BHARATH COLLEGE OF SCIENCE AND MANAGEMENT, THANJAVUR-5

PG & Research Department of Bio Technology

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UNIT 1: CENTRIFUGATION

- **METHODS OF CENTRIFUGATION**
- **CENTRIFUGATION PRINCIPLES AND APPLICATIONS**
- **TYPES OF CENTRIFUGE**
- **TYPES OF ROTORS**

Methods of centrifugation:

Centrifugation is a process that involves use of centrifugal force for separation of particles from mixture with the use of centrifuge**.** Depending upon the medium of suspension in which the separation is carried out it is of two types:

- Differential
- Density Gradient

Density Gradient centrifugation can be further divided into **rate zonal** and **isopycnic**.

Differential centrifugation: Separations carried out in a homogenous medium are known as differential centrifugation. It is most common type of centrifugation employed.Rate of particle sedimentation depends mainly on its size and the applied g-force. Most commonly used method for isolation of intracellular organelles from tissue homogenates because of its relative ease, convenience and time economy.

Process:

- Tissues are homogenized in a sucrose solution that contains buffer.
- The homogenate is placed in a centrifuge and spun at constant centrifugal force at constant temperature.
- After sometime, sediment forms at the bottom of centrifuge called pellet and overlying solution called supernatant.
- The overlying solution is then placed in another centrifuge tube which is then rotated at higher speeds in progressing steps.

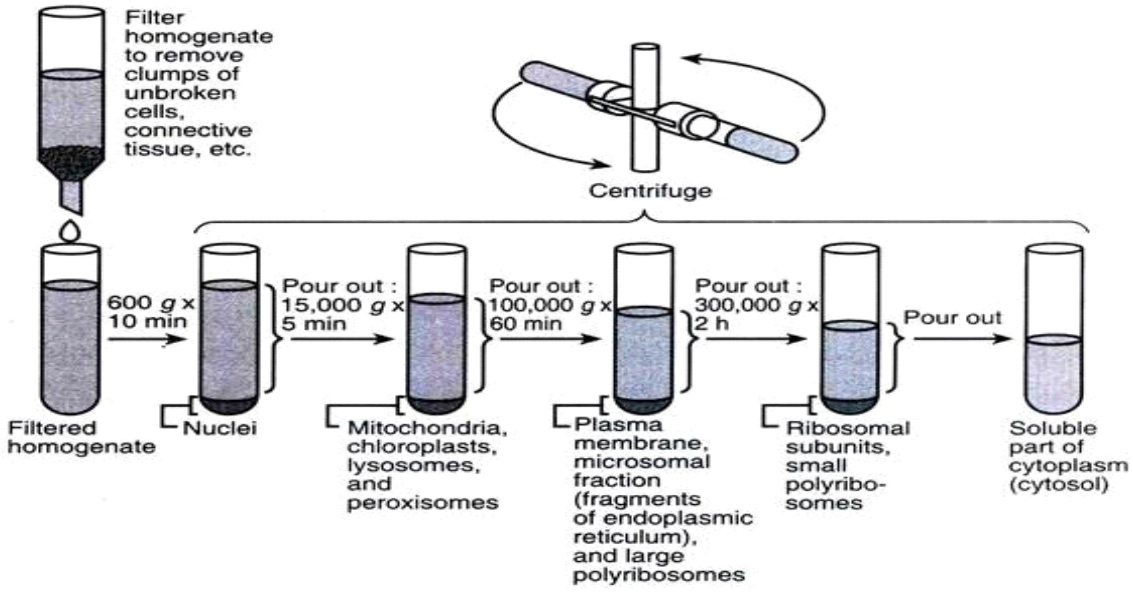


Fig. 5.13: Isolation of different cell organelles by differential centrifugation

Density gradient centrifugation:

Density Gradient Centrifugation: Centrifugation carried out in suspending medium such as sucrose or cesium chloride having density gradients. It permits separation of multicomponent mixture of macromolecules and measurement of sedimentation coefficient.The separation under centrifugal field is dependent upon buoyant densities of the particles. It is mainly used to purify viruses, ribosomes, membranes etc.

Process:

- A sucrose density gradient is created by gently overlaying lower concentrations of sucrose on higher concentrations in centrifuge tubes
- Particles of interest are placed on top of the gradient and centrifuge in ultra-centrifuges.
- Particle travel through gradient until they reach a point at which their density matches with density of surrounding sucrose.
- Fraction is removed and analyzed

Rate-Zonal: It is also known as band or gradient centrifugation. The gradient used has maximum density below that of least dense sedimenting particle. This involves careful layering of a thin layer of the sample solution on top of a preformed liquid density gradient whose density continuously increases towards the bottom of the sample tube. Components of mixture sediments/separates according to shape, size and density. Useful for separating particles which differ in size but not in density, separation of RNA-DNA hybrids and ribosomal subunits.

Isopycnic: Also called buoyant or equilibrium separation. Components are separated solely on the basis of their density & size only affects the rate of movement of molecules. The density of gradient medium must be greater than density of particles to be separated. In this method, components never sediment to the bottom of tube. Upon centrifugation, particles of a specific

density sediment until they reach a point where their density is same as gradient media (i.e. **equilibrium position**). This is to say the sample molecules move to the region where their density equals to the density of gradient.

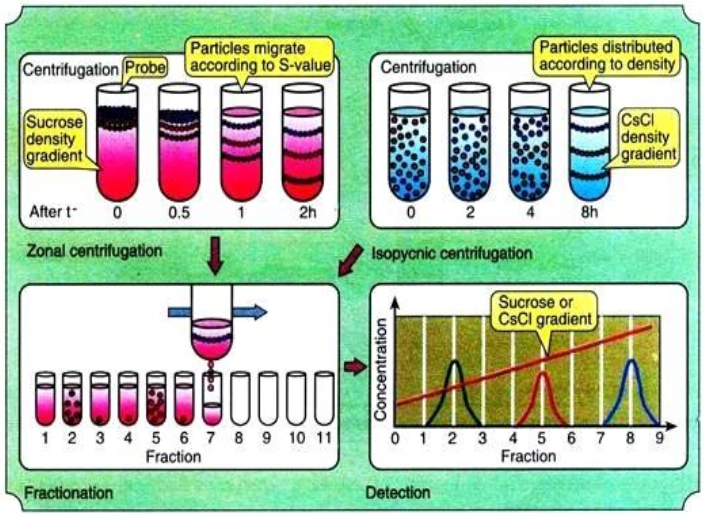


Fig. 5.15: Density gradient centrifugation

centrifugation principles and applications:

Centrifugation is a technique used for the separation of particles using a centrifugal field. The particles are suspended in liquid medium and placed in a centrifuge tube. The tube is then placed in a rotor and spun at a definitive speed. Rotation of the rotor about a central axis generates a centrifugal force upon the particles in the suspension.
Two forces counteract the centrifugal force acting on the suspended particles:

- Buoyant force: This is the force with which the particles must displace the liquid media into which they sediment.
- Frictional force: This is the force generated by the particles as they migrate through the solution.

Particles move away from the axis of rotation in a centrifugal field only when the centrifugal force exceeds the counteracting buoyant and frictional forces resulting in sedimentation of the particles at a constant rate.

Particles which differ in density, size or shape sediment at different rates. The rate of sedimentation depends upon:

1. The applied centrifugal field
2. Density and radius of the particle.
3. Density and viscosity of the suspending medium.

Angular velocity = w radians / second;
since one revolution = 360^o = 2p radians,
(r = radial distance of the particle from the axis of rotation)

As the centrifugal field acting on the particle is much greater than the Earth's gravitational field, CF is generally expressed relative to the Earth's gravitational field as multiples of g, the acceleration due to gravity (g= 980 cm/s^2). This expression relates relative centrifugal field (RCF) to the speed of the centrifuge (rpm) and and the radius of the rotor (r). For example, if a rotor with an average radius of 7 cm revolves at a speed of 20,000 rpm, a centrifugal field of 31,300 g is created.The sedimentation rate of velocity (v) of a particle can be expressed in terms of its sedimentation rate per unit centrifugal field. This is termed as sedimentation coefficient (s).

Application of centrifugation:

- To separate two miscible substances
- To analyze the hydrodynamic properties of macromolecules
- Purification of mammalian cells
- Fractionation of subcellular organelles (including membranes/membrane fractions) Fractionation of membrane vesicles
- The clarification and stabilization of wine
- Separation of urine components and blood components in forensic and research laboratories
- Aids in the separation of proteins using purification techniques such as salting out, e.g. ammonium sulfate precipitation.

Types of centrifuge:

There are four major types of centrifuges. They are:

1. Small Bench Centrifuges:

They are used to collect small amount of material that rapidly sediment like yeast cells, erythrocytes etc. They have maximum relative centrifugal field of 3000-7000 g.

2. Large Capacity Refrigerated Centrifuges:

They have refrigerated rotor chamber and have capacity to change rotor chambers for varying size. They can go up to maximum of 6500 g and use to sediment or collect the substances that sediment rapidly like erythrocytes, yeast cell, nuclei and chloroplast.

3. High Speed Refrigerated Centrifuges:

They can generate speed of about 60000g and are used to collect micro-organism, cellular debris, larger cellular organelles and proteins precipitated by ammonium sulphate.

4. Ultra Centrifuges:

(a) Preparative ultracentrifuge:

It can produce relative centrifugal force of about 600000g and its chamber is refrigerated, sealed and evacuated. It is employed for separation of macromolecules/ligand binding kinetic studies, separation of various lipoprotein fractions from plasma and deprotonisation of physiological fluids for amino acid analysis.

(b) Analytical ultracentrifuge:

It is capable of operating at 500000 g. Three kinds of optical systems are available in analytical ultracentrifuges: a light absorption system, and the alternative Schlieren system and Rayleigh interferometric system, both of which detect changes in the refractive index of the solution.

Types of rotor:

1. Swinging Bucket Rotors:

The swinging bucket rotor has buckets that start off in a vertical position but during acceleration of the rotor swing out to a horizontal position so that during centrifugation the tube and hence the solution in the tube, is aligned perpendicular to the axis of rotation and parallel to the applied centrifugal field, the tube returning to its original position during deceleration of the rotor.

2. Fixed Angle Rotors:

In fixed angles the tubes are located in holes in the rotor body set at a fixed angle between 14° and 40° to the vertical. Under the influence of centrifugal field, particles move radially outward and have only a short distance to travel before colliding with, and precipitating on, the outer wall of the centrifuge tube. A region of high concentration is formed that has a density greater than surrounding medium, with the result that the precipitate sinks and collects as a small compact pellet at the outermost point of the tube.

3. Vertical Tube Rotors:

They are considered as zero angle fixed angle rotors in which the tubes are aligned vertically in the body of the rotors at all times.

4. Zonal Rotors:

The zonal rotors may be of the batch or continuous flow type. The former being more extensively used than the latter, and are designed to minimize the wall effect that is encountered in swinging- bucket and fixed angle rotors, and to increase sample size.

5. Elutriator Rotors:

The elutriator is a kind of continuous flow rotor that contains recesses to hold a single conical shaped separation chamber, the apex of which points away from the axis of rotation, and a bypass chamber on the opposite side of the rotor that serves as a counter balance and to provide the fluid outlet.

UNIT II- CHROMATOGRAPHY

- **PRINCIPLES AND APPLICATIONS OF CHROMATOGRAPHY**
- **DIFFERENCE BETWEEN PARTITION AND ADSORPTION CHROMATOGRAPHY**
- **PRINCIPLE AND MECHANISM OF PAPER AND THIN LAYER CHROMATOGRAPHY**
- **ION EXCHANGE CHROMATOGRAPHY**
- **GEL PERMEATION CHROMATOGRAPHY**
- **AFFINITY CHROMATOGRAPHY**
- **GAS CHROMATOGRAPHY**
- **HPLC**

Principle and application of chromatography:

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.The Russian botanist Mikhail Tswett coined the term chromatography in 1906.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.A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties to

separate materials. It is a powerful separation tool that is used in all branches of science and is often the only means of separating components from complex mixtures.
Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase.The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights.Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into the mobile phase, and leave the system faster.
Three components thus form the basis of the chromatography technique.

- **Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface solid support".
- **Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."
- **Separated molecules.**

Application of chromatography:

Pharmaceutical sector

- To identify and analyze samples for the presence of trace elements or chemicals.
- Separation of compounds based on their molecular weight and element composition.
- Detects the unknown compounds and purity of mixture.
- In drug development.

Chemical industry

- In testing water samples and also checks air quality.
- HPLC and GC are very much used for detecting various contaminants such as polychlorinated biphenyl (PCBs) in pesticides and oils.
- In various life sciences applications

Food Industry

- In food spoilage and additive detection
- Determining the nutritional quality of food

Forensic Science

- In forensic pathology and crime scene testing like analyzing blood and hair samples of crime place.

Molecular Biology Studies

- Various hyphenated techniques in chromatography such as EC-LC-MS are applied in the study of metabolomics and proteomics along with nucleic acid research.
- HPLC is used in Protein Separation like Insulin Purification, Plasma Fractionation, and Enzyme Purification and also in various departments like Fuel Industry, biotechnology, and biochemical processes.

Difference partition chromatography and adsorption chromatography

Partition chromatography:

Partition chromatography is a process of separation which is based on the partition coefficient. Here the components of the mixture get distributed into two liquid phases. Here both the stationary phase and mobile phase are liquids. The components get partitioned in between two

phase due to the differences in partition coefficients.Polar molecules get partitioned into the polar phase to a maximum extent. The nonpolar molecules get partitioned into the non-polar liquid phase.This mode of *partition chromatography* applies to Liquid-liquid, liquid-gas chromatography and not to solid-gas chromatography. Gases are freely flow-able hence, for them partition works better than adsorption. Examples include paper chromatography, gas chromatography, high-performance thin layer chromatography (HPTLC), partition chromatography is the principle of separation.In paper chromatography, the paper is in the solid state, but the pores in between the paper contain moisture which acts as a stationary liquid phase.

Adsorption chromatography:

The sample components physically adsorb (stick) to the stationary phase. There is relatively no adsorption of the sample with the mobile phase. Mobile phase here just forces the sample particles to move over the stationary phase. It is a process of separation of components of a mixture based *on the relative differences in adsorption of components to the stationary phase* present in the chromatography column. Here the molecules or components of the mixture travel with different rates due to differences in their affinity towards stationary phase.Based on nature, polar compounds adsorb with greater intensity to the polar stationary phase while non-polar compounds remain suspended in the mobile phase. Hence during separation of components, the nonpolar component comes out of the column first while the polar components elute out last due to greater adsorption.This is exactly reverse on using a non-polar stationary phase.This adsorption chromatography applies to only solid-liquid or solid-gas chromatography. Because the adsorption phenomenon is an inherent property of solids and hence it is seen only with solid stationary phase chromatography.Examples for this type are Column chromatography, HPLC chromatography, Thin layer chromatography.

Thin layer chromatography:

Thin Layer Chromatography is a technique used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium foil, plastic, or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose, or silica gel.

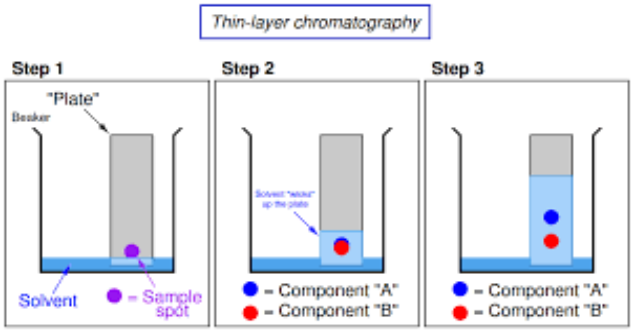
Principle:

Thin layer chromatography (TLC) depends on the separation principle. The separation relies on the relative affinity of compounds towards both the phases. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the other compounds travel fast. Therefore, the separation of the mixture is attained. On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques.

Procedure:

The stationary phase that is applied to the plate is made to dry and stabilize.

- To apply sample spots, thin marks are made at the bottom of the plate with the help of a pencil.
- Apply sample solutions to the marked spots.
- Pour the mobile phase into the TLC chamber and to maintain equal humidity, place a moistened filter paper in the mobile phase.
- Place the plate in the TLC chamber and close it with a lid. It is kept in such a way that the sample faces the mobile phase.
- Immerse the plate for development. Remember to keep the sample spots well above the level of the mobile phase. Do not immerse it in the solvent.
- Wait till the development of spots. Once the spots are developed, take out the plates and dry them. The sample spots can be observed under a UV light chamber.



Application of TLC:

- The qualitative testing of Various medicines such as sedatives, local anaesthetics, anticonvulsant tranquilisers, analgesics, antihistamines, steroids, hypnotics is done by TLC.
- TLC is extremely useful in Biochemical analysis such as separation or isolation of biochemical metabolites from its blood plasma, urine, body fluids, serum, etc.
- Thin layer chromatography can be used to identify natural products like essential oils or volatile oil, fixed oil, glycosides, waxes, alkaloids, etc
- It is widely used in separating multicomponent pharmaceutical formulations.
- It is used in the food industry, to separate and identify colours, sweetening agent, and preservatives
- It is used in the cosmetic industry.
- It is used to study if a reaction is complete

Paper Chromatography

Principle

The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The

two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the separation of mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.

Types of paper chromatography:

- Ascending Paper Chromatography – The techniques goes with its name as the solvent moves in an upward direction.
- Descending Paper Chromatography – The movement of the flow of solvent due to gravitational pull and capillary action is downwards hence the name descending paper chromatography.
- Ascending – Descending Paper Chromatography – In this version of paper chromatography movement of solvent occurs in two directions after a particular point. Initially, the solvent travels upwards on the paper which is folded over a rod and after crossing the rod it continues with its travel in the downward direction.
- Radial or Circular Paper Chromatography – The sample is deposited at the center of the circular filter paper. Once the spot is dried, the filter paper is tied horizontally on a Petri dish which contains the solvent.
- Two Dimensional Paper Chromatography – Substances which have the same r_f values can be resolved with the help of two-dimensional paper chromatography.

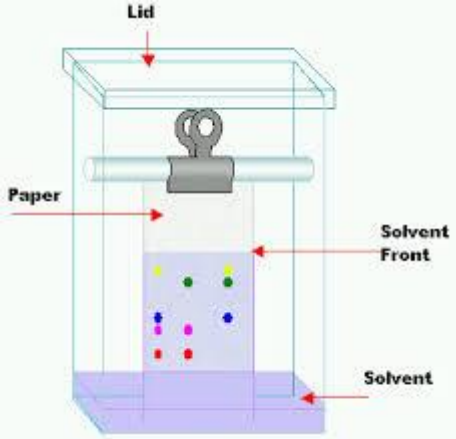
procedure

1.Selecting a suitable filter paper: Selection of filter paper is done based on the size of the pores, and the sample quality.

2.Prepare the sample: Sample preparation includes the dissolution of the sample in a suitable solvent (inert with the sample under analysis) used in making the mobile phase.

3.Spot the sample on the paper: Samples should be spotted at a proper position on the paper by using a capillary tube.

- **Chromatogram development:** Chromatogram development is spotted by immersing the paper in the mobile phase. Due to the capillary action of paper, the mobile phase moves over the sample on the paper.
- **Paper drying and compound detection**: Once the chromatogram is developed, the paper is dried using an air drier. Also, detecting solution can be sprayed on the chromatogram developed paper and dried to identify the sample chromatogram spots.



Paper Chromatography Applications

There are various applications of paper chromatography. Some of the uses of Paper Chromatography in different fields are discussed below:

- To study the process of fermentation and ripening.
- To check the purity of pharmaceuticals.
- To inspect cosmetics.
- To detect the adulterants.
- To detect the contaminants in drinks and foods.
- To examine the reaction mixtures in biochemical laboratories.
- To determine dopes and drugs in humans and animals.

Ion exchange chromatography

Principle

- Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers.
- The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on ion exchangers.
- In this process two types of exchangers i.e., cationic and anionic exchangers can be used.
- **Cationic exchangers** possess negatively charged group, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials, because their negative charges result from the ionization of acidic group.
- **Anionic exchangers** have positively charged groups that will attract negatively charged anions. These are also called "Basic ion exchange" materials
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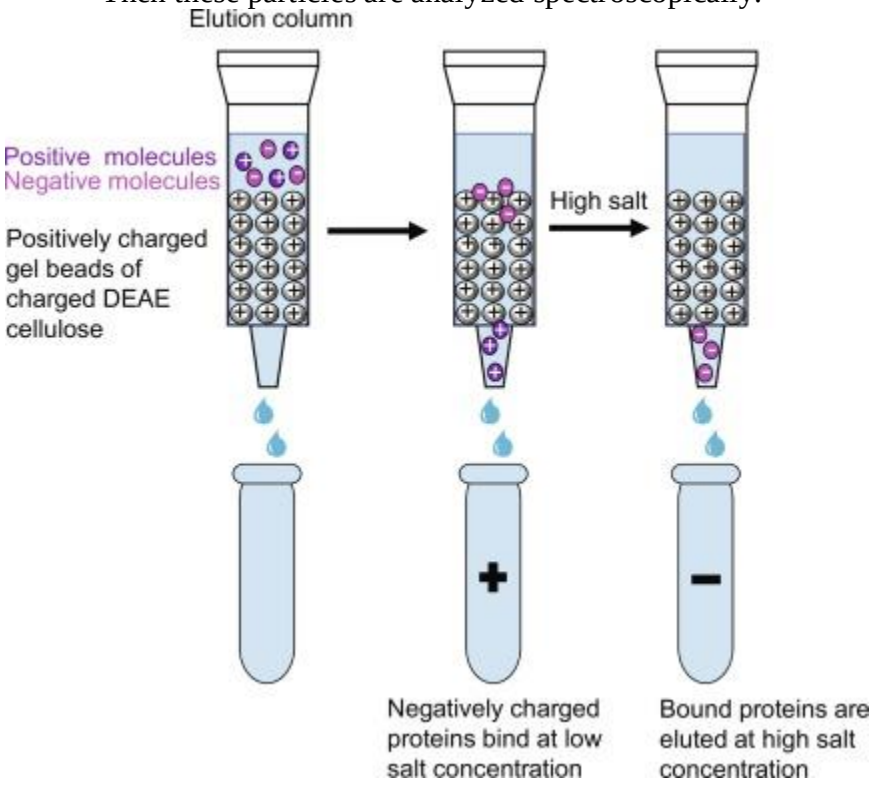
  Procedure:

- Ion exchange separations are carried out mainly in columns packed with an ion-exchanger.

- These ionic exchangers are commercially available. They are made up of styrene and divinyl benzene. Example. DEAE-cellulose is an anionic exchanger, CM-cellulose is a cationic exchanger.
- The choice of the exchanger depends upon the charge of particle to be separated. To separate anions "Anionic exchanger" is used, to separate cations "Cationic exchanger" is used.
- First the column is filled with ion exchanger then the sample is applied followed by the buffer. The tris-buffer, pyridine buffer, acetate buffer, citrate and phosphate buffers are widely used.
- The particles which have high affinity for ion exchanger will come down the column along with buffers.
- In next step using corresponding buffer separates the tightly bound particles.
- Then these particles are analyzed spectroscopically.



Applications :

- An important use of ion-exchange chromatography is in the routine analysis of **amino acid** mixtures.
- The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated and used in clinical diagnosis.
- This is most effective method for water purification.
- In the analysis of products of hydrolysis of nucleic acids. .
- To analyze lunar rocks and rare trace elements on Earth.

Gel permeation chromatography

- Gel permeation chromatography is also called as gel filtration or size exclusion chromatography.

- In size exclusion chromatography, the stationary phase is a porous matrix made up of compounds like cross-linked polystyrene, cross-like dextrans, polyacrylamide gels, agarose gels, etc.
- The separation is based on the analyte molecular sizes since the gel behaves like a molecular sieve.
- This technique is used for the separation of proteins, polysaccharides, enzymes, and synthetic polymers.
- As a technique, size exclusion chromatography was first developed in 1955 by Lathe and Ruthven.

Principle:

- It is a technique in which the separation of components is based on the difference in molecular weight or size.
- The stationary phase used is a porous polymer matrix whose pores are completely filled with the solvent to be used as the mobile phase.
- The molecules in the sample are pumped through specialized columns containing such microporous packing material (gel).
- The basis of the separation is that molecules above a certain size are totally excluded from the pores, while smaller molecules access the interior of the pores partly or wholly.
- The flow of the mobile phase hence will cause larger molecules to pass through the column unhindered, without penetrating the gel matrix, whereas smaller molecules will be retarded according to their penetration of the gel.

Procedure

It involves three major steps:

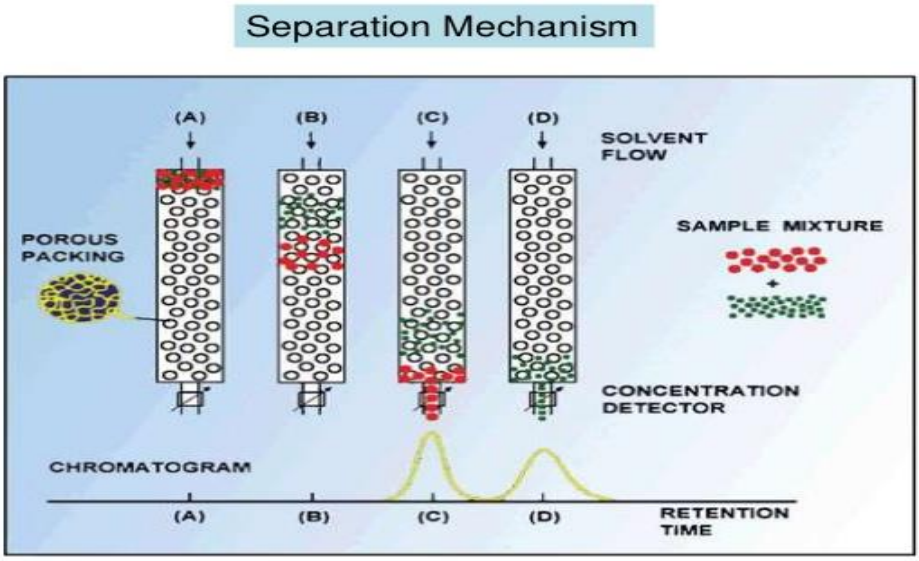
A. Preparation of column for gel filtration

It involves:

- Swelling of the gel
- Packing the column semi-permeable, porous polymer gel beads with a well-defined range of pore sizes.
- Washing: After packing, several column volumes of buffer solution is passed through the column to remove any air bubbles and to test the column homogeneity.

B. Loading the sample onto the column using a syringe

C. Eluting the sample and detection of components



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Application

- Proteins fractionation
- Purification
- Molecular weight determination.
- Separation of sugar, proteins, peptides, rubbers, and others on the basis of their size.
- Can be used to determine the quaternary structure of purified proteins.

Affinity chromatography:

- Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- It is a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase.
- Affinity chromatography is a type of liquid **chromatography** for the separation, purification or specific analysis of sample components.
- It utilizes the reversible biological interaction or molecular recognition called affinity which refers to the attracting forced exerted in different degrees between atoms which cause them to remain in combination.

Example: Enzyme with and inhibitor, antigen with an antibody etc.

- It was discovered by Pedro Cuatrecasas and Meir Wilcheck.

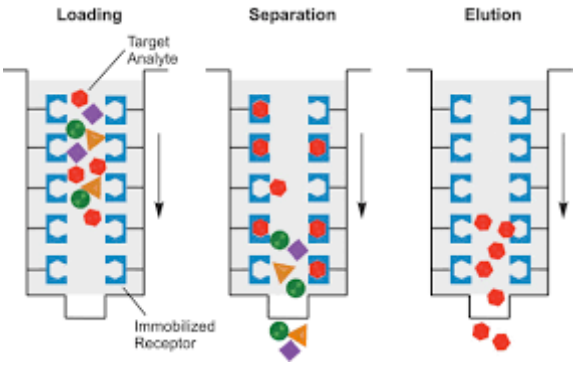
Principle:

- The stationary phase consists of a support medium, on which the substrate (ligand) is bound covalently, in such a way that the reactive groups that are essential for binding of the target molecule are exposed.

- As the crude mixture of the substances is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substances is eluted in the void volume of the column.
- Once the other substances are eluted, the bound target molecules can be eluted by methods such as including a competing ligand in the mobile phase or changing the pH, ionic strength or polarity conditions.

Procedure:

- **Preparation of Column**
- The column is loaded with solid support such as sepharose, agarose, cellulose etc.
- Ligand is selected according to the desired isolate.
- Spacer arm is attached between the ligand and solid support.
- **Loading of Sample**
- Solution containing a mixture of substances is poured into the elution column and allowed to run at a controlled rate.
- **Elution of Ligand-Molecule Complex**
- Target substance is recovered by changing conditions to favor elution of the bound molecules.



Application

Its major application includes:

- Separation of mixture of compounds.
- Removal of impurities or in purification process.
- In enzyme assays
- Detection of substrates
- Investigation of binding sites of enzymes
- In in vitro antigen-antibody reactions
- Detection of Single Nuceotide polymorphisms and mutations in nucleic acid

Gas chromatography:

- Gas chromatography differs from other forms of **chromatography** in that the mobile phase is a gas and the components are separated as vapors.
- It is thus used to separate and detect small molecular weight compounds in the gas phase.

- The sample is either a gas or a liquid that is vaporized in the injection port. The mobile phase for gas chromatography is a carrier gas, typically helium because of its low molecular weight and being chemically inert.
- The pressure is applied and the mobile phase moves the analyte through the column. The separation is accomplished using a column coated with a stationary phase.

Principle:

The equilibrium for gas chromatography is partitioning, and the components of the sample will partition (i.e. distribute) between the two phases: the stationary phase and the mobile phase. Compounds that have a greater affinity for the stationary phase spend more time in the column and thus elute later and have a longer **retention time (Rt)** than samples that have a higher affinity for the mobile phase.

Affinity for the stationary phase is driven mainly by intermolecular interactions and the polarity of the stationary phase can be chosen to maximize interactions and thus the separation.

Ideal peaks are Gaussian distributions and symmetrical, because of the random nature of the analyte interactions with the column.

Procedure:

Step 1: Sample Injection and Vapourization

- A small amount of liquid sample to be analyzed is drawn up into a syringe.
- The syringe needle is positioned in the hot injection port of the gas chromatograph and the sample is injected quickly.
- The injection of the sample is considered to be a "point" in time, that is, it is assumed that the entire sample enters the gas chromatograph at the same time, so the sample must be injected quickly.
- The temperature is set to be higher than the boiling points of the components of the mixture so that the components will vaporize.
- The vaporized components then mix with the inert gas mobile phase to be carried to the gas chromatography column to be separated.

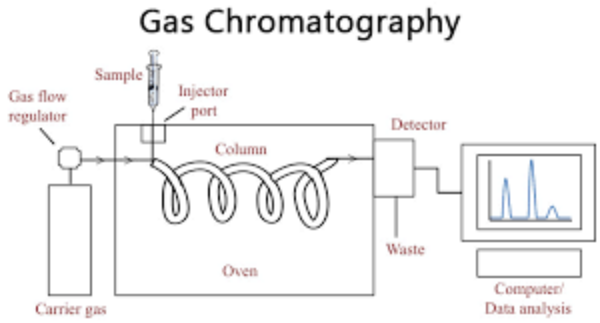
Step 2: Separation in the Column

- Components in the mixture are separated based on their abilities to adsorb on or bind to, the stationary phase.
- A component that adsorbs most strongly to the stationary phase will spend the most time in the column (will be retained in the column for the longest time) and will, therefore, have the longest retention time (Rt). It will emerge from the gas chromatograph last.
- A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will, therefore, have the shortest retention time (Rt). It will emerge from the gas chromatograph first.
- If we consider a 2 component mixture in which component A is more polar than component B then:
- component A will have a **longer retention time** in a polar column than component B
- component A will have a **shorter retention time** in a non-polar column than component B

Step 3: Detecting and Recording Results

- The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.

- The component that is retained the shortest time in the column is detected first. The component that is retained the longest time in the column is detected last.
- The detector sends a signal to the chart recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is recorded last.



Applications:

- GC analysis is used to calculate the content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances in soil, air or water.
- Gas chromatography is used in the analysis of:
  (a) air-borne pollutants
  (b) performance-enhancing drugs in athlete's urine samples
  (c) oil spills
  (d) essential oils in perfume preparation
- GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.
- Gas Chromatography is used extensively in forensic science.

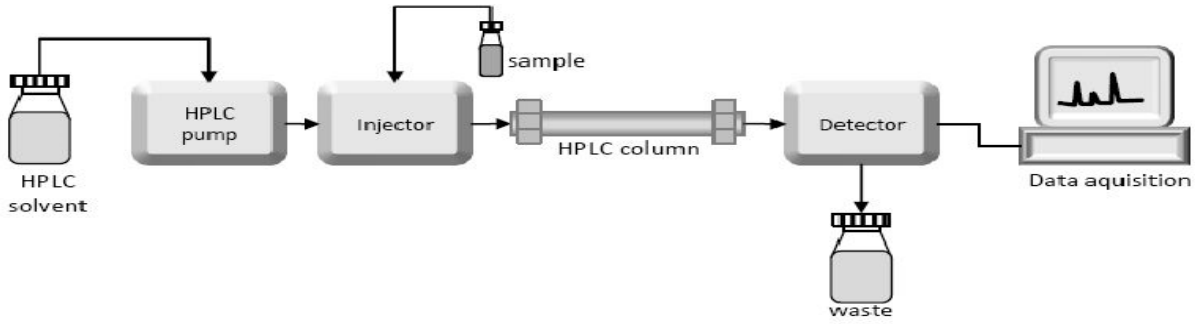
HPLC

- High performance liquid chromatography or commonly known as HPLC is an analytical technique used to separate, identify or quantify each component in a mixture.
- The mixture is separated using the basic principle of column **chromatography** and then identified and quantified by spectroscopy.
- In the 1960s the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.
- HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

Principle

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.

- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.
- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.



The Pump

- The development of HPLC led to the development of the pump system.
- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system.
- High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.
- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses".

Injector

- An injector is placed next to the pump.
- The simplest method is to use a syringe, and the sample is introduced to the flow of eluent.
- The most widely used injection method is based on sampling loops.
- The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Column

- The separation is performed inside the column.
- The recent columns are often prepared in a stainless steel housing, instead of glass columns.

- The packing material generally used is silica or polymer gels compared to calcium carbonate.
  The eluent used for LC varies from acidic to basic solvents.
- Most column housing is made of stainless steel since stainless is tolerant towards a large variety of solvents.

Detector

- Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation.
- The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences.
- This difference is monitored as a form of an electronic signal. There are different types of detectors available.

Recorder

- The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes.
- In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer-based data processor (integrator) is more common.
- There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non- visible to our eyes.

- When gas is present in the eluent, this is detected as noise and causes an unstable baseline.
- Degasser uses special polymer membrane tubing to remove gases.
- The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore.

Column Heater

The LC separation is often largely influenced by the column temperature.

- In order to obtain repeatable results, it is important to keep consistent temperature conditions.
- Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C).
- Thus columns are generally kept inside the column oven (column heater).

<u>Types of HPLC:</u>

- **Normal phase:**

Column packing is polar (e.g silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cis-trans isomers, and chiral compounds.

- **Reverse phase:**

The column packing is non-polar (e.g C18), the mobile phase is water+ miscible solvent (e.g methanol). It can be used for polar, non-polar, ionizable and ionic samples.

- **Ion exchange:**

Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.

- **Size exclusion:**

Molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

Applications:

- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals
- Separation and purification of biopolymers such as enzymes or nucleic acids

Unit III- ELECTROPHORESIS

1. PRNICIPLES AND TYPES OF ELECTROPHORESIS
2. AGAROSE GEL ELEECTRPHORESIS
3. SDS PAGE ELECTEROPHORESIS
4..ISOELECTROFOCUSSING

PRINCIPLE AND TYPES OF ELECTROPHORESIS

principle:

The term electrophoresis describes the migration of a charged particle under the influence of an electrical field. Many important biomolecules — such as peptide, proteins nucleotides and nucleic acids — possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-).Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge. The equipment required for electrophoresis consists basically of two items — a power pack, and an electrophoresis unit. Electrophoresis units are available for running either vertical or horizontal gel system

Types of electrophoresis:

1.Agarose Gel Electrophoresis
2.Polyacrylamide Gels
3.Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
4.Isoelectric Focusing and Two-Dimensional Gel Electrophoresis

Agarose gel electrophoresis:

principle:

Agarose gel electrophoresis is one of the most common electrophoresis technique which is relatively simple and straightforward to perform but possesses great resolving power. The agarose gel consists of microscopic pores that act as a molecular sieve which separates molecules based upon the charge, size and shape.Agarose gel electrophoresis is a powerful separation method frequently used to analyze DNA fragments generated by restriction enzymes, and it is a convenient analytical method for separating DNA fragments of varying sizes ranging from 100 bp to 25 kb. DNA fragments smaller than 100 bp are more effectively separated using polyacrylamide gel electrophoresis whereas pulse-field gel electrophoresis is used to separate DNA fragments larger than 25 kb. Agarose gel electrophoresis can also be used to separate other charged biomolecules such as RNA and proteins.

procedure:

Preparation of Agarose gel matrix

The centerpiece of agarose gel electrophoresis is the horizontal gel electrophoresis apparatus. The gel is made by dissolving agarose powder in boiling buffer solution.

The concentration of agarose in a gel depends on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%. The solution is then cooled to approximately 55℃ and poured into a casting tray which serves as a mold. A well-former template (often called a comb) is placed across the end of the casting tray to form wells when the gel solution solidifies.After the gel solidifies, the gel is submerged in a buffer-filled electrophoresis chamber which contains a positive electrode (anode) at one end, and a negative electrode (cathode) at the other. The volume of the buffer should not be greater than 1/3 of the electrophoresis chamber. The most common gel running buffers are TAE (40 mM Tris-acetate, 1 mM EDTA) and TBE (45 mM Tris-borate, 1 mM EDTA).

Sample preparation and loading

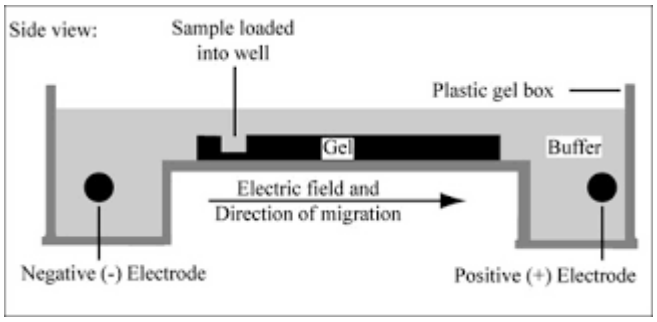
Samples are prepared for electrophoresis by mixing them with loading dyes. Gel loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol). Loading dyes used in gel electrophoresis serve three major purposes: add density to the sample, allowing it to sink into the gel. provide color and simplify the loading process.the dyes move at standard rates through the gel, allowing for the estimation of the distance that DNA fragments have migrated.

Applying electric current and separating biomolecules

A direct current (D.C.) power source is connected to the electrophoresis apparatus and electrical current is applied. Charged molecules in the sample enter the gel through the walls of the wells. Molecules having a net negative charge migrate towards the positive electrode (anode) while net positively charged molecules migrate towards the negative electrode (cathode). The buffer serves as a conductor of electricity and to control the pH, which is important to the charge and stability of biological molecules. Since DNA has a strong negative charge at neutral pH, it migrates through the gel towards the positive electrode during electrophoresis.

Visualization

The agarose gel will have to be post stained after electrophoresis. The most commonly used stain for visualizing DNA is ethidium bromide (EtBr)*.The exact sizes of separated DNA fragments can be determined by plotting the log of the molecular weight for the different bands of a DNA standard against the distance travelled by each band.



Application:

- The agarose gel electrophoresis is widely employed in molecular genetics, especially in PCR and PCR related techniques such as DNA fingerprinting, RFLP, AFLP, and RAPD analysis.

- Agarose gel electrophoresis is commonly used in the diagnosis of several diseases such as thalassemia, sickle cell anemia, hemophilia, and cystic fibrosis.

SDS PAGE ELECTROPHORESIS

SDS PAGE or Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis is a technique used for the separation of proteins based on their molecular weight. It is a technique widely used in forensics, genetics, biotechnology and molecular biology to separate the protein molecules based on their electrophoretic mobility.

principle:

The principle of SDS-PAGE states that a charged molecule migrates to the electrode with the opposite sign when placed in an electric field. The separation of the charged molecules depends upon the relative mobility of charged species.The smaller molecules migrate faster due to less resistance during electrophoresis. The structure and the charge of the proteins also influence the rate of migration. Sodium dodecyl sulphate and polyacrylamide eliminate the influence of structure and charge of the proteins, and the proteins are separated based on the length of the polypeptide chain.SDS is a detergent present in the SDS-PAGE sample buffer. SDS along with some reducing agents functions to break the disulphide bonds of proteins disrupting the tertiary structure of proteins.

procedure:

Preparation of the Gel

1.All the reagents are combined, except TEMED, for the preparation of gel.
2.When the gel is ready to be poured, add TEMED.

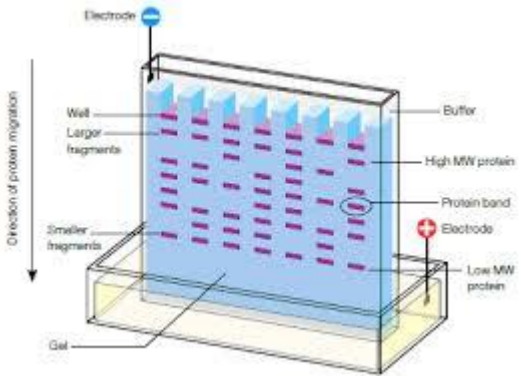
3.The separating gel is poured in the casting chamber.
4.Add butanol before polymerization to remove the unwanted air bubbles present.
5.The comb is inserted in the spaces between the glass plate.
6.The polymerized gel is known as the “gel cassette”

Sample Preparation

1. Boil some water in a beaker.
2.Add 2-mercaptoethanol to the sample buffer.
3.Place the buffer solution in microcentrifuge tubes and add protein sample to it.
4.Take MW markers in separate tubes.
5.Boil the samples for less than 5 minutes to completely denature the proteins.

Electrophoresis

1.The gel cassette is removed from the casting stand and placed in the electrode assembly.
2.The electrode assembly is fixed in the clamp stand.
3.1X electrophoresis buffer is poured in the opening of the casting frame to fill the wells of the gel.
4.Pippete 30ml of the denatured sample in the well.
5.The tank is then covered with a lid and the unit is connected to a power supply.
6.The sample is allowed to run at 30mA for about 1 hour.
7.The bands are then seen under UV light.



Application:

1.It is used to measure the molecular weight of the molecules.
2.It is used to estimate the size of the protein.
3.Used in peptide mapping
4.It is used to compare the polypeptide composition of different structures.
5.It is used to estimate the purity of the proteins.

ISOELECTROFOCUSSING:

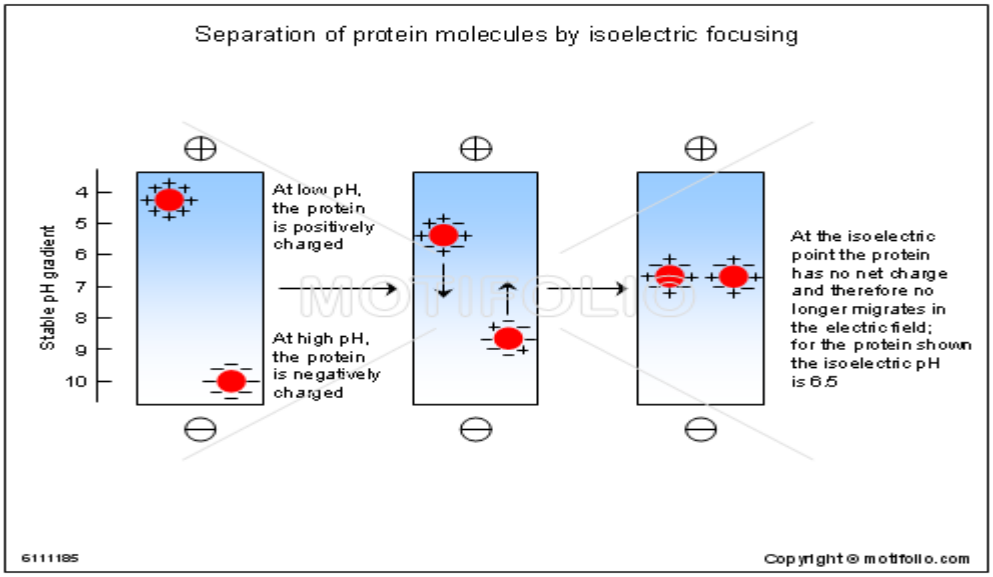
principle:

IEF, also known simply as electrofocusing, is a technique for separating charged molecules, usually proteins or peptides, on the basis of their isoelectric point (pI), i.e., the pH at which the molecule has no charge. IEF works because in an electric field molecules in a pH gradient will migrate towards their pI.The IPG strip consists of an acrylamide gel that contains wide pores to prevent a sieving effect based on protein mass, with a pH gradient. Various gradients are available, with wider gradients, such as pH 3-10 that are used for whole proteome analysis, and narrower ranges, such as pH 5-8 that are used for more specialist applications.The sample is usually combined with carrier ampholytes to assist in migration. Ampholytes are a mixture of charged molecules with a range of pIs that matches the pI range of the IPG strip. The migration of the ampholytes encourages the sample molecules to move along the pH gradient. Ampholyte mixtures of a variety of pI ranges are commercially available.

procedure:

1.The IPG strips are rehydrated (face down) in a denaturing buffer (>6 M urea) with detergent, usually including carrier ampholytes.

2.The strips are then placed in the IEF apparatus, filter paper wicks are placed over the ends of the gel.
3. Electrodes are placed on top and ready to run
4. visualize the result



UNIT IV-SPECTROSCOPY

1. BEER LAMBERT'S LAW
2. PRINCIPLE AND MECHANISM OF SPECTROPHOTOMETER

3. FLAME PHOTOMETRY
4. PRINCIPLE AND APPLICATION OF NMR AND ESR TECHNIQUES
5.OPTICAL ROTATORY DISPERSION

BEER LAMBERT'S LAW :

Beer-Lambert Law derivation helps us to define the relationship of the intensity of visible UV radiation with the exact quantity of substance present. The Derivation of Beer-Lambert Law has many applications in modern day science. Used in modern-day labs for testing of medicines, organic chemistry and to test with quantification. These are some of the fields that this law finds its uses in.

The Beer-Lambert law states that: for a given material sample path length and concentration of the sample are directly proportional to the absorbance of the light.
The Beer-Lambert law is expressed as: **A = εLc** where,

A is the amount of light absorbed for a particular wavelength by the sample
ε is the molar extinction coefficient
L is the distance covered by the light through the solution
c is the concentration of the absorbing species
Beer's law was stated by August Beer which states that concentration and absorbance are directly proportional to each other and Lambert law was stated by Johann Heinrich Lambert which states that absorbance and path length are directly proportional.
Beer-Lambert Law FormulaI **I0e−μ(x)**
Where,
I is the intensity
I0 is the initial intensity
x is the depth in meters
μ is the coefficient of absorption

Absorption of energy causes the absorption of light as well usually by electrons. Different forms of light such as visible light and ultraviolet light get absorbed in this process. Therefore, change in the intensity of light due to absorption, interference, and scattering leads to:

ΔI = I0 – IT
The following equations are necessary for us to obtain our ultimate derivative equation. Transmittance is measured as the ratio of light passing through a substance. It can be calculated as IT/I0. To calculate the of transmittance percentage we can do so by:

Percent TransmittanceAnother key metric is absorbance that is defined as the amount of light absorbed. This is usually calculated as the negative of transmittance and is given by:

Absorbance (A) The rate of decrease in the intensity of light with the thickness of the material the light is directly proportional to the intensity of the incident light. Mathematically, it can be expressed as:

As k'= Proportionality constant
Taking in the reciprocal of the equation we get,

Integrating the above equation we also get

In the above equation, b and C is the constant of integration and IT is the intensity being transmitted at the thickness. In order to solve the above equation with the constant of integration, we then get,

While solving for C in the equation will give us,

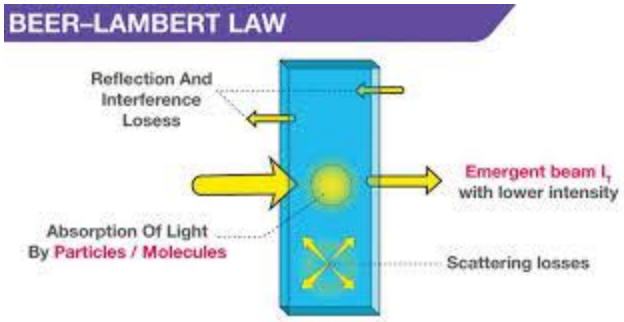
Converting to log10 we get,

Rearranging the equation

Lambert Derivation

Thus, Lambert's law was formed and it states that the monochromatic radiation changes exponentially and decreases when it passes through a medium of uniform thickness.

Beer Derivation
Thus, this concludes the derivation of Beer-Lambert law. This goes to show you that in order to derive a particular law, there are a lot of different equations that need to be found out first, to achieve the ultimate result.



PRINCIPLE AND MECHANISM OF SPECTROPHOTOMETER:

A spectrophotometer is an instrument that measures the amount of light absorbed by a sample.Spectrophotometer techniques are mostly used to measure the concentration of solutes in solution by measuring the amount of the light that is absorbed by the solution in a cuvette placed in the spectrophotometer.Scientist Arnold J. Beckman and his colleagues at the National Technologies Laboratory (NTL) invented the Beckman DU spectrophotometer in 1940.

Principle of spectrophotometer:

The spectrophotometer technique is to measure light intensity as a function of wavelength. It does this by diffracting the light beam into a spectrum of wavelengths, detecting the intensities with a charge-coupled device, and displaying the results as a graph on the detector and then on the display device.In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths.By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm.The spectrophotometer is useful for measuring the absorption spectrum of a compound, that is, the absorption of light by a solution at each wavelength.

Instrumentation:

The essential components of spectrophotometer instrumentation include:

A table and cheap radiant energy sourceMaterials that can be excited to high energy states by a high voltage electric discharge (or) by electrical heating serve as excellent radiant energy sources.A monochromator, to break the polychromatic radiation into component wavelength (or) bands of wavelengths. A monochromator resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.

1.Prisms:

A prism disperses polychromatic light from the source into its constituent wavelengths by virtue of its ability to reflect different wavelengths to a different extent

Two types of Prisms are usually employed in commercial instruments. Namely, 600 cornu quartz prism and 300 Littrow Prism.

2.Grating:

Gratings are often used in the monochromators of spectrophotometers operating ultraviolet, visible and infrared regions.

3.Transport vessels (cuvettes), to hold the sample

-Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as “CUVETTES”.

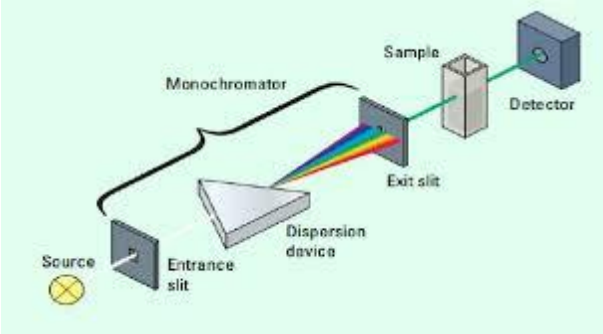
-Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes Quartz.

4.A Photosensitive detector and an associated readout system

Most detectors depend on the photoelectric effect. The current is then proportional to the light intensity and therefore a measure of it.

Radiation detectors generate electronic signals which are proportional to the transmitter light. These signals need to be translated into a form that is easy to interpret.

This is accomplished by using amplifiers, Ammeters, Potentiometers and Potentiometric recorders.



Application:

1.Detection of concentration of substances
2.Detection of impurities
3.Structure elucidation of organic compounds
4.Monitoring dissolved oxygen content in freshwater and marine ecosystems
5.Characterization of proteins
6.Detection of functional groups
7.The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations.

FLAME PHOTOMETRY

Flame photometry is one of the branches of atomic absorption spectroscopy. It is also known as flame emission spectroscopy. Currently, it has become a necessary tool in the field of analytical chemistry. Flame photometer can be used to determine the concentration of certain metal ions like sodium, potassium, lithium, calcium and cesium etc. In flame photometer spectra the metal ions are used in the form of atoms. The International Union of Pure and Applied Chemistry (IUPAC) Committee on Spectroscopic Nomenclature has named this technique as flame atomic emission spectrometry (FAES).

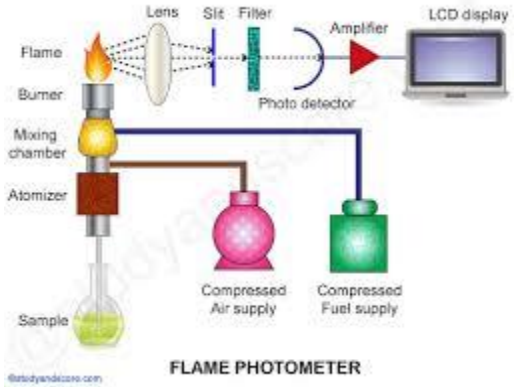
Principle:

The compounds of the alkali and alkaline earth metals (Group II) dissociate into atoms when introduced into the flame. Some of these atoms further get excited to even higher levels. But these atoms are not stable at higher levels.Hence, these atoms emit radiations when returning back to the ground state. These radiations generally lie in the visible region of the spectrum. Each of the alkali and alkaline earth metals has a specific wavelength**.**

procedure:

- Both the standard stock solution and sample solution are prepared in fresh distilled water.
- The flame of the photometer is calibrated by adjusting the air and gas. Then the flame is allowed to stabilize for about 5 min.
- Now the instrument is switched on and the lids of the filter chamber are opened to insert appropriate colour filters.
- The readings of the galvanometer are adjusted to zero by spraying distilled water into the flame.
- The sensitivity is adjusted by spraying the most concentrated standard working solution into the flame. Now the full scale deflection of the galvanometer is recorded.

- Again distilled water is sprayed into the flame to attain constant readings of galvanometer. Then the galvanometer is readjusted to zero.
- Now each of the standard working solutions is sprayed into the flame for three times and the readings of galvanometer are recorded. After each spray, the apparatus must be thoroughly washed.
- Finally sample solution is sprayed into the flame for three times and the readings of galvanometer are recorded. After each spray, the apparatus must be thoroughly washed.
- Calculate the mean of the galvanometer reading.
- Plot the graph of concentration against the galvanometer reading to find out the concentration of the element in the sample.



FLAME PHOTOMETER

Application

- Flame photometer can be applied both for quantitative and qualitative analysis of elements. The radiations emitted by the flame photometer are characteristic to particular metal. Hence with the help of Flame photometer we can detect the presence of any specific element in the given sample.
- The presence of some group II elements is critical for soil health. We can determine the presence of various alkali and alkaline earth metals in soil sample by conducting flame test and then the soil can be supplied with specific fertiliser.
- The concentrations of Na^+ and K^+ ions are very important in the human body for conducting various metabolic functions. Their concentrations can be determined by diluting and aspirating blood serum sample into the flame.
- Soft drinks, fruit juices and alcoholic beverages can also be analysed by using flame photometry to determine the concentrations of various metals and elements.

PRINCIPLE AND APPLICATION OF NMR AND ESR TECHNIQUES

NMR:

Nuclear magnetic resonance (NMR) spectroscopy is the study of molecules by recording the interaction of radiofrequency (Rf) electromagnetic radiations with the nuclei of molecules placed in a strong magnetic field.

Principle:

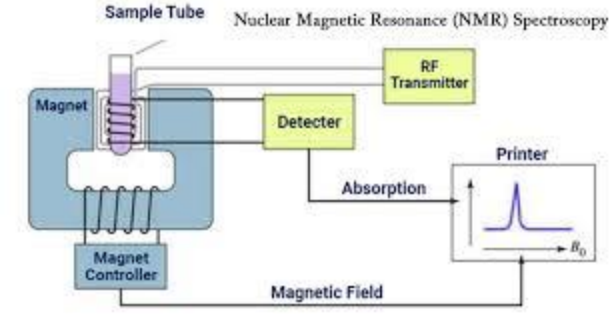
- All nuclei are electrically charged and many have spin.
- Transfer of energy is possible from base energy to higher energy levels when an external magnetic field is applied.
- The transfer of energy occurs at a wavelength that coincides with the radio frequency.
- Also, energy is emitted at the same frequency when the spin comes back to its base level.
- Therefore, by measuring the signal which matches this transfer the processing of the NMR spectrum for the concerned nucleus is yield.

Instrumentation:

- Place the sample in a magnetic field.
- Excite the nuclei sample into nuclear magnetic resonance with the help of radio waves to produce NMR signals.
- These NMR signals are detected with sensitive radio receivers.
- The resonance frequency of an atom in a molecule is changed by the intramolecular magnetic field surrounding it.
- This gives details of a molecule's individual functional groups and its electronic structure.
- Nuclear magnetic resonance spectroscopy is a conclusive method of identifying monomolecular organic compounds.
- This method provides details of the reaction state, structure, chemical environment and dynamics of a molecule.

This instrument consists of nine major parts. They are discussed below:

- Sample holder – It is a glass tube which is 8.5 cm long and 0.3 cm in diameter.
- Magnetic coils – Magnetic coil generates magnetic field whenever current flows through it
- Permanent magnet – It helps in providing a homogenous magnetic field at 60 – 100 MHZ
- Sweep generator – Modifies the strength of the magnetic field which is already applied.
- Radiofrequency transmitter – It produces a powerful but short pulse of the radio waves.
- Radiofrequency – It helps in detecting receiver radio frequencies.
- RF detector – It helps in determining unabsorbed radio frequencies.
- Recorder – It records the NMR signals which are received by the RF detector.
- Readout system – A computer that records the data.



Application:

- NMR spectroscopy is a Spectroscopy technique used by chemists and biochemists to investigate the properties of organic molecules, although it is applicable to any kind of sample that contains nuclei possessing spin.

- For example, the NMR can quantitatively analyze mixtures containing known compounds. NMR can either be used to match against spectral libraries or to infer the basic structure directly for unknown compounds.
- Once the basic structure is known, NMR can be used to determine molecular conformation in solutions as well as in studying physical properties at the molecular level such as conformational exchange, phase changes, solubility, and diffusion

ESR:

Electron Spin Resonance (ESR) also known as Electron Magnetic Resonance (EMR) or Electron Paramagnetic Resonance (EPR) is a branch of absorption spectroscopy in which radiations having frequency in the microwave region (0.04 – 25 cm) is absorbed by paramagnetic substances to induce transitions between magnetic energy levels of electrons with unpaired spins.ESR is based on the fact that atoms, ions, molecules or molecular fragments which have an odd number of electrons exhibit characteristic magnetic properties. An electron has a spin and due to spin there is magnetic moment.

Since its discovery in 1944 by E.K. Zavoisky, EPR spectroscopy has been exploited as a very sensitive and informative technique for the investigation of different kinds of paramagnetic species in solid or liquid states.

Priniciple:

The phenomenon of electron spin resonance (ESR) is based on the fact that an electron is a charged particle. It spins around its axis and this causes it to act like a tiny bar magnet. When a molecule or compound with an unpaired electron is placed in a strong magnetic field The spin of the unpaired electron can align in two different ways creating two spin states ms = ± ½.

The alignment can either be along the direction (parellel) to the magnetic field which corresponds to the lower energy state ms = – ½ Opposite (antiparallel) to the direction of the applied magnetic field ms = + ½

The two alignments have different energies and this difference in energy lifts the degeneracy of the electron spin states. The energy difference is given by:

$\Delta E = E+ - E- = hv = gm\beta B$

Where,

h = Planck's constant (6.626 x 10-34 J s-1)

v = the frequency of radiation

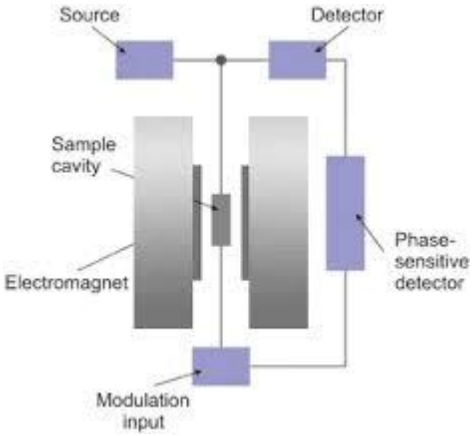
ß = Bohr magneton (9.274 x 10-24 J T-1) B = strength of the magnetic field in Tesla

g = the g-factor which is a unit less measurement of the intrinsic magnetic moment of the electron, and its value for a free electron is 2.0023.

An unpaired electron can move between the two energy levels by either absorbing or emitting a photon of energy {\displaystyle h\nu } hv such that the resonance condition, hv = Δ E, is obeyed. This leads to the fundamental equation of EPR spectroscopy

Instrumentation:

- Although the equation permits a large combination of frequency and magnetic field values, the great majority of EPR measurements are made with microwaves in the 9000–10000 MHz (9–10 GHz) region.
- EPR spectra can be generated mostly by keeping the photon frequency fixed while varying the magnetic field incident on a sample.
- A collection of paramagnetic centers, such as free radicals, is exposed to microwaves at a fixed frequency.
- By increasing an external magnetic field, the gap between the and energy states is widened until it matches the energy of the microwaves.
- At this point the unpaired electrons can move between their two spin states. Since there typically are more electrons in the lower state, due to the Maxwell–Boltzmann distribution, there is a net absorption of energy.
- It is this absorption that is monitored and converted into a spectrum.



Application

ESR spectrometry is one of the main methods to study transition metal containing metalloproteins.

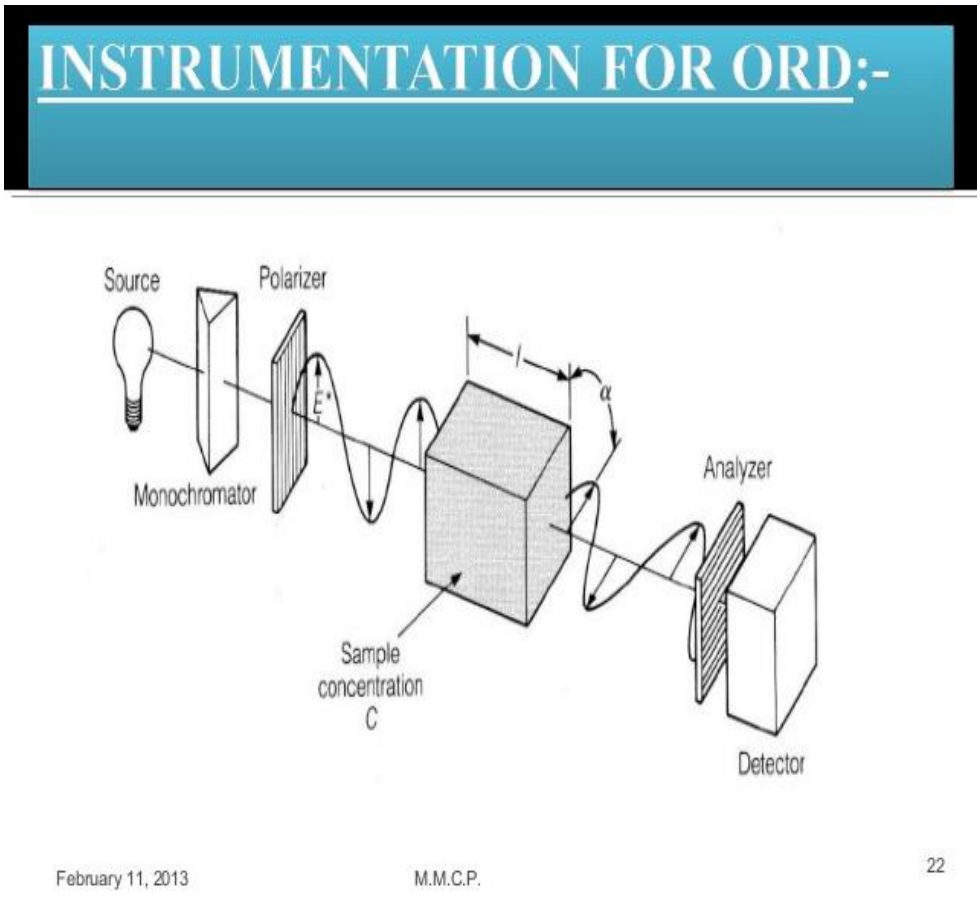
- To determine the rate of catalysis
- To know about the active site geometry
- To study denaturation and protein folding
- In studies relating to enzyme-ligand interaction
- In Biological Systems
- Study of Free Radicals

OPTICAL ROTATORY DISPERSION

As plane polarized light passes through a substance, the velocities of two circularly polarized components are reduced. If they are reduced to the same extent, the substance is optically inactive; if not, the substance is optically active. After passing through an optically active substance, there is a phase difference and there will be a rotation of the plane of polarization.

The difference is expressed in terms of different refractive indices. The optical rotation α at a given wavelength of incident light λ is directly proportional to the difference between the refractive indices of the two circularly polarized components. The optical rotation of an optically active substance is expressed as the specific rotation [α], the molar rotation [M], and the mean residual rotation [M]. For data analysis, two equations are described: the Drude equation and the Moffit-Yang equation.

If the intensity of absorption (not the refractive index n) is used as a function of the orientation of the plane of polarization, we have a phenomenon called circular dichroism. As in the case of refractive indices, for an optically active substance, the intensities of the left and right circularly polarized components are not equal. The differences Δ ε is the difference in molar absorptivities of the left- and right- circularly polarized components. Circular dichroism is usually measured as a dichroic ratio, which is the ratio of the optical densities of an absorption band in the direction of the polarized light (parallel over perpendicular) to a specified direction in the sample. It depends on the angle θ. The tangent of θ is the ratio of the minor axis b to the major axis a of the ellipse, written as [θ], called the molar ellipticity.



UNIT V- CRYSTALLOGRAPHY

1. PRINCIPLE AND INSTRUMENTATION OF X-RAY CRYSTALLOGRAPHY
2. BRAGG'S EQUATION
3. CONCEPT AND DETERMINATION OF CRYSTAL STRUCTURE

PRINCIPLE AND INSTRUMENTATION OF X-RAY CRYSTALLOGRAPHY

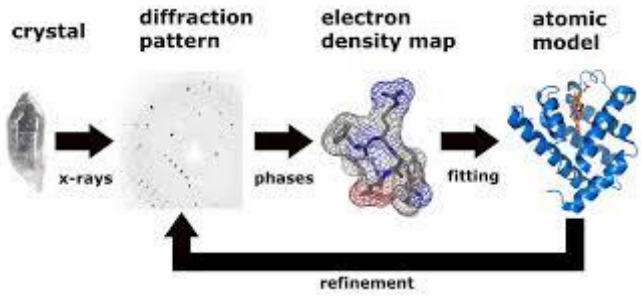
X-ray crystallography is a technique used for determining the high-resolution, three-dimensional crystal structures of atom and molecules and has been fundamental in the development of many scientific fields. In its first decades of application, it is mainly used for determining the size of atoms, the lengths and types of chemical bonds, the atomic-scale differences among various materials, as well as the crystalline integrity, grain orientation, grain size, film thickness and interface roughness of the related materials, especially minerals and alloys. This method could also reveal the structure and function of many biological molecules like vitamins, drugs, proteins and nucleic acids

Principle:

In a single-crystal X-ray diffraction measurement, a crystal is mounted on a goniometer, which is used to position the crystal at selected orientations. The crystal is illuminated with a finely focused monochromatic beam of X-rays, leading to a diffraction pattern of regularly spaced spots known as reflections. X-ray crystallography works in a manner of elastic scattering with the outgoing X-rays having the same energy and wavelength as the incoming X-rays, which get an altered direction after diffraction. A crystallographer can then produce a three-dimensional picture of the density of electrons within the crystal by measuring the angles and intensities of these diffracted beams under the assistance of the mathematical method Fourier transforms. From this electron density, the mean positions of the atoms, chemical bonds, crystallographic disorder, and some other information in the crystal can be determined. Poor resolution or even errors may occur if the crystals are too small, or not uniform enough in their internal makeup.

Instrumentation:

The technique of single crystal X-ray crystallography has three basic steps. The first and usually most difficult step is to produce an adequate crystal of the studied material. The crystal should be sufficiently large with all dimensions larger than 0.1 mm, pure in composition and regular in structure, and have no significant internal imperfections such as cracks or twinning. The crystal is subsequently placed in an intense beam of X-rays, usually of a single wavelength, to produce regular reflection pattern. The angles and intensities of diffracted X-rays are measured with each compound having a unique diffraction pattern. Previous reflections disappear and new ones appear along with the gradual rotation of the crystal, and the intensity of every spot is recorded at every orientation of the crystal. Multiple data sets may have to be collected since each set covers slightly more than half a full rotation of the crystal and typically contains tens of thousands of reflections. Ultimately, these collected data are combined computationally with complementary chemical information to obtain and refine a model from the arrangement of atoms within the crystal. The final refined model of the atomic arrangement is called a crystal structure and usually stored in a public database.



BRAGG'S EQUATION:

Bragg's Law Statement

Bragg's law is a special case of Laue diffraction which determines the angles of coherent and incoherent scattering from a crystal lattice. When X-rays are incident on a particular atom, they make an electronic cloud move just like an electromagnetic wave. The movement of these charges radiates waves again with similar frequency, slightly blurred due to different effects and this phenomenon is known as Rayleigh scattering.

The same process takes place upon scattering neutron waves via nuclei or by a coherent spin interaction with an isolated electron. These wave fields which are re-emitted interfere among each other either destructively or constructively creating a diffraction pattern on a film or detector. The basis of diffraction analysis is the resulting wave interference and this analysis is known as Bragg diffraction.

Bragg Equation

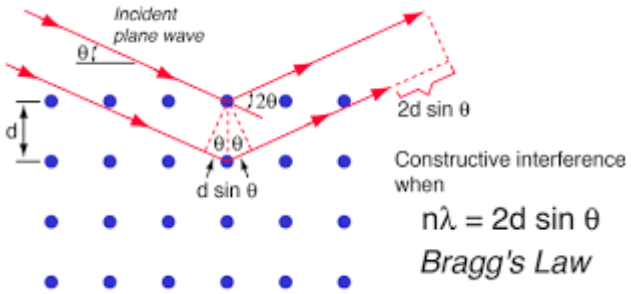
According to Bragg Equation: $\lambda = 2d \sin\Theta$

Therefore, according to the derivation of Bragg's Law:

The equation explains why the faces of crystals reflect X-ray beams at particular angles of incidence (Θ, λ).

The variable d indicates the distance between the atomic layers and the variable Lambda specifies the wavelength of the incident X-ray beam.

n as an integer.This observation illustrates X-ray wave interface which is called as X-ray diffraction (XRD) and proof for the atomic structure of crystals.



Applications of Bragg's Law

There numerous Bragg's law applications in the field of science. Some common applications are given in the points below.

- In the case of XRF (X-ray fluorescence spectroscopy) or WDS (wavelength dispersive spectrometry), crystals of known d-spacings are used as analyzing crystals in the spectrometer.

- In XRD (X-ray diffraction) the interplanar spacing or d-spacing of a crystal is used for characterization and identification purposes.

CONCEPT AND DETERMINATION OF CRYSTAL STRUCTURE

A crystal structure is made of atoms. A crystal lattice is made of points. A crystal system is a set of axes. In other words, the structure is an ordered array of atoms, ions or molecules.
Crystal structure:
Crystal Structure is obtained by attaching atoms, groups of atoms or molecules. This structure occurs from the intrinsic nature of the constituent particles to produce symmetric patterns. A small group of a repeating pattern of the atomic structure is known as the unit cell of the structure. A unit cell is the building block of the crystal structure and it also explains in detail the entire crystal structure and symmetry with the atom positions along with its principal axes. The length, edges of principal axes and the angle between the unit cells are called lattice constants or lattice parameters.

Crystal Systems

A Crystal System refers to one of the many classes of crystals, space groups, and lattices. In crystallography terms, lattice system and crystal, the system are associated with each other with a slight difference. Based on their point groups crystals and space groups are divided into seven crystal systems.

The Seven Crystal Systems is an approach for classification depending upon their lattice and atomic structure. The atomic lattice is a series of atoms that are organized in a symmetrical pattern. With the help of the lattice, it is possible to determine the appearance and physical properties of the stone. It is possible to identify so as to which crystal system they belong to. In a Cubic System crystals are said to represent the element earth.