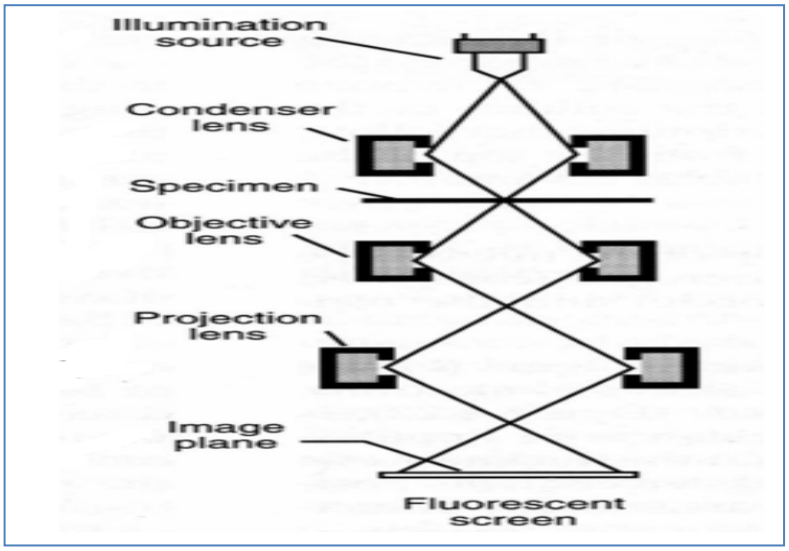

Fig. 5.2 : Image of TEM

- **Objectives lens :** In the magnetic coil which produce first magnified image of the object. It also focuses the electron which is reflected by the object and form first image.
- **Project lens :** It is also electromagnetic coil which magnify the first image formed by objective lens, it produce first image.
- **Photographic plates :** Florescence screen is used for observing image of object. Final image can be captured on the photographic film. Such photographs are known as electron micrograph.

Sample Preparation :

The TEM sample should be thin (50-100mn), because, electron, have limited power to penetrate the electron. So, special care must be taken while preparing sample for image. For enhancing the image, there should be added some specific heavy metal such as uranium, lead or osmium. The water molecules have to be removed from the specimen because electron beam only can proceed and focus in vacuum. In TEM there is no need to mention physical, environment or condition in imaging. For TEM analysis extensive specimen preparation is require because there can be issues of interpreting of image because of artifacts from specimen preparation.

Application :

TEM is most commonly used microscopy compare to other microscopy. It is highly useful in observation and detection of internal structure of cell and it has great resolving power. TEM offer very powerful magnification and resolution. TEM has a wide range of applications and can be utilized in a variety of different scientific, educational and industrial fields. TEM provide information on element and compound structure. Images are high qualified and detailed.

5.3.2 SCANNING MICROSCOPY (SEM)

A scanning electron microscopy (SEM) is a type of electron microscopy that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample.

Basic principle :

Scanning electron microscopy (SEM) is non-destructive techniques to surface analysis and that use in determination of morphology of solid particles. The instrumentation of SEM is somewhat similar to TEM. SEM has electron gun and lens system. In SEM, the first beam of electron (5 to 20 nm) is making at 3-30 kV for scanning a selective area of specimen. In specimen the electron does not pass but it focuses on the surface on specimen. The beam is moved rapidly back and forth by beam deflector scan the surface of specimen. When the beam hits to the surface of

specimen it induces the molecules of specimen to higher level. Due to this, the secondary electrons are emitted from metabolic surface. These secondary electrons are collected by the positively charged grid. The signal form the gird is transmitted to the television tube, which scan and form the image on the screen. The amount of secondary electron produce depends on the angle of specimen. The resolving power of SEM is found less than TEM and it has maximum magnification upto 20000 times. The spatial resolution of the SEM depends on the size of the electron spot, which in turn depends on both the wavelength of the electrons and the electron-optical system which produces the scanning beam.

The basic difference in SEM and TEM is; TEM form image from electron that are transmitted through the specimen whereas the SEM forms the image from electrons that have bounced of the surface of specimen.

Instrumentation :

SEM has an electron optical system to generate electron probe for illumination of image. There is a secondary electron detector to detect the secondary electron, an image display unit, and an operating system to perform various operations shown in Fig.5.3. The electron operating system and space surrounding the specimen kept in vacuum.

- **Electron gun :** Several types of electron guns are used in SEM system and the qualities of electrons beam they produced vary considerably. Tungsten electron gum generally used in SEM to produce electron beam. The election beam used as source of energy ranging from 0.2 keV to 40 keV. It is focused by one or two condenser lenses to a spot about 0.4 nm to 5 nm in diameter. The beam passes through pairs of scanning coils or pairs of deflector plates in the electron column, typically in the final lens, which deflect the beam in the x and y axis so that it scans in a faster fashion over a rectangular area of the sample surface.

- **Electron Lenses :** SEM generally used magnetic lens, which focus or condense the electron beam in the plan of object. SEM uses the electron lens to magnify and demagnify the electron beam. It consist of both condense and objective lens. The condense lens converged and collimate the beam of electrons whereas focus the electron beam into a probe point at the specimen surface and to supply further demagnification

- **Sample Stage :** The sample stage for the SEM can perform the following movements like horizontal movement(X, Y), vertical movement (X,Y), specimen tilting (T) and rotation (R). The X and Y movement are use for the selection of a field of view.

- **Detectors for all signals of interest :** The detector is used for the detection of primary and secondary detector emitted from the specimen. Florescence substance is coated in the tip of detector and high voltage about 10 kV is applied on it.

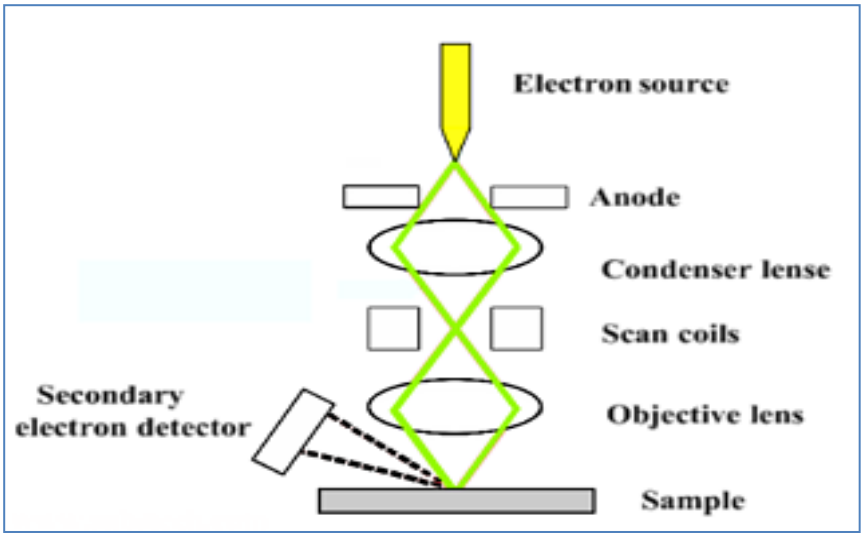

Fig. 5.3 : Image of scanning electron microscopy

- **Display/Data output devices:** The output signal of secondary electron detector is amplified and then transfer to the display unit. Since the scanning on the display unit is synchronized with electron probe scan, brightness variation, which depends on the number of secondary electron which appear on the display unit, thus forming the SEM image.
- **Infrastructure Requirements :**
  - Power Supply
  - Vacuum System
  - Cooling system
  - Vibration-free floor
  - Room free of ambient magnetic and electric fields

Applications :

SEM provides surface morphology of specimen. It is an essential research tool in life science, biology, medical and forensic science and metallurgy. It has wide-array of applications, including industrial and technological applications. The SEM is very useful non destructive techniques for characterization of biological and chemical sample. The very precise measurement of very small material about 50 nm in size is obtained through SEM. The high resolution image is produced by scanning of electron on the surface of specimen. It give various information to recognize chemical composition of materials such as follows:

- Acquiring elemental maps or spot chemical analyses using EDS.
- Discrimination of phases based on mean atomic number using BSE.

- Compositional maps based on differences in trace element "activators" using CL.
- Measurement of very small features and objects down to 50 nm in size
- Back scattered electron images (BSE) can be used for rapid discrimination of phases in multiphase samples.

5. 4 SCANNING TUNNELING MICROSCOPY

Scanning Tunnelling Microscopy (STM) was developed in 1981 by Gerd Binnig and Heinrich Rohrer. STM was firstly used to imaging structure of a crystal of gold. The development of STM provided a different and fresh area for monitoring science of surfaces. The process of STM dependents upon concept of quantum tunneling. As the tip which is conducting moved closer towards surface of the sample that is monitored, a biased (potential difference) is provided in between two surfaces that results in electrons to undergo tunnelling through vacuum between them. The resultant current that tunnels is a function of tip position, applied voltage, and local density of states (LDOS) of sample. STM can be an ambitious technique, since it requires extremely dirt free and stable surfaces, sharp tips, excellent vibration control, and sophisticated electronic assembly. The components necessary for assembling an STM are highly sophisticated and are very expensive.

Basic Principle :

STM works on the concept of quantum tunnelling. Quantum tunnelling or tunnelling is a quantum mechanical phenomenon wherein a particle tunnels through a barrier. Fig. 5.4 clearly depicts the principle of quantum tunnelling effect. The atoms escape the surface of the solid specimen forming an atomic cloud hovering over the surface. As the other surface approaches, there occurs an overlapping of atomic clouds and an atomic exchange takes place. This plays a crucial role in several physical phenomena, such as the nuclear fusion. STM has a very high resolution. The lateral resolution is equal to 0.1 nm and 0.01 nm (depth). The high resolution of STMs enables researchers to examine surfaces at an atomic level. It is used only under ultra-high vacuum conditions; at temperatures ranging from 0 K to over 1273 K. Figure 3 depicts the STM instrument.

In STM a tunnelling current (I_t) is generated on application of voltage V that is applied between tip and sample surface and its magnitude is given by the following equation:

$$I_t = Ve\text{-}Cd$$

Where, V = Bias Voltage.

C = Constant (characteristic of the composition of the conductors).

d = Spacing between the lowest atom on the tip and the highest atom on the sample.

From the above equation we can say that the tunnelling current decreases exponentially with thedistance between tip and the sample surface. This decrease in tunnelling current with the distancecauses the tunnelling current to become significant only at small tip-sample separation. Thus, leading to high resolution in STM.

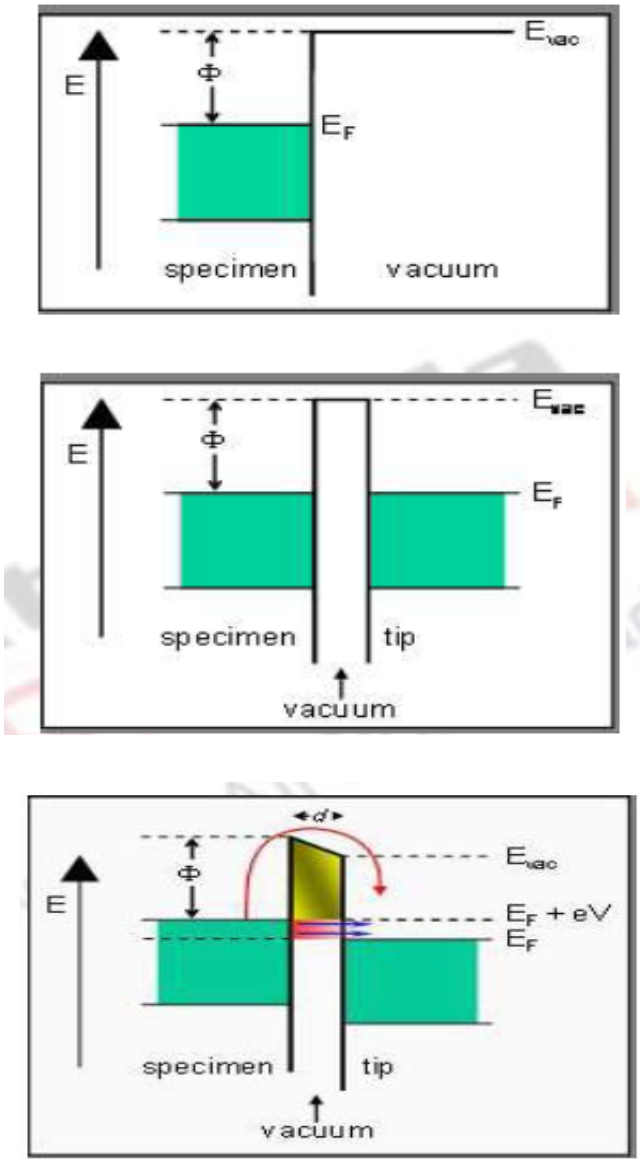

Fig 5.4 : Principle of quantum tunnelling effect

Source: www.eng.utah.edu/~lzang/images/Lecture_6_STM.pdf

Instrumentation :

The essential components of a typical Scanning Tunnelling Microscopy instrument includes

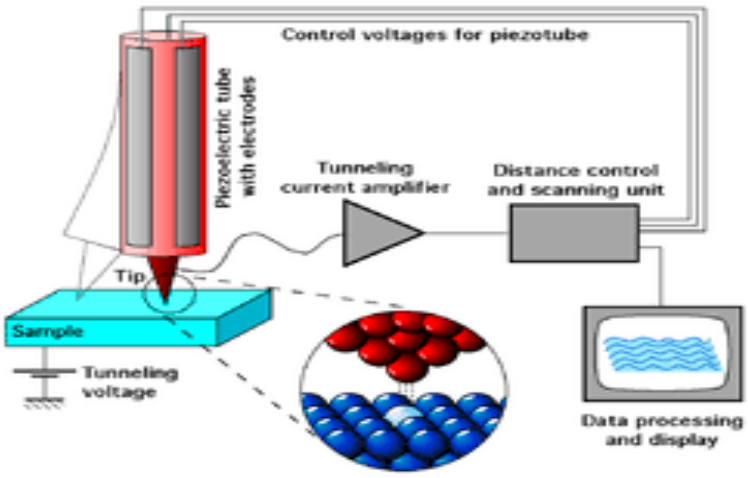

Fig. 5.5 : Schematic representation of STM instrument

Source: www.eng.utah.edu/~lzang/images/Lecture_6_STM.pdf

1. **Sharp probe tip.**
2. **A coarse positioning unit-** Brings tip-sample separation within the tunnelling range. A computer system automatically brings the sample within the tunnelling range of the tip. The devices that has been used for appropriate positioning mechanism are:
   - **Screws :** Can be used with lever, gear or spring reduction mechanism.
   - **Clamp-step :** This device can be used by clamping one foot to a base, expanding the body, clamping the other foot to the base while releasing the first foot and then contracting the body just like an inchworm.
   - **Stick-slip :** These devices are generally configured as a sample holder resting on a piezoelectric plate which is configured for shear motion.
   - **Vibration isolation stage**.-A vibrational isolation system is used in order to avoid any external vibrations interacting with the sample.
3. **Piezoelectric Scanner.**
4. **Computer.**

A sharp tip of a tungsten, platinum-iridium or gold needle is placed at a few angstroms distance (4-7 Å) from the sample surface. A small bias voltage is applied between the probe tip and the sample surface, causing electrons to tunnel across the gap. As the probe is scanned in a raster manner over the surface of the sample, it registers variations or changes in the tunnelling current, and this

information is processed to provide a topographical image ofthe surface of the specimen. Hence, one could get a 3-D atomic scale map of the sample surface.

Applications of Scanning Tunnelling Microscopy

1. The various applications that has been studied of Scanning Tunnelling Microscopy (STM) includes:
2. STM provides a detailed image of the structure of the specimen. Arrangement of each atoms on to the metal surfaces have also been precisely documented and presented.
3. It is used in studying friction, surface roughness, defects etc. It is also widely used in determining surface reactions in materials. Absorption and diffusion of various species have been examined and studied in well detail and thereby reported.
4. It is a very important tool in research particularly in the field of semiconductors and microelectronics.
5. The silicon surfaces have also been studied and monitored quiet extensively and vigorously in comparison to any other material.

5.5 SPECIFIC STAINING OF BIOLOGICAL MATERIALS

Cell staining is a technique used for the main purpose of increasing contrast through changing the color of some of the parts of the structure being observed thus allowing for a clearer view. There are a variety of microscopic stains that can be used in microscopy.

The staining of biological materials is carried out either in-vivo or in-vitro condition. In-vivo staining refers to the staining of a biological matter while it is still alive; in-vitro staining refers to a staining technique where the biological matter is non-living. The following are common stains explaining techniques, preparations and procedures for each:

1. **Haematoxylin and Eosin Staining :**

   The Haematoxylin and Eosin staining are very useful in the examination of thin slices of biological tissue. Contrast is created by the stains where Haematoxylin turns the nuclei blue while eosin turns the cytoplasm as well as other parts pink or red.

Haematoxylin :

At 70-80 C degrees centigrade the 10 grams of haematoxylin crystals kept in 500 ml of water (70- 80) and mix to dilute completely. The 20 grams of alum takes in separate flask mix with 500 ml of hot tap water (70- 80 degrees). After that two mixtures together and add 1 gram of crystal. In this mixture the alum used as a mordant thymol prevents fungal growth. The

mixture is then kept in a translucent flask away from direct sunlight for one week. This is covered with a paper towel that allows for air circulation (early maturation). The solution is then put in to a dark flask and topped tightly after one week, and stored in a dark place for 3 weeks.

Eosin (1000ml) :

In the eosin process the 10 grams of eosin crystals takes and add to mix in 1000 ml of hot tap water at 70- 80 degrees. The mixed solution should be properly mixed to dilute and stored in a dark flask. This can be used directly. In the eosin process carried out in following steps such as:

- A rehydrated section is stained in a solution of haematoxylin for 20 to 40 minutes
- The section is then washed in tap water for about 3 minutes until it turns blue,
- The section is the differentiated in 70percent ethanol that contains 1 percent of HCL for about 5 seconds to remove excess dye and allow the nuclear to emerge, This is then washed in tap water,
- Stain with eosin for 10 minutes,
- Then wash for about 1 to 5 minutes in tap water,
- Dehydration, clear and mount on a rack

2. Acid and Basic Fuchsin Stain :

Acid fuchsin and basic fuchsin is very useful in plasma and nucleus staining, respectively. The magenta red dye use as acid while the magenta dye largely used as basic fuchsin. The Acid fuchsin and basic fuchsin is also referred to as acid fast staining. The acid fast bacteria have a waxy substance (mycolic acid) on their cell wall that makes them impermeable to staining procedures. The term acid fast is used since they resist decolourization with acid alcohol. Carbol fuchsin, the primary stain contains phenol, which helps solubilize the cell wall whereas heat is used to increase the penetration of the stain. On using alcohol to decolorize, cells will be decolorized except for acid fast ones. Methylene blue is used as the counterstain to counterstain any cell that was decolorized. At the end of the procedure, acid fast cells remain red/pink while non-acid fast cells retain a blue color. Preparation of carbol fuchsin by mixing two solutions:

3. Wright's Stain :

This is a Romanowsky type of metachromatic stain that is prepared by mixing specially treated methylene blue dye with eosin. The acidic portion of the stain unites with the basic components of the cells such as hemoglobin, and thus they are referred to as eosinophilic and are stained pink or red. The acidic components of the cell, such as the nucleic acids on the other hand take the basic dye and stain blue or purple. PH has to be controlled using a buffer of 6.4 to 6.7 to avoid poor staining.

Preparation :

- Measure 1.0 grams of wright's stain powder and 400 ml of methanol (methyl alcohol),
- Add a few glass beads to assist in dissolving and add the ethanol to the stain,
- Mix well at intervals until the powder has completely dissolved (do this by warming in 37 C water bath to aid in the dissolving),
- Label the bottles and mark it as flammable and toxic,
- Tightly atop and store at room temperature in the dark

Procedure :

- Prepare a fill of the sample and allow drying on a slide,
- Prepare three containers, and fill one with one step Wright's Stain and the other two with distilled water,
- Keep the stain tightly covered when not in use to avoid evaporation (always replace the stain once it becomes insufficient)
- Always replace distilled water once iridescent scum start forming on the surface, or when it starts turning blue,
- Dip the slide in the stain for 15 to 20 seconds,
- Dip the slide in distilled water in the second container for 15-45 seconds,
- Dip the slide in container 3 for 25 seconds using quick dips,
- Wipe the back of the slide,
- Dry the slide on a vertical position, on the absorbent surface and avoid blotting the smear,
- Apply oil to examine microscopically,

4. Gram Staining *:*

It is largely used to differentiate bacteria species as either gram positive or gram negative. This is achieved through the chemical properties of bacterial cell walls, where different colors are displayed after staining. This technique is based on the fact that the gram positive cell wall has a strong attraction for crystal violet following the addition of iodine as compared to the cell wall of gram negative. Iodine is the mordant, and forms a complex with crystal violet, which is easily washed off from the gram negative cell wall using ethyl alcohol.

5.6 SUMMARY

Microscopy is most useful instruments invented more than 400 years ago. The most microcopes use light or lens which is curved pieces of glass or plastics to magnify the objects. Anton von Leeuwenhoek (1660) firstly used simple microscopy or magnifies glass to see the single-celled animals and even some larger bacteria with a simple microscopy. The microscopy that has more than one lens is called compound microscopy that magnifies the objects of about 1500 times. One basis, on contrast image, the light microscopy may be bright field, dark field, phase contrast and florescence microscopy. The bright field microscopys have both deflected and undeflected lights are collected by the objectives lens. Phase contrast microscopy is a form of microscopy used to view unstained cells growing in tissue culture and for testing cells or cell organelles. The dark field spectroscope is useful to determine the outline of specimen in liquid media such as living spermatozoa, microorganism etc. Fluorescence is popular microscopy because of its ability to achieve highly specificity. In transmission electron microscopy, the electron pass through the specimen and produce image while in scanning electron microscopy, the electron reflected back from the specimen (secondary electron of same frequency) collected on florescence screen and thus image is produce. Working of STM is dependent upon concept of quantum tunnelling. Quantum tunnelling or tunnelling is a quantum mechanical phenomenon wherein a particle tunnels through a barrier. STM provides a detailed image of the structure of the specimen. Arrangement of each atoms over metal surfaces have also been precisely documented and presented.

5.7 TERMINAL QUESTION

Q.1. What is microscopy? Define its use in biochemistry

Answer:----------

Q.2. Write the application of electron microscopy.

Answer:----------

Q.3. Discuss about instrumentation of scanning electron microscopy.

Answer:----------

Q.4. What is scanning electron microscopy? How it is useful in biological sample analysis?

Answer:----------

Q.5. Write the principle and instrumentation of Scanning Tunnelling Microscopy.

Answer:----------

Q.6. Discuss about instrumentation of transmission electron microscopy.

Answer:----------

5.8 FURTHER READING

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UNIT-6 X-RAY DIFFRACTION AND NMR

6.1 INTRODUCTION

This unit covers the basic features of X-Ray Differaction (XRD) and Nuclear magnetic resonance (NMR). The XRD has identification of a crystalline material and can provide information on unit cell dimensions. The crystalline substances act as three-dimensional diffraction, first distinguished by Max von Laue through gratings of X-ray wavelengths. X-ray diffraction is now a common technique for the study of crystal structures and atomic spacing. The NMR spectrum is obtained when the nuclei has odd number of proton in their nucleus. The splitting of signal determines the position and number of proton in any organic compounds. In this unit, all aspect of NMR has been described in detail. The basic principle, instrumentation and application of NMR are:

Objectives:

- to discuss the basic structure of XRD and NMR

- to learn phenomenon and working principle of NMR
- to study components and features of XRD and NMR
- To develop the basic understanding of analytical tools that is very useful in detection or characterization of biological samples.

6.2 X-RAY DIFFRACTION OVERVIEW

The powder sample of verity of organic compounds like natural product, steroids, vitamins, antibiotics etc. are best analyzed by XRD. A quantitative identification of crystal is also possible since X-ray diffraction pattern is unique for each crystal like fingerprint. X ray spectroscopy is non destructive techniques to analysis of crystalline material. X-ray absorption, X-ray diffraction and X-ray florescence are the three main field of X-ray spectroscopy. Amongst three techniques, the X ray diffraction (XRD) is most useful in elucidation of structure of compounds. The X-ray diffraction based on its scattering by crystal is extremely important as compared to other. By X- ray diffraction, one can be identify the crystal structure of determine the particle size. The XRD is scarcely used for quantitative analysis but in some time it is also useful in quantitative analysis. The X ray has significance aspect due to much shorter wavelength of about < 0.01 – 10 nm.

The atomic planes of a crystal cause an incident beam of X-rays to interfere with one another as they come out from the crystal. This phenomenon is called X-ray diffraction and it is represented by symbolϖ. X-ray powder diffraction (XRD) is an important tool for identification of phase of crystalline material and its unit cell dimensions. When the monochromatic ray generated by cathode ray tube is collimated to concentrate and directed toward the sample, it produces constructive interference after satisfy with the conditions of Bragg's Law ($n\lambda=2d \sin\theta$). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted. The average crystalline size of particles was measured by Scherrer's equation as expressed in Eq. (6.1):

$$D_{hkl} = \frac{k\,x\,\lambda}{\beta_{hkl} x\, Cos\theta_{hkl}} \qquad (6.1)$$

where D_{hkl} is the particle size (nm), perpendicular to the normal line of plane, k is (0.9) β_{hkl} is the full width at half maximum of the (hkl) diffraction peak , θ is the bragg angle of (hkl) peak, and λ is the wavelength of X-ray (λ = 0.1540 nm).

6.2.1 PRINCIPLE

In XRD, the interaction of electromagnetic radiation with matter causes the electron in the exposed sample to oscillate. The secondary

waves that are produced by accelerated electron have same frequency as incident radiation. This super-position of waves gives rise to phenomenon of interference. The interference depends on the displacement of two waves. The amplitude of these waves either reinforces or cancels each other out. Thus the maximum interference is called constructive interference and the cancelling is called destructive interference. The interference gives rise to dark and white ring line or spot. Since the distance between atom or ions is of the order of 10^{-1} m (1 A˚) different methods are used to determine structure at atomic level. In this case X ray region of electromagnetic radiation is required. X-rays are produced within a closed tube under vacuum atmosphere. The most prevalent type of diffraction to X-ray crystallography is known as Bragg diffraction, which is defined as the scattering of waves from a crystalline structure. Formulated by William Lawrence Bragg, the equation of Bragg's law (equation 1) relates wavelength to angle of incidence and lattice spacing. Because of the nature of diffraction, waves will experience either constructive or destructive interference with other waves. In the same way, when an X- ray beam is diffracted off a crystal, the different parts of the diffracted beam will have seemingly stronger energy, while other parts will have seemed to lost energy.

$$n\lambda = 2dsin\theta$$

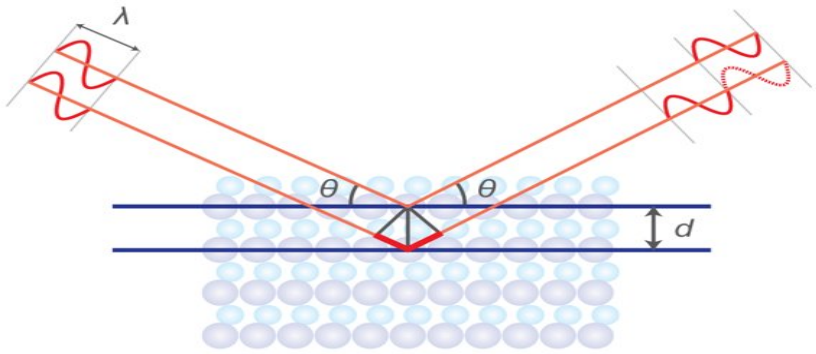


Fig. 6.1 : Schematic representation of Bragg's law interference

where *n* is an integer called the order of reflection, λ is the wavelength of x-rays, *d* is the characteristic spacing between the crystal planes of a given specimen and θ is the angle between the incident beam. By measuring the angles, θ, under which the constructively interfering x-rays leave the crystal, the inter planar spacings *d*, of every single crystallographic phase can be determined.

6.2.2 INSTRUMENTATION

X-ray for chemical analysis is carried out by rotating anode generator pr synchrotron facilities. X-rays are the key component in this instrument because it has characteristic character to indentify the object. In rotating anode generators, a rotating metal target is bombarded with high-energy (10–100 keV) electrons that knock out core electrons. An electron

in an outer shell fills the hole in the inner shell and emits the energy difference between the two states as an X-ray photon. Common targets are copper, molybdenum and chromium, which have strong distinct X-ray emission at 1.54 A˚ , 0.71 A˚ and 2.29 A˚ , respectively. However, in the x-ray emission copper is used because it has strong target distinct x ray emission at 1.54 A˚. That superimposed on a continuous spectrum known as Bremasstrahlung. In synchrotrons, electron is accelerated in ring thus producing a continuous spectrum of x rays that converted into single beam by Monochromator.

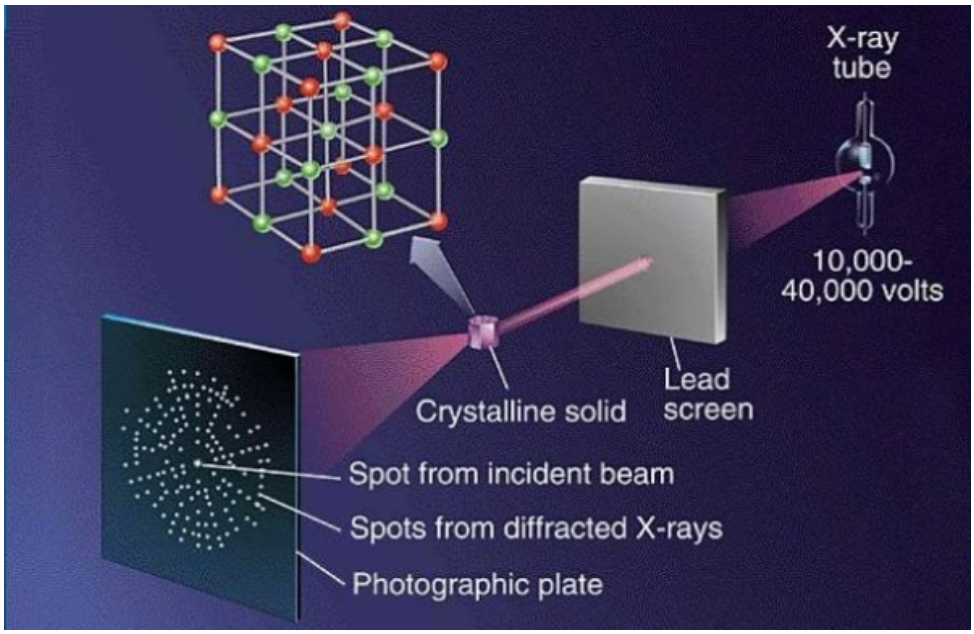


Fig. 6.2 : Schematic representation of x-rays production

6.2.3 APPLICATION

Generally in single crystal and powder crystal XRD are used in practice. We know that solid is made-up of particles by regular arrangement. A crystal is a solid in which atoms or molecules are packed in a particular arrangement within the unit cell which is repeated indefinitely along three principal directions in space. Crystals can be formed by a wide variety of materials, such as salts, metals, minerals and semiconductors, as well as various inorganic, organic and biological molecules. The crystal is subjected to X-ray produced by rotating anode generator or synchrotron facilities. The diffraction pattern of regularly spaced spots known as reflection is recommended a detector. Some of biological sample such as DNA and cytoskeleton components does not makes crystalline form, but they form fibber in long structure which are parallel to each other. Generally two class of fiber diffraction such as crystalline fiber (A form DNA) and non crystalline fiber (B from DNA) is distinguished.

A form of DNA, the molecules packed to form these micro crystals randomly arranged the axis. While B form of DNA molecules packed to form this micro crystals are arranged parallel to other but random orientation around the common axis. However, XRD is a nondestructive technique to identify crystalline phases and orientation. Multi components mixture of crystal will be analyzed by XRD with need of extensive sample preparation. In this types of XRD study all types of crystalline orientation are equally represented. The data of powder XRD is compared with known standard or to database (Powder diffraction file) for identification of individual components. Powder diffraction is a rapid method to analyse multicomponent mixtures without the need for extensive sample preparation. Instead of using single crystals, the solid material is analysed in the form of a powder where, ideally, all possible crystalline orientations are equally represented.

6.3 NUCLEAR MAGNETIC RESONANCE OVERVIEW

NMR stands for Nuclear Magnetic Resonance it is another form of absorption spectroscopy because under magnetic condition the sample absorbs certain wavelength of electromagnetic radiations. NMR is an analytical tool to determine the structure and purity of a sample as well as its molecular structure. Most studies in organic chemistry involve the use of ^{1}H, but NMR spectroscopy with ^{13}C, 15N and ^{31}P isotopes is frequently used in biochemical studies. The resonance condition in NMR is satisfied in an external magnetic field of several hundred mT, with absorptions occurring in the region of radio waves (frequency 40 MHz) for resonance of the ^{1}H nucleus. However, in the NMR the magnet involved is not electron but nuclei of atom of element. Once the basic structure is known, NMR can be used to determine molecular conformation in solution in which the studying physical properties such as conformational exchange, phase changes, solubility, and diffusion are determined. The nuclei of molecules give rise to spectrum which absorbs electromagnetic radiation under magnetic condition. Most of the study shows in organic chemistry involve the use of ^{1}H NMR but NMR study with ^{13}C, ^{15}N and ^{31}P is frequently used in biochemical study. While the proton of nuclei is put under magnetic conditions. The proton present in nuclei shows spin due to absorption of radio wave and act like small magnet. Resonance in this small magnate show spin and process is called NMR in other word we can say that, the nuclear magnetic resonance is the phenomenon of nucleolus in which proton and neutron spin about the axis due to electromagnetic radiation.

All nuclei carry a charge. In some nuclei charge “spin” on the nuclear axis and it circulation of nuclear charge generates a magnetic dipole along the axis. The angular moment of spinning charge can be describe in term of quantum spin numbers ***I;*** these have number values of 0, ½,1, 3/2 and so on**.** The intrinsic magnitude of generated dipole is expressed in term of magnetic moments μ. The H-nucleus is the most

commonly studied by NMR spectroscopy because of its high natural abundance of 99.985% and its presence in the majority of organic compounds. NMR studying ^{1}H atom is called Hydrogen or proton NMR spectrum. The proton NMR spectrum gives the information about the number of different types of protons and also the chemical environment around it.

6.3.1 ^{13}C NMR

Carbon forms the backbone of the organic molecules therefore C NMR is also important and gives valuable information about the molecule. The ^{13}C NMR is generated in the same fundamental principle such as proton ^{P}NMR spectrum. Only 1.1 % of naturally occurring carbon is ^{13}C and actually an advantage because of less coupling. In ^{13}C NMR spectrum is occurs directly due to present of carbon skeleton in the molecule and the proton present in molecules does not play direct role. The ^{12}C do not absorb radio frequency energy but the other isotope i.e. ^{13}C absorbs the energy but the ^{13}C has a natural abundance of only about 1%. Therefore the sensitivity of ^{13}C is less than the 1H NMR and requires longer time to record

There is some point regarding ^{13}C NMR as follows:

- The number of signals tell us how many different carbons or set of equivalent carbons
- The splitting of a signal tells us how much hydrogen is attached to each carbon. (N+1 rule)
- The chemical shift tells us the hybridization (sp^3, sp^2, sp) of each carbon.

6.3.2 PRINCIPLE

The principle behind NMR is that many nuclei have charge "spin" when they have odd number of proton in their nuclei. When an external magnetic field applied and the nuclear spin occurs, the energy transfer is possible between the base energy to a higher energy level. The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in several ways and processed in order to yield an NMR spectrum. The molecular environment of proton governs the value to applied external field at which the nucleuses resonate. This gives recorded as chemical shift. The chemical field rises from the applied field including secondary field of about 0.15 – 0.2 mT at proton by interacting with adjacent bonding electron.

Nuclear magnetic moment $\mu = g_N u_N [I(I+1)]^{1/2}$

Where I is angular momentum

Also nuclear spin angular momentum $= [I(I+1)]^{1/2} \frac{h}{2\pi}$

$$\Delta E = h\nu\, g u_N B_o \quad \text{(bore-Einstein)}$$

g_N = Nuclear g factor, characteristic nucleus

μ_N = nuclear magnetron = 5.05X 10^{-27} J T^{-1}

B_o = External magnetic field in tesla

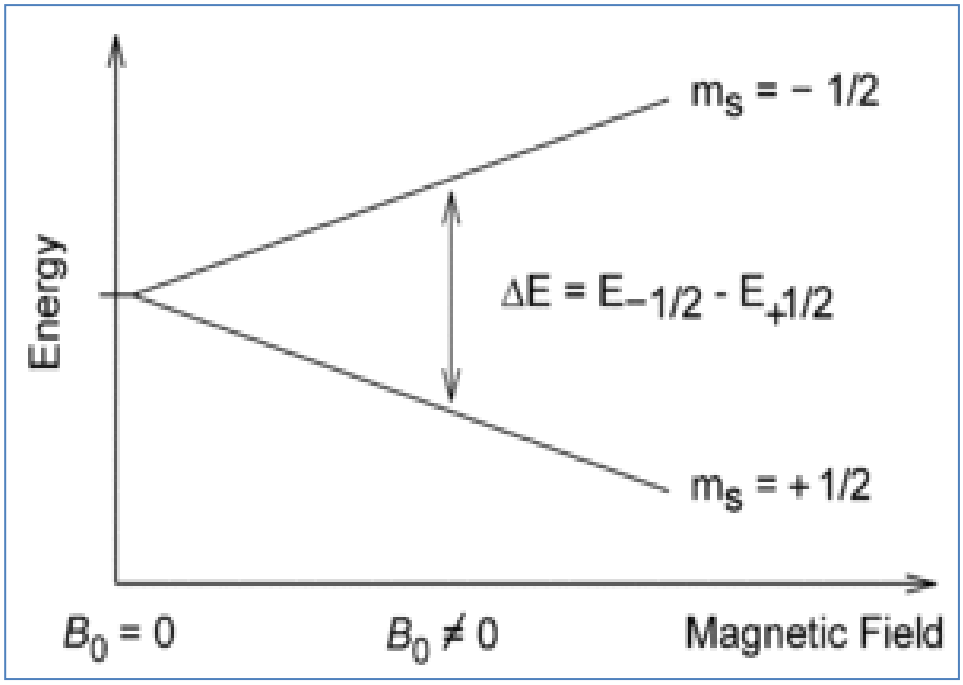


Fig. 6.3 : Representing the phenomenon of NMR in ^{1}H molecule

6.3.3 INSTRUMENTATION

In NMR the samples in solution are contained in sealed tubes which are rotated rapidly in the cavity to eliminate irregularities and imperfections in sample distribution. In solid samples, the number of spin–spin interactions is greatly enhanced due to intermolecular interactions that are absent in dissolved samples due to translation and rotation movements. As a result, the resonance signals broaden significantly. However, high-resolution spectra can be obtained by spinning the solid sample at an angle of 54.7˚. Advanced computer facilities are needed for operation of NMR instruments, as well as analysis of the acquired spectra.

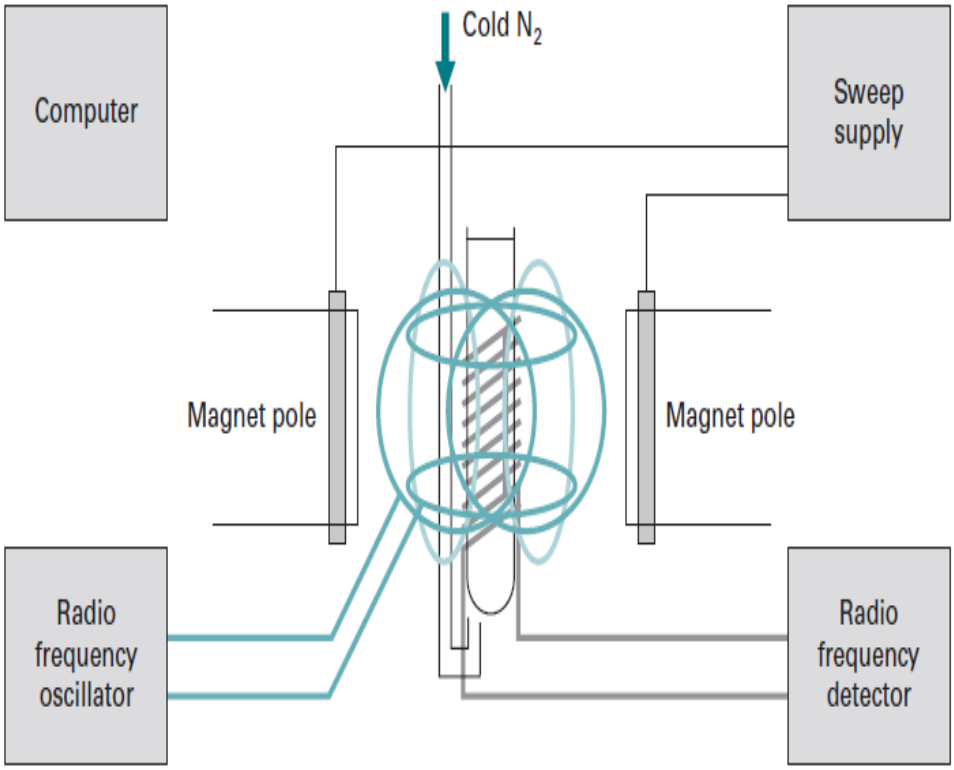


Fig. 6.4 : Schematic diagram of an NMR spectrometer with cryoprobe

6.3.4 NMR ACTIVE NUCLEI

We know the nuclei are built up of protons and neutrons each possessing an angular momentum due to its motion about the centre of the nucleus. The total angular momentum of a nucleus in its ground state is called as nuclear spin. This is characterized by a spin quantum number I, which may be integral, half-integral or 0. Only those nuclei which have non-zero spin number can absorb/emit radiofrequency (RF) radiation and hence are NMR active nuclei.

1. If the number of neutrons and the number of protons are both even, then the nucleus has NO spin (I = 0). For example ^{12}C, 16O, ^{32}S have no NMR signal because of even number of proton and neutrons.

2. If the number of neutrons and the number of protons are both odd, then the nucleus has an integer spin (I = 1, 2, 3 and so on). Example: ^{2}H, ^{10}B, ^{14}N are NMR active.

3. If the number of neutrons plus the number of protons is odd (odd mass number or we can say even number of protons/neutrons), then the nucleus has a half-integer spin (I = n/2, where n is an odd integer; I = 1/2, 3/2, 5/2 and so on) Example: ^{1}H, ^{13}C, ^{15}N, ^{31}P are NMR active.

6.3.5 NMR PHENOMENON

Only those nuclei will show NMR phenomenon whose spin number I is greater than 0 (zero). In the nucleolus when the number of proton is equal to the number of neutron are paired the spin will be cancelled due to parallel and anti-parallel spin. Thus the net result of spin will be zero and such nuclei will not show NMR phenomenon. For example molecules of carbon and oxygen (C atomic number = 12, O atomic number = 16) have some number of proton and neutron in their nucleus. Whereas as those molecules that have not paired proton and neutron or having unequal number of proton and neutron in their nucleus shows NMR phenomenon because such nuclei will have resultant spin (I) more than zero. Thus, resulting nuclei carry charge, then get spin and generate magnetic field. Thus we can say that nuclei act as a tiny bar magnet.

The magnitude and direction of the magnetic field generate by this nuclei is described by a vector called magnetic moment or magnetic dipole. The nuclei is placed in strong magnetic field it spin states shows two stages.

α-spin state : In this state nuclear spin aligned in the same direction as that of applied magnetic field. α-spin state is of lower energy.

β-spin state : In this state nuclear spins aligned themselves in the opposite direction of applied magnetic field. β-spin state is of higher energy.

When the electromagnetic radiation of proper frequency is passed, a nucleus with α-spin state absorbs this radiation and converted into higher energy state that is β-spin state. This process is called flipping of nucleus. When the proton is placed at magnetic field then it starts processing a certain frequency in the radio wave region and thus it will capable of taking up one of the two orientations with respect to the axis of the external field.

- Alignment with the field
- Alignment against the field

If the proton is processing in the aligned orientation. It can pass into the opposed orientation by absorbing higher energy. It come backs to lower energy aligned orientation by losing energy. The transition between two energy states is called flipping of proton. The transition between two energy states can be brought about by the absorption of electromagnetic radiation in the radio wave region and resulting single peaks is obtained.

6.3.6 SOLVENT USED IN NMR

The spectrum of NMR depends on the nature of solvent of samples. The solvent should have following character

- It should be chemically inert
- It should be magnetically isotropic in nature
- It should be free from any hydrogen (^1H) atom

Some solvent that are most commonly used in NMR study are

- Carbon tetrachloride (CCl_4)
- Carbon disulphide (CS_2)
- Deuterochloroform ($CDCl_3$)
- Deutero dimethyl sulfoxide $(CH_3)SO$
- Deuterobenzene (C_6D_6)

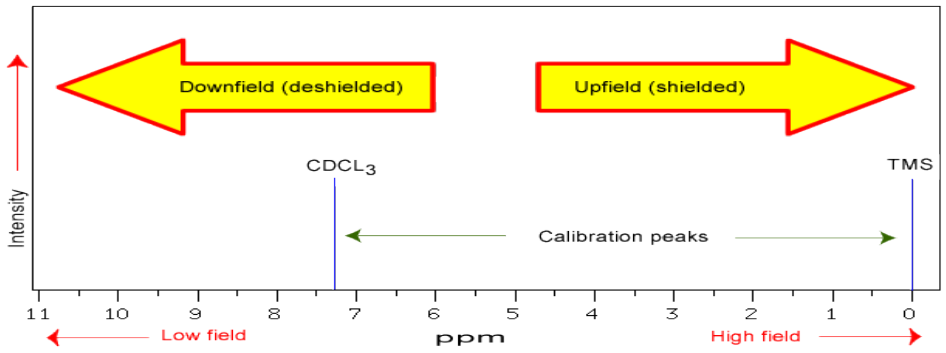
6.3.7 CHEMICAL SHIFT AND SIGNALS IN NMR

The chemical shift is the position of NMR signals which arises due to shielding and deshielding of electron in chemical bond is called chemical shift. Different types of proton in the compound have different electronic environment. The proton gets absorb at different applied magnetic field. Chemical shift are measured in reference to a particular standard.

The shielding and deshielding proton by electron produced very small change in the strength of applied magnetic field. This small change in the field strength can't be determined accurately. Therefore, chemical shift of proton are measured with reference to a solvent tetra methyl silane. Chemical shift δ is usually expressed in parts per million (ppm) by frequency, because it is calculated from:

$$\delta = \frac{v_{sample} - v_{ref}}{v_{ref}}$$

Where ν_{sample} is the absolute resonance frequency of the sample and ν_{ref} is the absolute resonance frequency of a standard reference compound, measured in the same applied magnetic field B_0. Since the numerator is usually expressed in hertz, and the denominator in megahertz, δ is expressed in ppm. When molecule is placed in external magnetic field electron will produce secondary magnetic field.



Down-field	Up-field
← Deshielding	Shielding →
← Down-field	Up-field →
← High frequency	Low frequency →
← High chemical shift values	Low chemical shift values →

Secondary magnetic field → opposed → shielding → up-field

Secondary magnetic field → reinforced → shielding → up-field

Up-field/down-field shift is known as chemical shift.

TMS (tetra methyl silane) is considered has reinforce compound because it has following characteristic characters

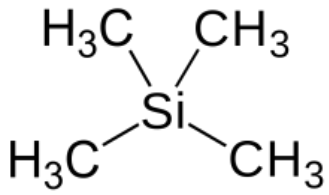


Fig 6.5 : Tetra methyl silane

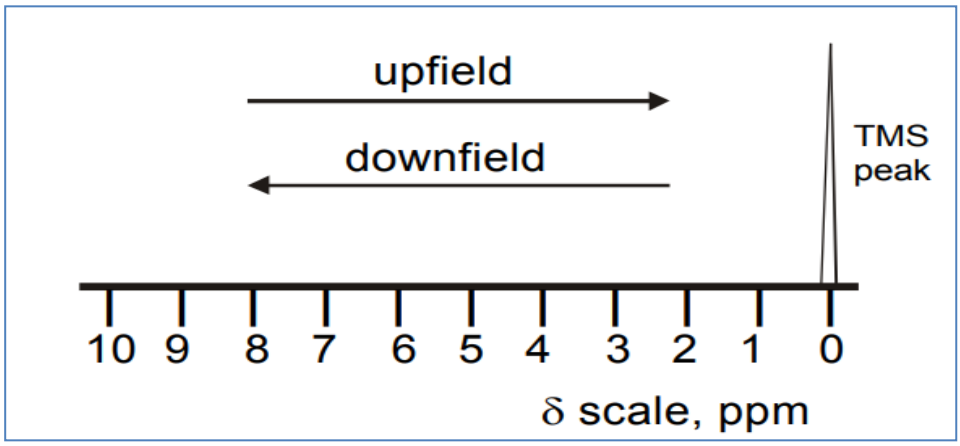


Fig. 6.6 : Chemical shift in TMS

- It has zero delta values because it highly shielded
- TMS has highest shielding than other organic compounds because it absorb at higher field. The reason behind this is that silicon is more electro positive (1.8) than carbon(2.5)
- It is easy miscible with many other organic compound

- It highly volatile in nature (boiling point = 27 ˚C) so sample can be recorded easily.
- It does not take part in any chemical reaction.

The number of signals in NMR spectrum occurs by different set of chemically equivalent proton in molecules. The proton which has the same chemical environment is called equivalent proton and they show the chemical shift. The protons have equivalent chemical environment gives same signal whereas those protons have different chemical environment produces different signals. In other word we can say that the each signal in the NMR spectrum represents a set of equivalent proton. Thus, the NMR signal tells us the kind of proton present in the molecules and it position reveal the nature of proton. Chemically equivalent proton must be sterochemically equivalent and also have same chemical shift. Magnetically equivalent proton have same chemical shift and same coupling constant (J) to every other nucleolus in the molecules. Often, magnetically equivalent protons are chemically equivalent. For example CH_3CH_2OH gives three signals.

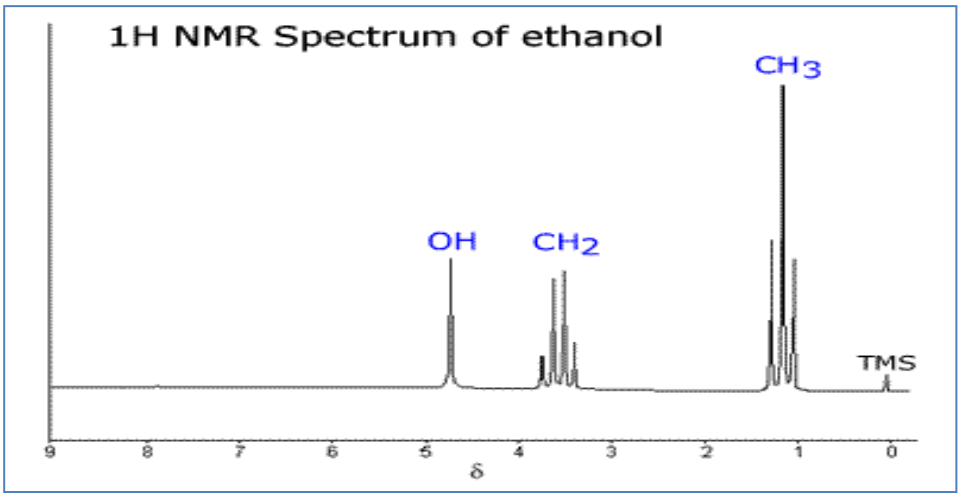


Fig. 6.7 : NMR spectrum of ethanol

6.3.8 APPLICATIONS

NMR spectroscopy is the use of NMR phenomena to study the physical, chemical, and biological properties of matter. Chemists use it to determine molecular identity and structure. In each carbon the multiplicity of the signal depends upon how many protons are attached to it. The ^{13}C-^{13}C coupling is not possible while ^{1}H-^{13}C coupling is possible.

Molecular structure determination :

The NMR is very useful method to structure determination for organic compounds. When the proton or carbon exhibit in the similar chemical environment it gives similar signal. In this case the chemical shift provides a clue about the environment of a particular proton or carbon, and thus allows conclusions as to the nature of functional groups. Spin–spin interactions allow conclusions as to how protons are linked with the carbon skeleton. For structure determination, the fine structure usually is the most useful information because it provides a unique criterion while chemical shifts of some groups can vary over an extended range. The structures of proteins up to a mass of about 50 kDa can be determined with biomolecular NMR spectroscopy. The development of magnets with very high field strengths (currently 900 MHz) continues to push the size limit. Heteronuclear multidimensional NMR spectra need to be recorded for the assignment of all chemical shifts (1H, 13C, 15N). For inter proton NOEs, 13C- and 15N-edited 3D NOESY spectra are required.

Magnetic resonance imaging :

The NMR spectroscopy is very useful in the imaging of live samples because the proton is one of the more sensitive nuclides and is present in all biological systems abundantly. The ^{1}H NMR spectroscopy has significance role in the imaging of live samples. The most important compound in biological samples in this context is water. It is distributed differently in different tissues, but constitutes about 55% of body mass in the average human. In NMR, the resonance frequency of a particular nuclide is proportional to the strength of the applied external magnetic field. If an external magnetic field gradient is applied then a range of resonant frequencies are observed, reflecting the spatial distribution of the spinning nuclei.

Magnetic resonance imaging (MRI) can be applied to large volumes in whole living organisms and has a central role in routine clinical imaging of large-volume soft tissues. The number of spins in a particular defined spatial region gives rise to the spin density as an observable parameter. This measure can be combined with analysis of the principal relaxation times (T_1 and T_2). The imaging of flux, as either bulk flow or localized diffusion, adds considerably to the options available. In terms of whole-body scanners, the entire picture is reconstructed from images generated in contiguous slices. MRI can be applied to

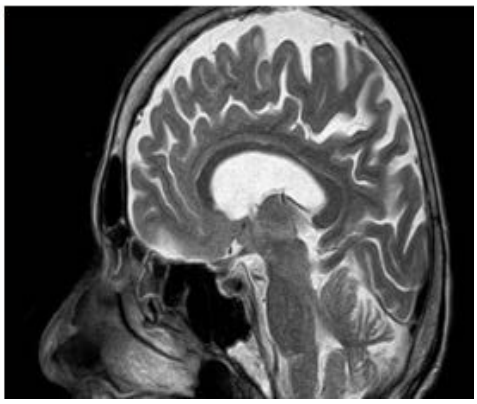


Fig. 6.8 : MRI Image of brain

the whole body or specific organ investigations on head, thorax, abdomen, liver, pancreas, kidney and musculoskeletal regions (Fig. 6.8). The use of contrast agents with paramagnetic properties has enabled investigation of organ function, as well as blood flow, tissue perfusion, transport across the blood– brain barrier and vascular anatomy. Resolution and image contrast are major considerations for the technique and subject to continuing development. The resolution depends on the strength of the magnetic field and the availability of labels that yield high signal strengths.

6.4 SUMMARY

In this unit you have learn that-

The XRD and NMR would be leading to biochemistry regarding characterization and detection any biological compounds. The body of organism contains groups of chemicals, and every chemical have different characteristic character and gives their performances accordingly. The chemical phenomenon creates abnormality in organism due to presence of various functional groups or different chemical structure. Thus, the instrumentation techniques are found very useful in understanding of chemical nature and structure of compounds. The NMR with learning of XRD and NMR techniques student would create new idea to utilize these techniques for characterization of biological samples.

6.5 TERMINAL QUESTIONS

Q.7. What do you understand about XRD? Write the application of XRD.

Answer:----------

Q.8. Write the phenomenon of NMR.

Answer:----------

Q.9. XRD work on which law. Discuss briefly.

Answer:----------

Q.10. Describe the principle and instrumentation of ^{1}HNMR .

Answer:----------

Q.11. XRD work on which law. Discuss briefly.

Answer:----------

Q.12. What is signal in NMR spectrum? Describe the principle of ^{13}C NMR.

Answer:----------

6.6 FURTHER READINGS

1. Principles and techniques of biochemistry and molecular biology; cambridge university press, seventh edition, edited by keith wilson and john walker,
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3. Y R Sharma, Elementary organic spectroscopy; S Chand publication, Fifth edition 2013.
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Notes