BPT 111/NSB 111/MSB 100
BASIC OF BIOMEDICAL SCIENCE

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Outline

- **Biomedical Methods:**
  - Sub-cellular fractionation
  - Separation procedures
    - Salt fractionation
    - Chromatography
    - Gel filtration
    - Electrophoresis

Subcellular Fractionation

- **Subcellular fractionation** refers to disintegrating intact cells into their **integral parts, the cellular organelles.**
- **Subcellular fractionation** is the process of isolating nuclear, cytosolic and mitochondrial fractions of high purity from tissues.
- **Subcellular fractionation** allows for different cellular proteins and organelles to be studied and characterized.
- Subcellular fractionation can be used for a wide variety of **cell types for sample preparation before omics analysis.**

- **For cell biologists**, the key objective is to isolate each cellular organelle to a high degree of purity even if the quantify is small.
- This is necessary for undertaking electron microscopic studies to reveal ultrastructure of organelles.
- **On the other hand**, **biochemists** are more **interested in macromolecular** composition (such as proteins, lipids and carbohydrates) and hallmark enzymes of each subcellular fraction and therefore they seek pure fractions in sufficient quantity.
- The **technology of the subcellular fractionation** was pioneered by the *Christian De Duve*, *Camillo Golgi* and *George Palade* with significant contributions made by *Albert Claude*.

- Based on requirements one has to choose an appropriate method and adapt it to the specific requirements of the experiment.
- **Broadly,** there are two methods to **perform subcellular fractionation using centrifugation**.

They are:

1. Differential centrifugation
2. Equilibrium density centrifugation

1. Differential Centrifugation

- **Subcellular compartments/organelles** vary **in physical parameters such as mass, size and density.**
- Differential centrifugation exploits the sedimentation behavior of particles varying in their densities.
- The particles are separated based on their differential sedimentation velocities in a medium of considerably **small density (ρ2)** and **low viscosity**.
- The **density of the medium** is critical because a *high-density medium* will prevent sedimentation of cellular organelles and the organelles will float as predicted by the equation given below.

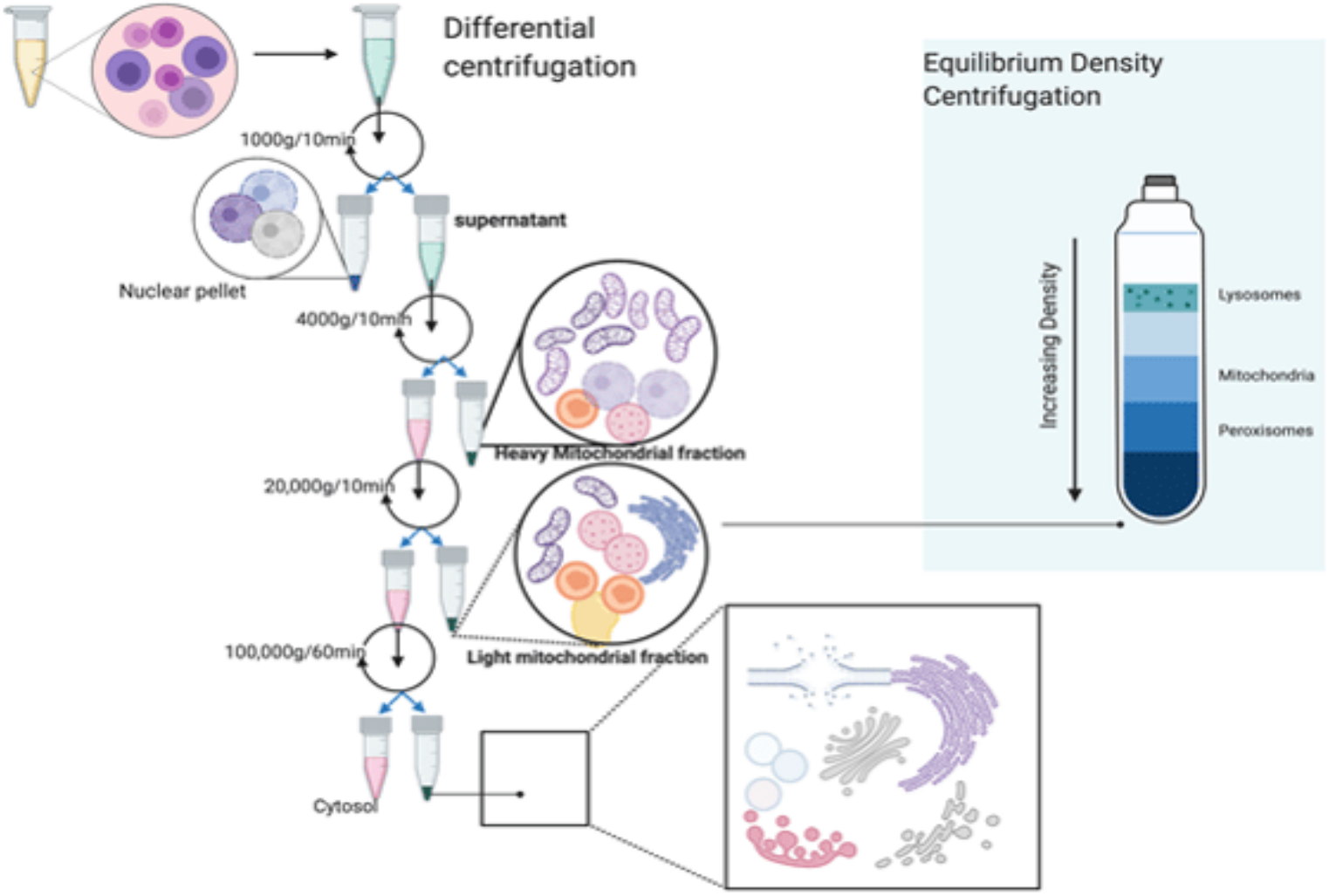
$$N = \frac{d^2(\rho 1 - \rho 2)}{18\mu} g$$

- ν =Sedimentation velocity
- d=diameter of distance
- ρ1=density of particle
- ρ2=density of medium
- g=centrifugal force
- μ=Viscosity of medium

- Often sucrose (0.25M) is used as a medium, this is because sucrose has the '**right' density** to bring **about sedimentation of cellular organelles**.
- Moreover, **sucrose at 0.25 M** doesn't have a high osmolarity and viscosity.
- The **consideration of osmolarity** is important because hyperosmotic concentrations of the medium can lead to osmotic-shrinkage of organelles.
- The **presence of ions and detergents** can also change the sedimentation behaviour of subcellular organelles.
- The **differential centrifugation** leads to separation of cellular lysate into a pellet and a supernatant fraction.
- At **each step,** the pellet fractions are made up of heavier fractions that settle and make a pellet at a given 'g-force' and time interval whereas supernatant is made of parts that could not be pelleted at that g-force and time values.

- **Another important factor** is the **volume** of the sample to be fractionated.
- The resolution achieved in differential centrifugation is **volume dependent**.
- Particles at the top of the tube have to travel maximum distance to make a pellet at the bottom of the tube and are exposed to lower values of g force.
- However, the **heavier particles** near the bottom of the tube are exposed to **higher values of 'g-force'** and have the **shortest distance to travel.**
- This **leads to low yield** and also contamination for lighter or smaller particles.

The scheme of *differential centrifugation* to obtain different subcellular organelles



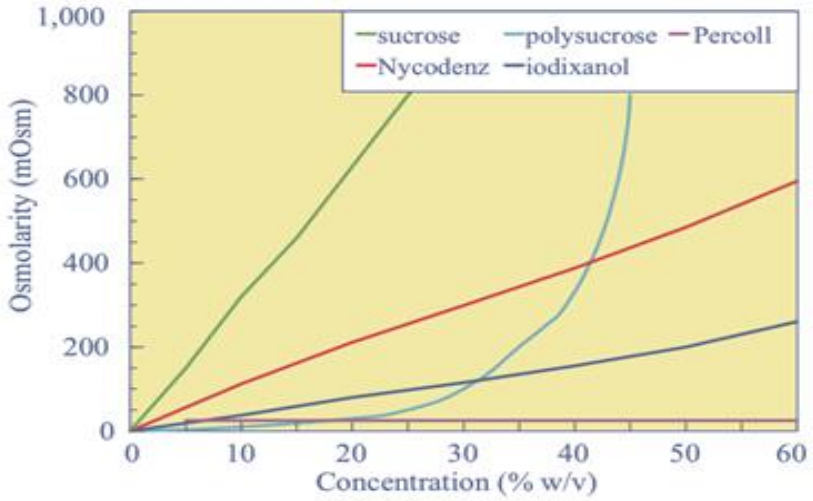
2. Equilibrium Density Centrifugation

- **Equilibrium density centrifugation** also known as **gradient centrifugation** is often employed to separate cellular components that have closely related densities.
- **A non-ionic medium** of low density is chosen which also has a **low osmolarity** and **viscosity** for similar reasons explained earlier.
- **Usually**, **Sucrose** and **Glycerol** have been medium of choice but lately synthetic mediums such as **Nycodenz, Iodixanol** and **Percol** have been also used with great success and better resolving ability.

- **Sucrose and glycerol** are limited by the fact that if their concentrations are increased (to adjust the medium density, **ρ2 see equation above**) it leads to a quick jump in osmolarity which is not suitable for obtaining intact organelles.
- Even at high concentrations **Percol, Nycodenz** and **Iodixanol** don't have a sharp change **in osmolarity**.

  - ν =Sedimentation velocity
  - d=diameter of distance
  - ρ1=density of particle
  - ρ2=density of medium
  - g=centrifugal force
  - μ=Viscosity of medium

$$N = \frac{d^2(\rho 1 - \rho 2)}{18\mu} g$$



- For **equilibrium density centrifugation**, the samples are spun at high '**g-force'** values (40,000-50,000) for almost 60 minutes, this generate zones of varying densities inside the tube and the organelles or subcellular fractions are separated on **basis of their densities in the gradient medium**, each organelle in a **zone equal to its density**.

Subcellular Fractionation Protocol

(1) The first step in cell fractionation is tissue disruption and cell lysis.

- This step allows you **to dis-aggregate and break open the cells with minimal damage to the cellular fraction of interest**.
- **Three basic methods can be used for tissue and cell lysis:**
    1) ***Homogenization,***
    2) ***Sonication,***
    3) ***Osmotic lysis.***
- The choice of the method depends on cell type and the subcellular fraction of interest.
- The most commonly used method for **animal and plant tissues** is **homogenization.**
- This process involves the use of **a mechanical homogenizer acting like a pestle and mortor/blende**r to break the tissue apart and lyse cells.
- To disrupt and lyse prokaryotic cells **sonication is used**. This process uses ultrasound to disrupt cellular membranes.
- **Osmotic lysis** is used when the cells of interest are **vulnerable to osmotic stress, such as red blood cells.**

(2) Centrifugation

- **Following** the initial disruption and lysis of cells, centrifugation is carried out.
- Centrifugation ensures the cellular components settle at the bottom of the tube.
- During centrifugation, the lysate is rotated at a certain speed, known as RPM – rotations per minute.
- This rotation imposes a force on the particles perpendicular to the axis of rotation and is known as **RCF – relative centrifugal force**, expressed as a multiple of the force of Earth's gravitational force (x g).

- **The part of the centrifuge that holds the centrifugation tubes is called the centrifuge rotor.**
- **Centrifuges** can have a number of different rotors.
- **There are three types of centrifuge rotors:** *fixed angle rotors, swinging bucket rotors, and vertical rotors*.
- **Fixed-angle and swinging-bucket rotors** are the most commonly used.
- As the name suggests in a **fixed-angle rotor** the centrifuge tubes are spun at a fixed angle, which is ideal for pelleting cells and subcellular components.
- **Swinging-bucket rotors** enables the tubes to swingout perpendicular to the axis of rotation as the rotor rotates and is commonly used in density-gradient centrifugation schemes.

(3) Differential Centrifugation

- **Differential centrifugation** is the sequential centrifugation of a cell lysate at progressively increasing centrifugation force, isolating cellular components of decreasing size and density.
- The **separation of the cellular components** is based on their sedimentation rate through the centrifugation medium and therefore is dependent on the size and shape of the cellular components.
- **Differential centrifugation** results in the production of a pellet following **each centrifugation step**.
- The **pellet** contains a mixture of cellular components of roughly the **same size and density**.
- Following each centrifugation step you can remove the supernatant and centrifuge again, allowing you to pellet other cellular components of a lesser size and density.

Cell Separation

- **Cell separation**, also commonly referred to as cell **isolation or cell sorting**, is a process to isolate one or more specific cell populations from a heterogeneous mixture of cells.
- There are a number of cell separation methods available, each with its own pros and cons.
- **Cell separation** has been performed using **various techniques** that take advantage of the different **physicochemical characteristics** of cells, such as cell size, cell density, electrostatic and hydrophobic qualities, the expression of cell-specific surface markers, and adherence to tissue culture plastic.

Cell separation Approaches

- **Cell separation methods typically take one of the three following approaches:**
- ***Positive selection*** is when the cell type of interest is targeted by the removal mechanism and retained for downstream applications.
- This approach involves targeting the desired cell population with an **affinity molecule specific to a surface marker of the cell,** leaving behind unwanted cells in the sample.
- ***Negative selection*** is when unwanted cell types are labeled with affinity molecules such as antibodies or proteins that target specific cell markers or populations and then removed, leaving one cell type untouched.
- The untouched cell sample is then collected for downstream applications.

- ***Cell depletion*** is the third and simplest approach in which a single cell type is removed from a biological sample.
- This strategy is typically used to remove large quantities of a single common contaminant, such as red blood cells (RBCs) or dead cells.
- If a sample is heavily saturated with residual RBCs after the cell separation process, RBC depletion kits can be used to further purify the sample.

Cell Separation Methods and Technologies

- ***There are several different technologies used to isolate cell populations.***
- **These technologies** are usually based on one or more properties unique to the targeted cell type—such as ***size, density, electric charge, shape, or protein expression***—to label those cells for removal.

Buoyancy Activated Cell Sorting (BACS)

- *Buoyancy activated cell sorting* (*BACS*) is a **negative selection process** that involves sorting cells with buoyant microbubbles.
- The **microbubbles** are coated with affinity molecules that attach to target cells and lift them to the surface of the solution.
- Once the cells are at the top, they can be removed from the sample through vacuum aspiration, leaving behind the enriched sample at the bottom.
- Microbubbles can also be used for the depletion of RBCs.

- **Microbubbles** allow researchers to increase the scalability of their experiments and expand their diagnostics to rare cell populations.
- This innovative method can be custom-tailored with a variety of bio-analytes to target specific cell groups while maintaining a **high purity, yield, recovery, and viability.**
- **BACS** is fast, easy, and inexpensive in comparison to the other methods, and preserves cell health and physiology for downstream applications.
- It can be used in conjunction with other techniques to further purify a sample or by itself as a standalone isolation method.

Magnetic Based Cell Sorting (MACS)

- *Magnetic based cell sorting* is a form of immunomagnetic separation that involves binding magnetic particles to target cells through an affinity molecule/surface marker interaction.
- Then, the sample is subjected to a magnetic field that suspends cells in a liquid solution, letting other cells flow through freely.
- Depending on the cells being targeted, MACS can be a **positive or negative selection method**.

Protocol for Cell Isolation Using Magnetic Beads

- There are **two variations of** magnetic cell sorting, column-based and column-free cell sorting.
- **Both strategies** use magnetic beads coated with specific molecules that bind to surface markers on target cells in the sample.
- **In column-based magnetic sorting**, the sample is passed between two columns that create a magnetic field.
- When turned on, this magnetic field catches the labeled cells which are bound to the magnetic beads.
- **When the field is turned off, the labeled cells are released for easy collection or removal**.
- **In column-free magnetic sorting**, the sample is placed inside a tube and subjected to a magnetic field that pulls the beads to the side of the container.
- The unwanted cells are then poured off while target cells are suspended. ***Then the magnetic field is turned off leaving behind only the desired cells in the tube.***

Fluorescence Activated Cell Sorting (FACS)

- *Fluorescence activated cell sorting* (*FACS*), or *FACS analysis* is a specialized type of flow cytometry that involves labeling targeted cells with fluorescent markers and running the sample through a flow cytometer device.
- Then, cells are identified and sorted one by one based on the color of their markers into isolated cell populations.

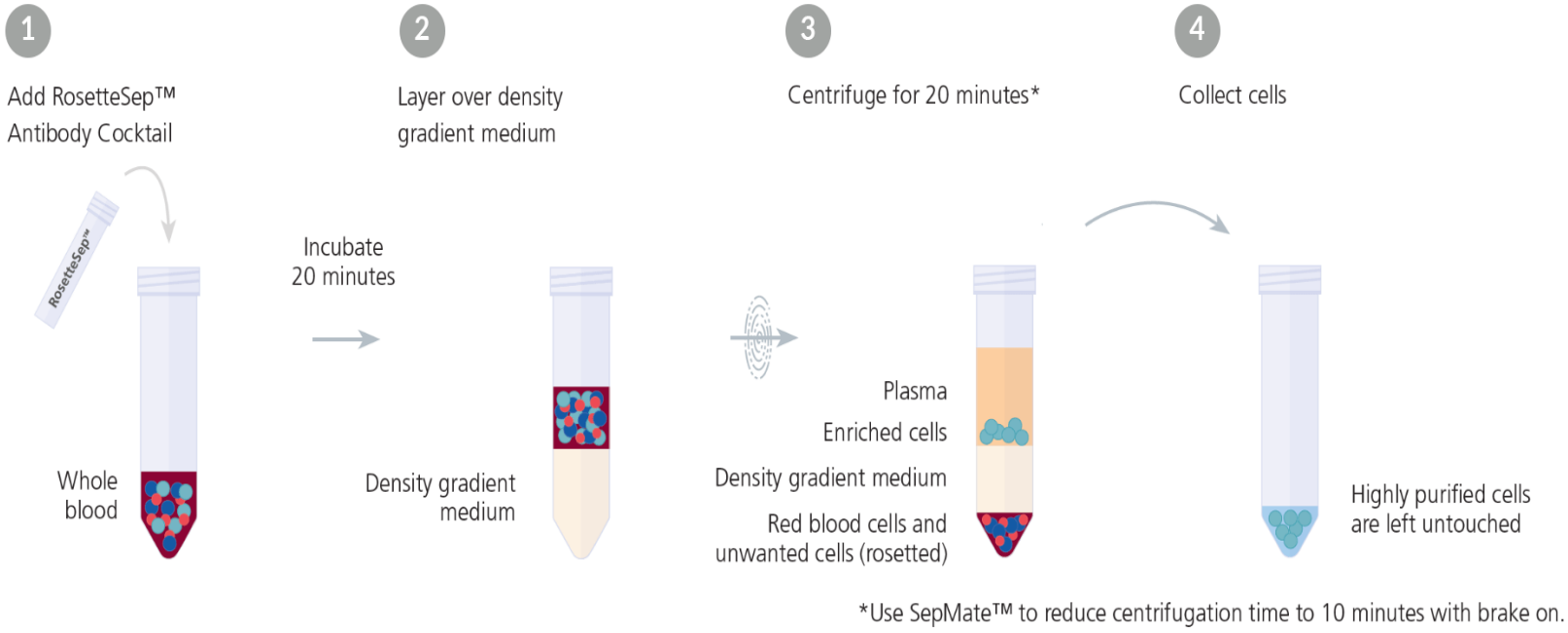
Protocol for Fluorescence Activated Cell Sorting (FACS)

- The flow cytometer does a majority of the work in FACS.
- The *flow cytometer* is a complex machine made up of ***three systems***: ***(1) Fluidics*** that transport and organize cells; ***(2) optics***—lenses, lasers, filters, and other components—that generate a photocurrent to detect cell characteristics; and ***(3) electronics*** that digitize data for processing.
- Fluorescent dye specific to certain cell types can also be purchased to assist in sorting certain populations.

Immunodensity Cell Separation

- Immunodensity cell separation, also referred to as **erythrocyte rosetting**, is a **negative selection** method that uses a combination of antibody-based labeling and density gradient centrifugation.
- With this method, antibodies are added to a whole blood sample, labeling the unwanted cells and cross-linking them to red blood cells.
- This results in the f**ormation of complexes called immunorosettes** that are much denser than the mononuclear cells being isolated.
- During centrifugation, the unwanted cells pellet with the red blood cells, leaving the target cells in a layer above the density medium.

- **Immunodensity cell** separation doesn't require any specialized equipment **beyond a centrifuge**, can be easily incorporated into established density gradient centrifugation protocols, and can be used to isolate specific cell subsets directly from whole blood.
- However, the technique is **limited to negative selection**, relies on the operator's blood sample layering technique, and requires a high concentration of red blood cells in the starting sample.



*Use SepMate™ to reduce centrifugation time to 10 minutes with brake on.

Sedimentation

- Sedimentation works on the basis that gravity will cause larger and denser components to sediment faster than materials that are smaller and less dense.
- The largest and densest components in a sample can be pelleted through an initial low-force centrifugation due to their high rate of sedimentation.
- The **supernatant can then be spun again**.
- Through successive centrifugations, components with an increasingly lower rate of sedimentation can be isolated.
- **Leukocytes are commonly separated from erythrocytes through dextran sedimentation.**
- Sedimentation is inexpensive but generally results in lower purity than other methods.

Adhesion

- The unique adhesion profiles of different cell types can be used to separate target cells from heterogeneous populations.
- By choosing suitable growth factors and cell culture plates to selectively favor or inhibit adhesion, adherent cells can be separated from cells in suspension.
- **Macrophages** are inherently adherent and often isolated from peripheral blood and bone marrow by adhesion.
- **Mononuclear cells** can be cultured with serum and a differentiation cocktail, promoting the formation of an adherent monolayer of macrophages.
- After removing the supernatant containing unwanted cells, the macrophages can be isolated.
- Alternatively, cells that naturally grow in suspension or have lost anchorage dependency can be isolated by culturing the heterogeneous cell population in plates designed for ultra-low attachment.
- Without a surface to adhere to, adherent cells will fail to survive and the target cells will remain in suspension.

Salt Fractionation

- **Salting out** (***also known as salt-induced precipitation, salt fractionation, anti-solvent crystallization, precipitation crystallization, or drowning out***) is a purification technique that **utilizes** the **reduced solubility of certain molecules (Proteins) in a solution of very high ionic strength.**

Fractionation of proteins

- The first step in purifying intracellular proteins is preparing a crude extract.
- The extract will contain a complex mixture of proteins from cell cytoplasm, and additional components such as macromolecules, cofactors and nutrients.
- The debris are removed by centrifugation and supernatant (crude protein extract) recovered.
- Crude preparations of extracellular proteins may be obtained by removing cells by centrifugation.

- The extract can be subjected to treatments that separate proteins into different fractions based on several properties such as size, charge etc **and this process is known as fractionation.**
- Fractionation helps in the removal of any other contaminating material and also in the enrichment of the desired protein fraction.
- **Early fractionation steps utilize the difference in protein solubility.**
- The **solubility of a protein** depends on the concentration of dissolved salts, polarity of the solvent, pH, temperature.
- Some or all of these variables can be manipulated to precipitate specific proteins from the solutions while others remain soluble.

Fractionation of proteins by precipitation with salt

- A common step to purify a protein from a crude extract is by precipitation in a **solution with high osmotic strength (i.e. salt solutions).**
- Protein precipitation is usually done using **ammonium sulfate as the salt.**
- Different proteins precipitate in different **ammonium sulphate concentrations**, thus separating the overall protein into several fractions.
- High molecular weight proteins precipitate in **lower ammonium sulfate concentrations.**
- Salt fractionation of protein does not usually lead to a highly purified protein.
- As mentioned above, it helps in elimination of unwanted proteins and in further concentrating the sample.
- **Salts in the solution are then required to be removed by dialysis or gel exclusion chromatography**.

Salting out with Ammonium Sulphate

- **Salting out** is an effective means for purification which explores the **reduced solubility of proteins** present in a solution of very high ionic strength causing certain proteins to precipitate.
- **Figure** below shows **salting in** and **salting out processes.**

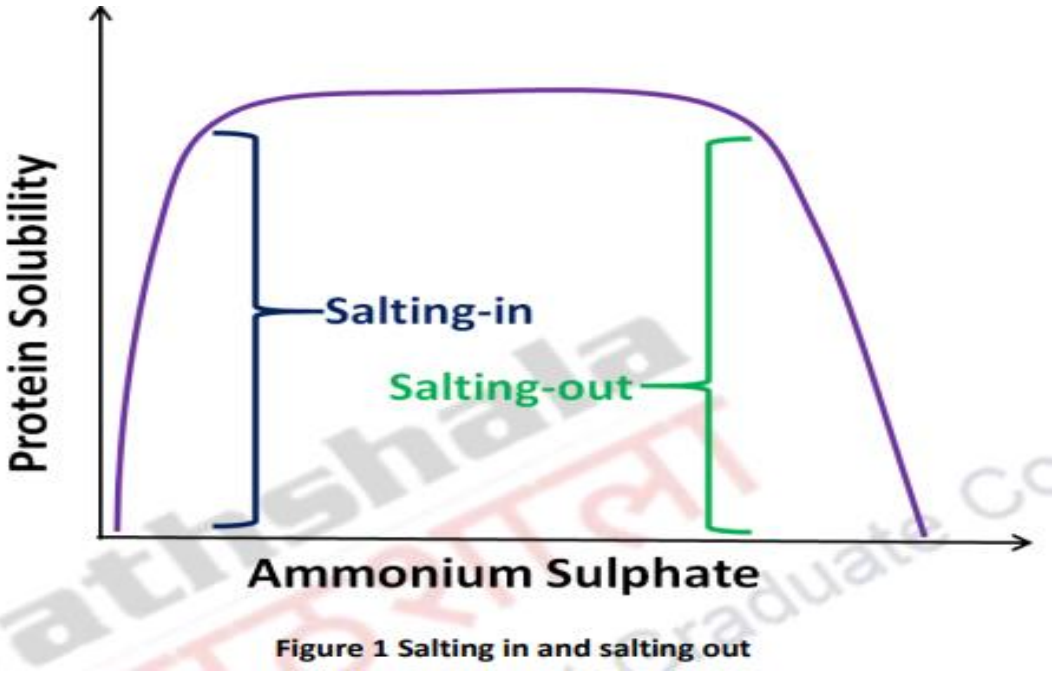
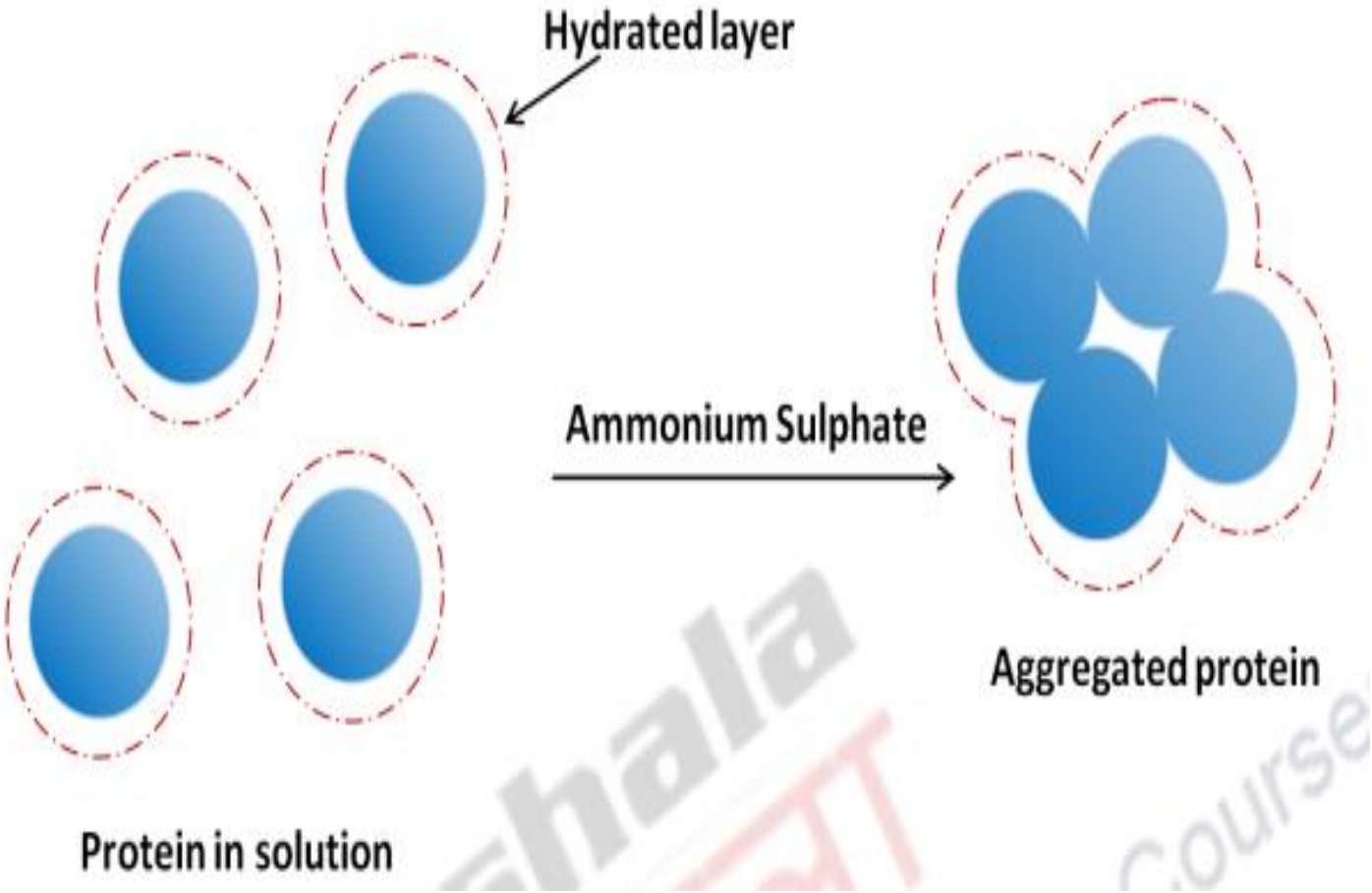


Figure 1 Salting in and salting out

- **Each protein molecule** in solution is uniformly layered by an essential "**layer of hydration**" by water molecules which enable the molecule to repel each other and stay in solution.
- **As more and more salt** is added to the protein, the solubility of the salt added gradually tends to become higher than protein.
- **Owing** to the increased affinity of salt molecules for water over protein molecules**, the hydration shell around the protein molecule is thus gradually displaced by the increasing ionic concentration in the solvent.**

- In other words, the protein molecules are thus "**stripped off**" their hydration layer, allowing hydrophobic interaction between proteins (interactions between hydrophobic patches on protein surface) to predominate which leads to aggregation of the protein molecules and precipitation.
- It is important to note that **salting out** occurs at high salt concentration.
- Use of salt at very high concentrations also cause a further increase in surface tension, inducing the protein to aggregate, resulting in salt-precipitation.

Effects of salt on protein precipitation



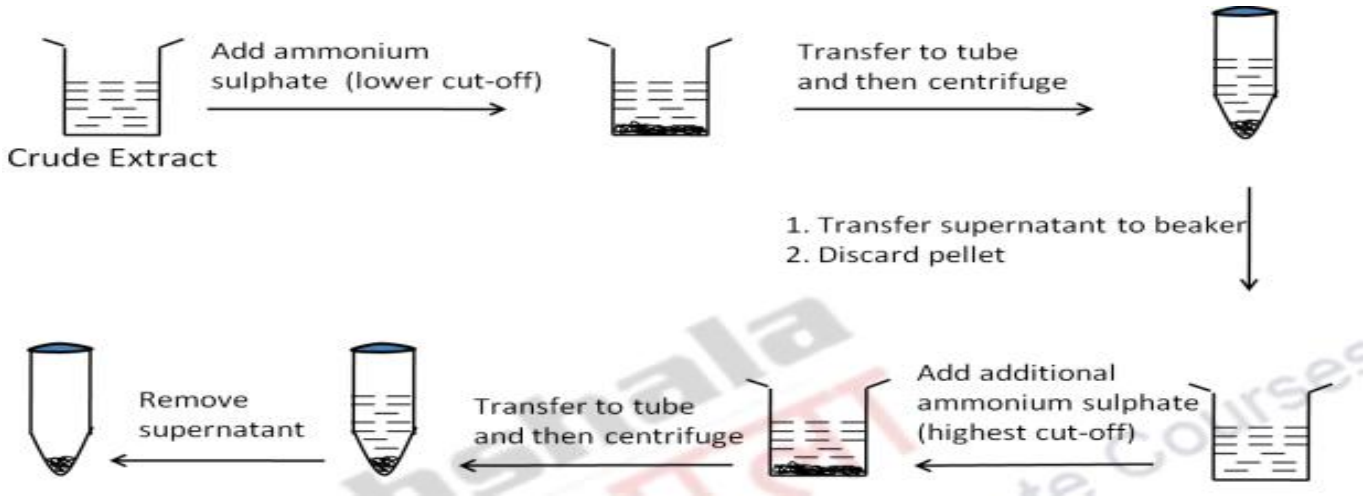
How does fractionation take place

- Fractionation of protein mixtures by the stepwise increase in the ionic strength of the salt being used for protein precipitation can prove to be an effective strategy of obtaining partially purified enzymes.
- **For example**, the salt concentration in a solution containing several proteins can be adjusted to just below the precipitation point of the protein (to be purified).
- This eliminates many unwanted proteins.
- **Precipitated proteins** may be removed by filtration or centrifugation, following which the salt concentration is increased further in the remaining supernatant solution to precipitate desired protein.

- Once the **desired protein is precipitated** along with many other proteins, which precipitated in presence of similar ionic strength, the precipitate is removed to obtain a fairly more concentrated fraction that now contains the desired protein.
- Salt concentration may be further increased in the supernatant again to precipitate remaining proteins.
- This is how salt fractionation of proteins takes place.
- **At very high salt concentrations**, i.e., when the protein solution is saturated with salt, all proteins may precipitate all together completely.

Steps in salt fractionation

- The **stepwise precipitations** of proteins by addition of increasing amounts of **ammonium sulphate** to the crude extract, with intermittent centrifugation steps is known as **ammonium sulphate cuts.**
- Amounts of solid ammonium sulphate which is to be added to a given volume of protein extract to achieve desired percentage saturation.
- Ammonium sulphate in solid powdered form, should be added slowly, in small batches, with continuous stirring, at low temperature, to allow for a uniform increase in concentration and ensure rapid equilibration.
- After all the **ammonium sulphate** that has been weighed out has been added and solubilized, the salt containing protein mixture is allowed to stand for sometime to allow for precipitated proteins to settle down which can be removed by centrifugation.



Chromatography

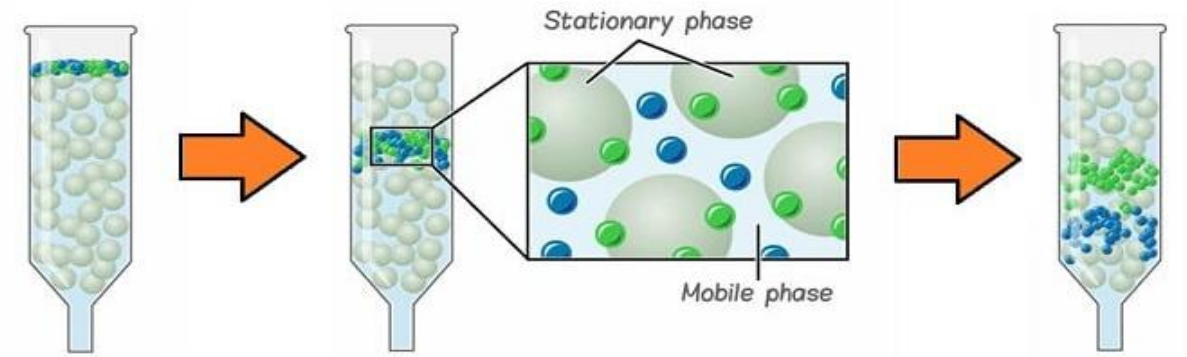
- Chromatography is the technique for the **separation, purification, and testing of compounds.**
- The term ***"chromatography"*** is derived from Greek, chroma meaning, ***"colour,"*** and graphein meaning "***to write."***
- **Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.**
- 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 a very useful technique as it allows the separation of components of a mixture on the basis **of their nature, structure, size, and other properties.**
- **Chromatography**, in general, is based on the principle that components of a mixture are separated when the mixture added to a mobile phase is moved through a stationary phase (which mostly is a solid surface), resulting in some components of the mixture being attached to the stationary phase.
- **At the same time**, the rest is passed along with the mobile phase.
- **Thus, there are two essential components of all chromatography techniques.**

Essential components of all chromatography techniques

Stationary phase

- **The stationary phase** in chromatography is the phase that is either a solid or liquid particle attached to a glass or a metal surface on which the components of the mixture to be separated is **absorbed selectively**.
- The **term stationary refers** to the fact that this phase remains stationary while the other phase moves.
- Most substances used as stationary phases are porous, thus allowing the attachment of components during chromatography.
- The **stationary phase** to be selected for a chromatographic process depends on the nature of the components to be separated and the type of chromatography.
- **Depending** on the type of chromatography gel beads, thin uniform paper, silica, glass, some gases, or even liquid components are used as a stationary phase.



Mobile Phase

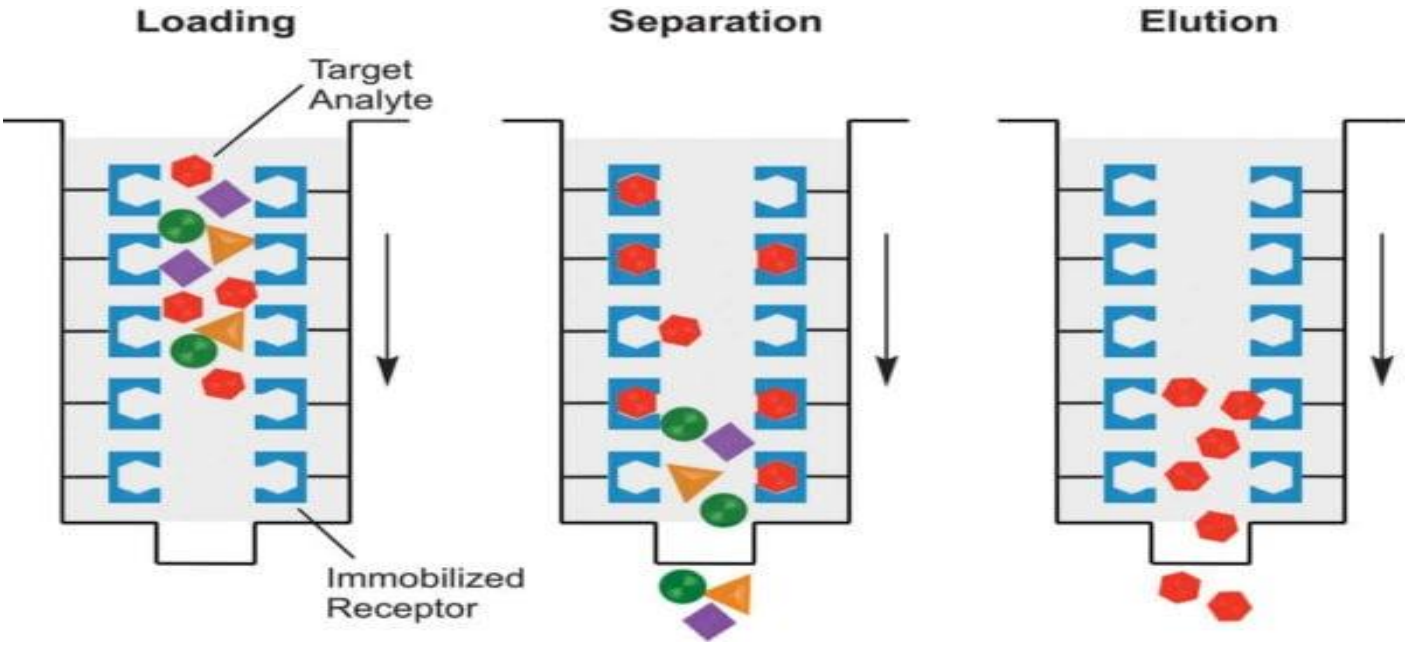
- The **mobile phase** in chromatography is the phase that is either liquid or gas that is passed through a chromatographic system where the components of the mixture are separated at different raters by adsorbing them to the stationary phase.
- The **mobile phase** is the solvent that carries the mixture as it moves down the stationary phase.
- The **term mobile** indicates that the phase is moving down the chromatographic system, whereas the other phase remains stationary.
- **Substances used** as mobile phases are selected for a chromatographic process depending on the nature of the components to be separated and the **type of chromatography**.
- **Alcohol, water, acetic acid, acetone,** or **some gases** are the commonly used mobile phase in **different chromatographic techniques.**

Types of Chromatography

- **Affinity chromatography**
- Affinity chromatography is a separation technique where the components of a mixture are separated **based on their affinity towards the stationary phase of the system.**
- **Principle of Affinity chromatography**
- This chromatography technique is based on the principle that components of a mixture are separated when the element having an affinity towards the stationary phase binds to the stationary phase.
- **In contrast,** other components are eluted with the mobile phase.
- The substrate/ ligand is bound to the stationary phase so that the reactive sites for the binding of components are exposed.
- Now, the mixture is passed through the mobile phase where the components with binding sites for the substrate bind to the substrate on the stationary phase while the rest of the components are eluted out with the mobile phase.
- The components attached to the stationary phase are then eluted by changing the pH, ionic strength, or other conditions.

Steps of Affinity Chromatography

- The **column** is prepared by loading it with solid support **like agarose or cellulose**, onto which the substrate/ ligand with the spacer arm, is attached.
- The mobile phase containing the mixture is poured into the column at a constant rate.
- Once the process is complete, the **ligand-molecule comp**lex is eluted from the stationary phase by changing the conditions that favor the **separation of ligand and components of the mixture.**



2. Anion exchange chromatography

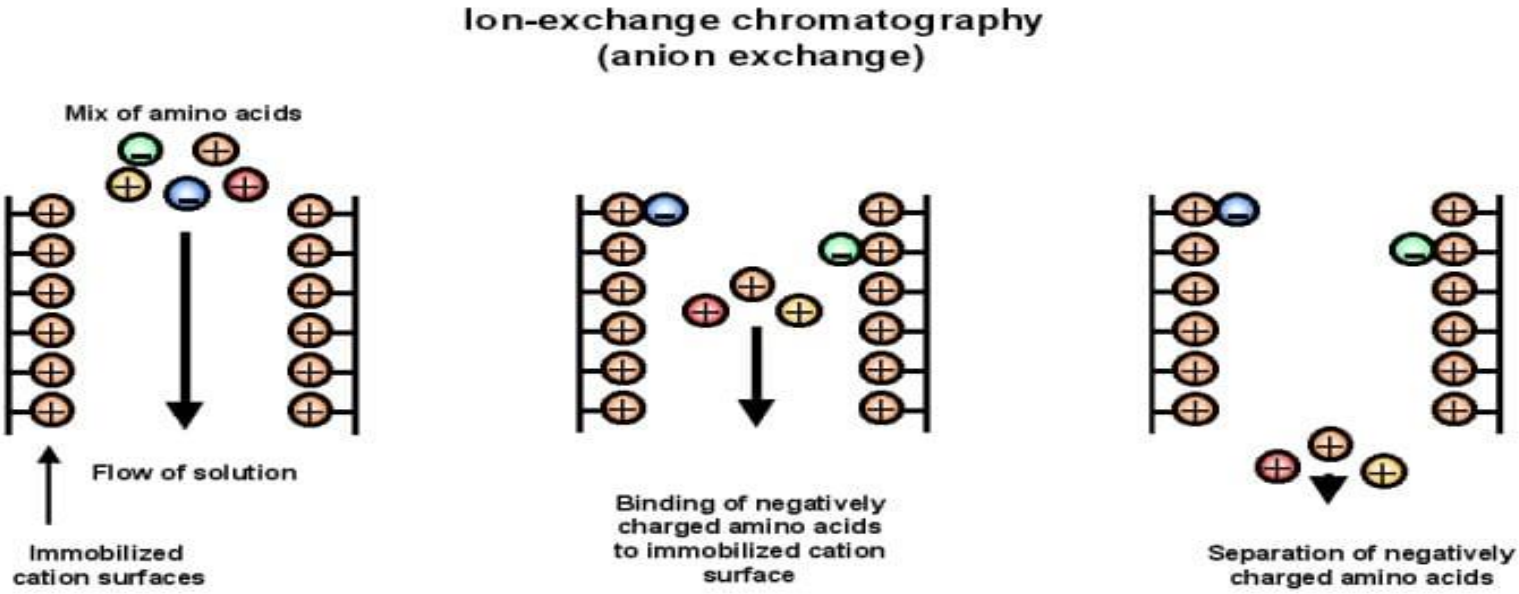
- **Anion exchange** chromatography is the **separation technique for negatively charged molecule**s by their interaction with the **positively charged stationary phase** in the form of ion-exchange resin.

Principle of Anion exchange chromatography

- This technique is based on the principle of attraction of positively charged resin and the negatively charged analyte.
- Here the exchange of positively charged ions takes place to remove the negatively charged molecules.
- The **stationary pha**se is first coated with positive charges where the components of the mixture with negative charges will bind.
- **An anion exchange resin** with a higher affinity to the negatively charged components then binds the components, displacing the positively charged resin.
- The **anion exchange resin-component complex** then is removed by using different buffers.

Steps of Anion exchange chromatography

- **A column** packed with **positively charged resin** is taken as the stationary phase.
- The mixture with the charged particles is then passed down the column where the negatively charged molecules bind to the positively charged resins.
- The **anion exchange resin** is then passed through the column where the negatively charged molecules now bind to the anion exchange resin displacing the positively charged resin.
- Now an **appropriate buffer is applie**d to the column to separate the complex of anion exchange resins and the charged molecules.



Cation exchange chromatography

- Cation exchange chromatography is the separation technique for positively charged molecules by their interaction with negatively charged stationary phase in the form of ion-exchange resin.

Principle of Cation exchange chromatography

- This technique is based on the principle of attraction of negatively charged resin and the positively charged analyte.
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- The stationary phase is first coated with negative charges where the components of the mixture with positive charges will bind.
- A cation exchange resin with a higher affinity to the positively charged components then binds the components, displacing the negatively charged resin.
- The **cation exchange resin-component complex** then is removed by using different buffers.

Steps of Cation exchange chromatography

- A column packed with **negatively charged resin is taken as the stationary phase.**
- The mixture with the charged particles is then passed down the column where the positively charged molecules bind to the negatively charged resins.
- The **cation exchange resin** is then passed through the column where the positively charged molecules now bind to the cation exchange resin displacing the negatively charged resin.
- Now an appropriate buffer is applied to the column to separate the complex of cation exchange resins and the charged molecules.

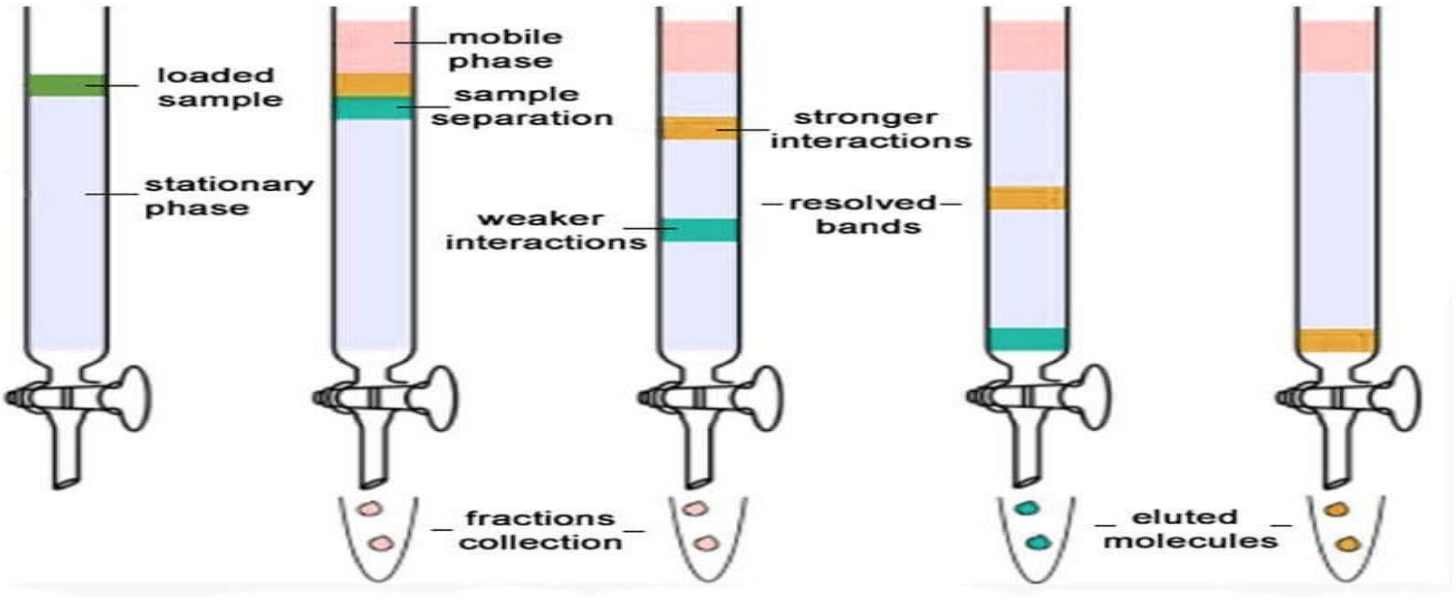
Column Chromatography

- **Column chromatography** is the separation technique where the components in a mixture are separated on the basis of their differential adsorption with the stationary phase, resulting in them moving at different speeds when passed through a column.
- It is a solid-liquid chromatography technique in which the stationary phase is a solid & mobile phase is a liquid or gas.

Principle of Column chromatography

- This technique is based on the principle of differential adsorption where different molecules in a mixture have different affinities with the absorbent present on the stationary phase.

- The molecules having higher affinity remain adsorbed for a longer time decreasing their speed of movement through the column.
- However, the molecules with lower affinity move with a faster movement, thus allowing the molecules to be separated in different fractions.
- Here, the stationary phase in the column chromatography also termed the absorbent, is a solid (mostly silica) and the mobile phase is a liquid that allows the molecules to move through the column smoothly.



Steps of Column chromatography

- The column is prepared by taking a **glass tube** that is dried and coated with a thin, uniform layer of stationary phase (cellulose, silica).
- Then the sample is prepared by adding the mixture to the mobile phase.
- The sample is introduced into the column from the top and is allowed to pass the sample **under the influence of gravity.**
- The molecules bound to the column are separated by elution technique where either solution of the same polarity is used (isocratic technique), or different samples with different polarities are used (gradient technique).
- The separated molecules can further be analyzed for various purposes.

Flash chromatography

- Flash chromatography is a separation technique where smaller sizes of gel particles are used as stationary phase, and pressurized gas is used to drive the solvent through the column.

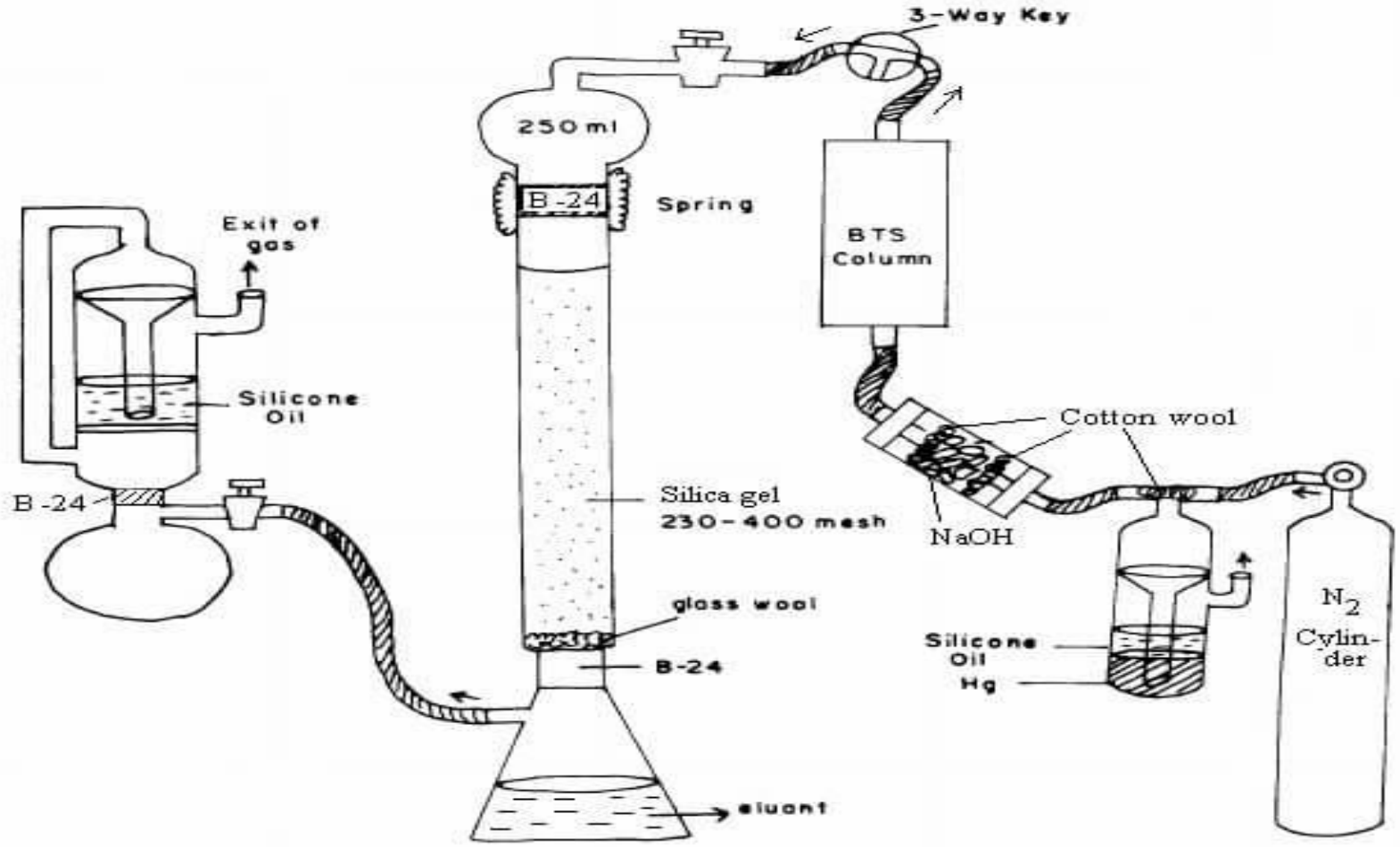
Principle of Flash chromatography

- The principle of flash **chromatography is similar to that of column chromatography**, where the components are separated on the basis of their differential adsorption to the stationary phase.
- The sample applied is passed by using a pressurized gas that makes the process faster and more efficient.
- Molecules bind to the stationary phase on the basis of their affinity while the rest of the solvent is eluted out by applying the pressured gas which quickens the process.
- Here, **the stationary phase is solid, the mobile phase and the elution solution are liquid, and an additional pressurized gas is used.**

Steps of Flash chromatography

- The **column is prepared** by taking a glass tube that is dried and coated with a thin, uniform layer of stationary phase (cellulose, silica).
- The bottom and top of the column are packed with cotton wool to prevent the gel from escaping.
- Then the sample is prepared by adding the mixture to the mobile phase.
- The sample is introduced into the column from the top, and a pumped sample is used to pass the sample at a constant rate.
- The molecules bound to the column are separated by elution solution where either solution of the same polarity is used (isocratic technique), or different samples with different polarities are used (gradient technique).
- The elution solvent is applied with a constant minimum pressure required to move the solute down the column.
- The separated molecules can further be analyzed for various purposes.

Instrumentation of Flash chromatography



Flash Chromatography apparatus

Gas Chromatography

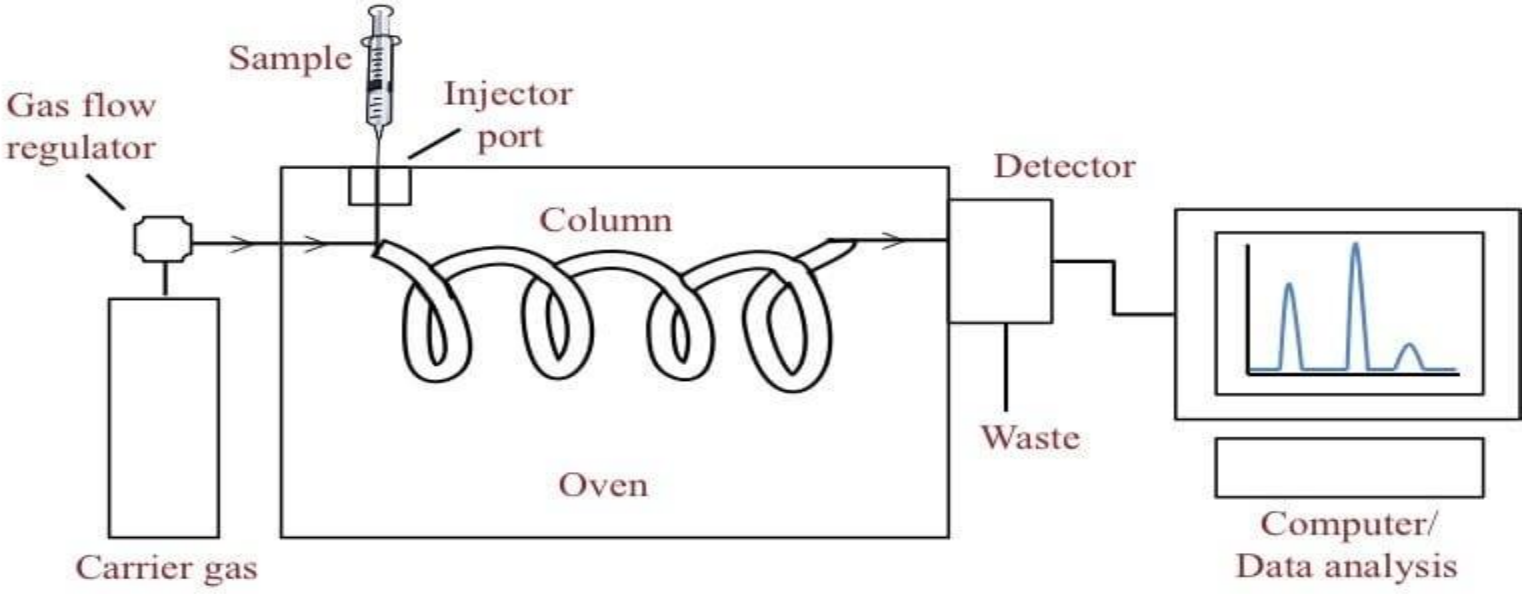
- Gas chromatography is a separation technique in which the molecules are separated on the basis of their retention time depending on the affinity of the molecules to the stationary phase.
- The sample is **either liquid or gas** that **is vaporized in the injection point**.

Principle of Gas chromatography

- Gas chromatography is based on the principle that components having a higher affinity to the stationary phase have a higher retention time as they take a longer time to come out of the column.
- However, the components having a higher affinity to the mobile phase have less retention time as they move along with the **mobile phase**.
- The mobile phase is a gas, mostly helium, that carries the sample through the column.
- The sample once injected in converted into the vapor stage is then passed through a detector to determine the retention time.
- The components are collected separately as they come out of the stationary phase at different times.

Steps of Gas Chromatography

- The **sample** is injected into the column where it is vaporized into a gaseous state.
- The **vapourised component** than mixes with the mobile phase to be carried through the rest of the column.
- The column is set with the stationary phase where the molecules are separated on the basis of their affinity to the stationary phase.
- The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.



Gel filtration chromatography/ Gel permeation chromatography/ Size exclusion chromatography/ Molecular sieve chromatography

- Gel-filtration chromatography is a form of partition chromatography used to separate molecules of different molecular sizes.
- **This technique** has also frequently been referred to by various other names, including gel-permeation, gel-exclusion, size- exclusion, and molecular- sieve chromatography.

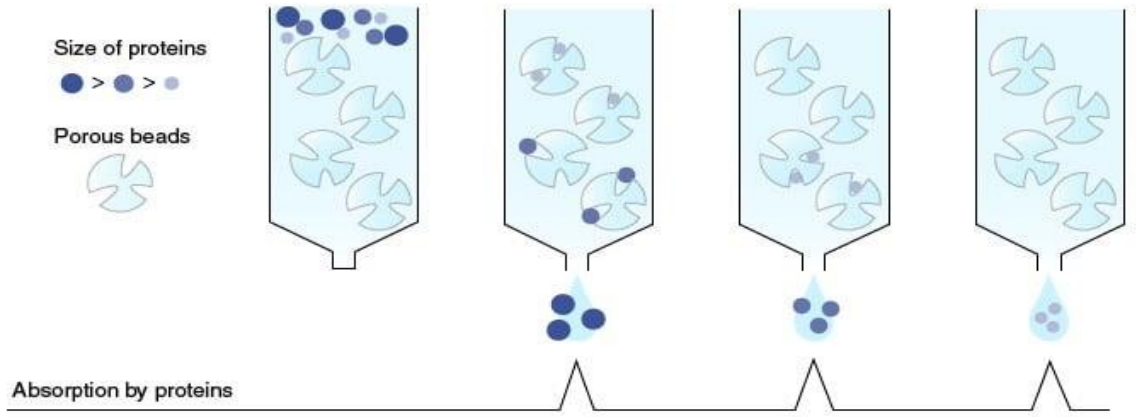
Principle

- Molecules are partitioned between a mobile phase and a stationary phase as a function of their relative sizes.
- The stationary phase is a matrix of porous polymer which have **pores of specific sizes**.

- When the sample is injected with the mobile phase, the mobile phase occupies the pores of the stationary phase.
- If **the size of the molecules is appropriate enough to enter the pores, they remain in the pores partly or wholly**.
- **However**, molecules with a larger size are retained from entering the paores, causing them to be moved with the mobile phase, out of the column.
- If the mobile phase used in an **aqueous solut**ion, the process is termed **gel filtration chromatography**.
- If the mobile phase used is an **organic solvent**, it is termed as **gel permeation chromatography**.

Steps

- The column is filled with semi-permeable, porous polymer gel beads with a well-defined range of pore sizes.
- The sample, mixed with the mobile phase, is then injected into the column from the top of the column.
- The molecules bound to the column are separated by elution solution where either solution of the same polarity is used (isocratic technique), or different samples with different polarities are used (gradient technique).
- **Elution conditions** (pH, essential ions, cofactors, protease inhibitors, etc.) can be selected, which will complement the requirements of the molecule of interest.

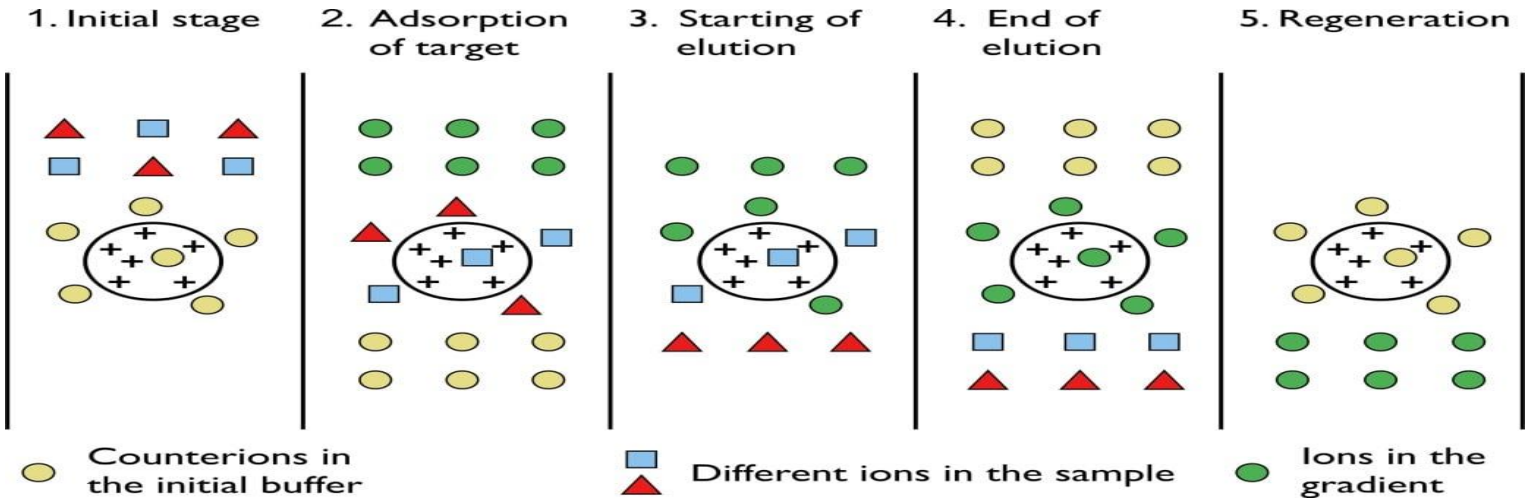


Ion exchange chromatography

- Ion exchange chromatography is the **separation technique for charged molecules** by their interaction with the oppositely charged stationary phase in the form of ion-exchange resin.
- **Principle of Ion exchange chromatography**
- This technique is based on the principle of attraction of charged resin and the oppositely charged analyte.
- Here the exchange of **negatively/ positively charged ions** takes place to remove the charged molecules.
- The **stationary phase** is first coated with particular charges where the components of the mixture with opposite charges will bind.
- A **cation or anion exchange resin** with a higher affinity to the charged components then binds the components, displacing the oppositely charged resin.
- The **cation or anion exchange resin-component complex** then is removed by using different buffers.

Steps of Ion exchange chromatography

- A column packed with charged resin that can either be positively charged or negatively charged is taken as the stationary phase.
- The mixture with the charged particles is then passed down the column where the charged molecules bind to the oppositely charged resins.
- If a **cation exchange resin** is used, the **positively charged molecules now bind** to the cation exchange resin displacing the negatively charged resin.
- **Similarly,** if an **anion exchange resin is use**d, the negatively charged molecules bind to the anion exchange resin displacing the positively charged resin.
- Now an appropriate buffer is applied to the column to separate the complex of charged exchange resins and the charged molecules.



Liquid chromatography

- Liquid chromatography is a separation technique where the mobile phase used is liquid, and the separation can take place either in a column or a plain surface.

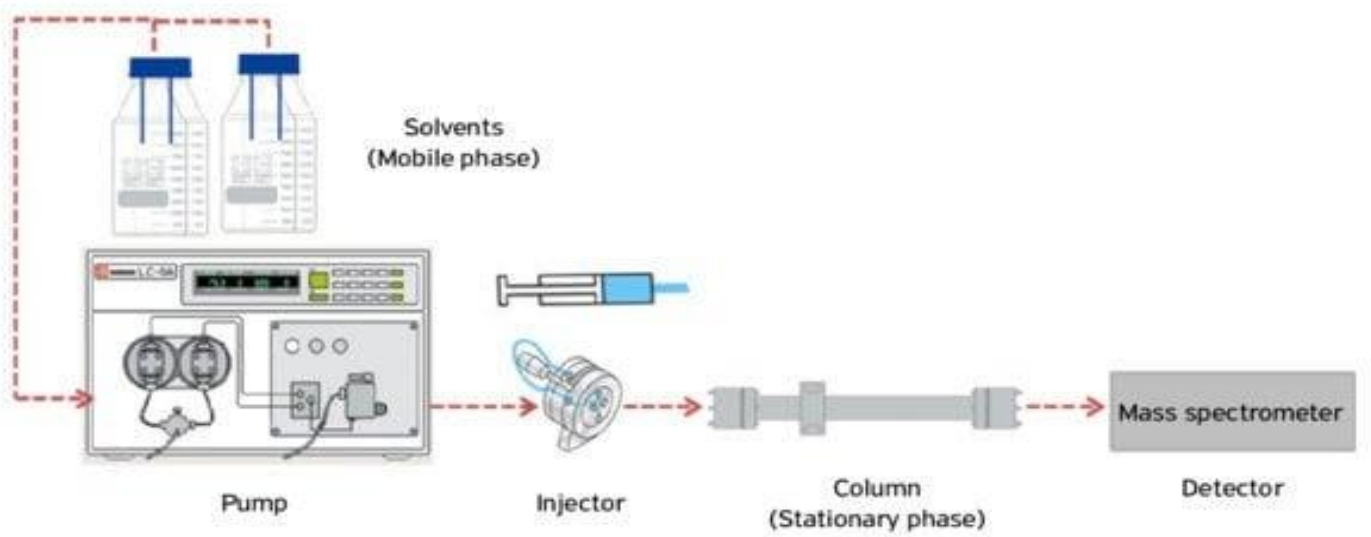
Principle of Liquid chromatography

- The process of liquid chromatography is based on the principle for the affinity of the molecules to the mobile phase.
- If the components to be **separated have a higher affinity to the mobile phase**, the molecules move along with the mobile phase and come out of the column faster.
- **However**, if the components have a **lower degree of interaction with the mobile phase**, the molecules move slowly and thus come out of the column later.
- Thus, if two molecules in a **mixture have different polarities and the mobile phase is of a distinct polarity**, the two molecules will move at different speeds through the stationary phase.

Steps of Liquid chromatography

- The **column or paper** is prepared where the stationary phase (cellulose or silica) is applied on the solid support.
- The sample is added to the liquid mobile phase, which is then injected into the chromatographic system.
- The mobile phase moves through the stationary phase before coming out of the column or the edge of the paper.
- An elution solution is applied to the system to separate the molecules from the stationary phase.

Instrumentation of Liquid chromatography



Paper chromatography

- Paper chromatography is a separation technique where the separation is performed on a **specialized paper.**

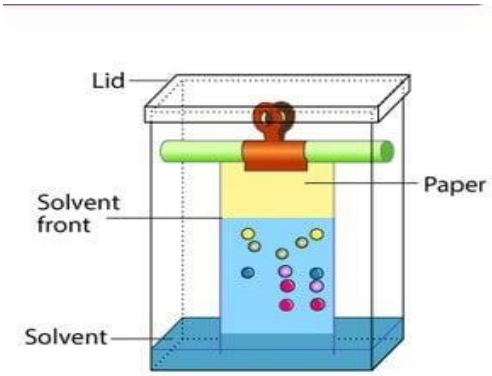
Principle of Paper chromatography

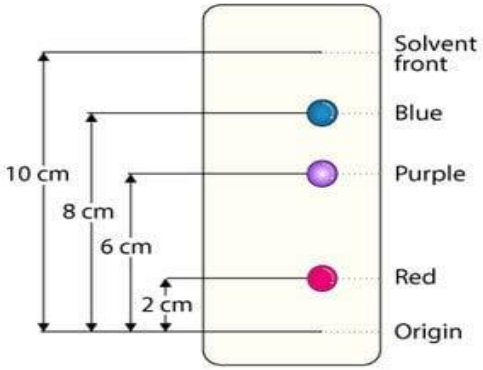
- Paper chromatography is of **two types based on two different principles.**
- **The first** is the paper adsorption chromatography that is based on the varying degree of interaction between the molecules and the stationary phase.
- The molecules having higher affinity remain adsorbed for a longer time decreasing their speed of movement through the column.
- **However**, the molecules with lower affinity move with a faster movement, thus allowing the molecules to be separated in different fractions.

- The **second type of paper chromatography** is the paper partition chromatography.
- It is based on the principle that the moisture on the cellulose paper acts as a stationary phase for the molecules moving with the mobile phase.
- The separation of the molecules is thus based on how strongly they adsorb onto the stationary phase.
- An **additional concept of 'retention factor'** is applied during the separation of molecules in the paper chromatography.
- The **retention value** for a molecule is determined as a ratio of distance traveled by the molecule to the distance traveled by the mobile phase.
- The retention value of different molecules can be used to differentiate those molecules.

Steps of Paper chromatography

- The **stationary phase** is selected as a fine quality cellulosic paper.
- Different combinations of organic and inorganic solvents are taken as the mobile phase.
- **About 2-200 µl of** the sample solution is injected at the baseline of the paper, and it is allowed to air dry.
- The sample loaded paper is then carefully dipped into the mobile phase not more than the height of 1 cm.
- After the mobile phase reaches near the edge of the paper, the paper is taken out.
- The retention factor is **calculated, and the separated components are detected by different techniques**.





Thin-layer chromatography (TLC)

- Thin-layer chromatography is a separation technique where the stationary phase is applied as a thin layer on a solid support plate with a liquid mobile phase.

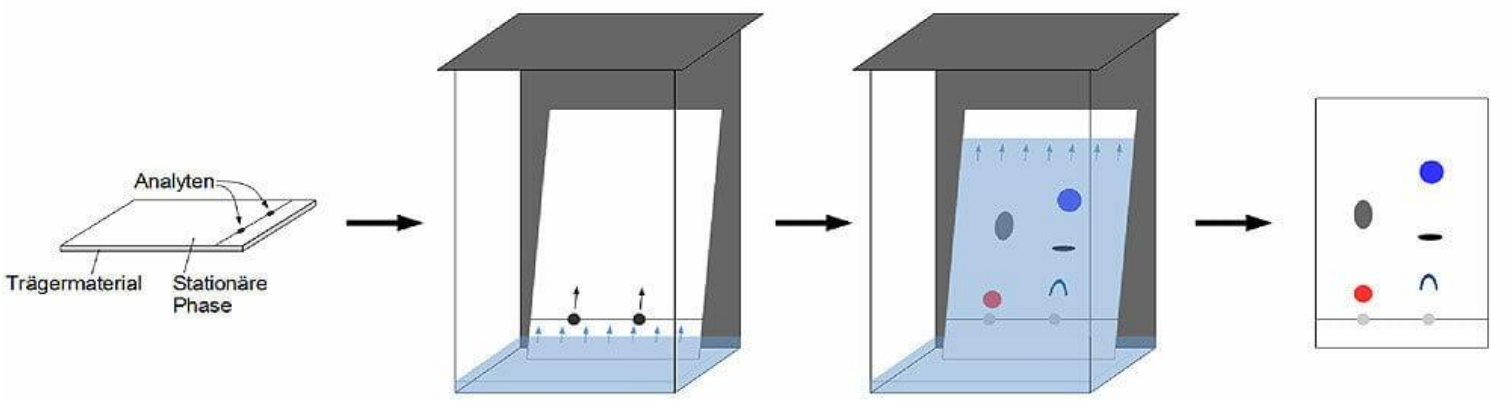
Principle of Thin-layer chromatography (TLC)

- This chromatography technique is based on the principle that components of a mixture are separated when the component having an affinity towards the stationary phase binds to the stationary phase.
- In contrast, other components are eluted with the mobile phase.

- The **substrate/ ligand** is bound to the stationary phase so that the reactive sites for the binding of components are exposed.
- Now, the mixture is passed through the mobile phase where the components with binding sites for the substrate bind to the substrate on the stationary phase while the rest of the components are eluted out with the mobile phase.
- After separation, the molecules are seen as spots at a different location throughout the stationary phase.
- The detection of molecules is performed by various techniques.

Steps of Thin-layer chromatography (TLC)

- The **stationary phase is uniformly applied** on the solid support (**glass, thin plate or aluminum foil**) and dried.
- The sample is injected as spots on the stationary phase about **1 cm above the edge of the plate.**
- The sample loaded plate is then carefully dipped into the mobile phase not more than the height of 1 cm.
- After the mobile phase reaches near the edge of the plate, the plate is taken out.
- The retention factor is calculated as in paper chromatography, and the separated components are detected by different techniques.



Electrophoresis

- **Electrophoresis** is a laboratory technique used to separate DNA, RNA, or protein molecules based on their size and electrical charge.
- An electric current is used to move molecules to be separated through a gel.
- Pores in the gel work like a sieve, allowing smaller molecules to move faster than larger molecules.

Working Principle of Electrophoresis

- **Charged macromolecules** placed in an electric field move in the direction of the positive or negative pole.
- The movement ultimately depends on the charge of the macromolecules.
- **For example**: Since nucleic acid is a negatively charged particle, it tends to move in the direction of the anode.
- The entire **electrophoresis procedure has two varieties;** they are **capillary electrophoresis and slab electrophoresis.**
- **Proteins**, if negatively charged, will move towards the anode and the cathode if they have a positive charge.
- **Because smaller molecules** migrate faster than **larger molecules**, scientists can easily measure the travelled distance and make use of logarithms for determining **the size of the partic**les.

The Principle of Electrophoresis

- The reason electrophoresis works is owed to one of the fundamental equations in the physics of electromagnetism: force equals electric charge times the strength of the field at that point. This assumes the form:
- **$F = qE$,**
- **Where F = force, q = electric charge, and E = electric field strength.**
- This equation implies that the **higher the charge on a particle**, the stronger the force that results from the application of a given electric field.
- **This means** that two particles of the same mass but different charges will move at different rates through the field.
- In **addition, the speed** at which any charged molecule moves is **dependent on its charge-to-mass ratio**.
- Together, these properties and relationships make it possible for scientists to separate the components of critical bio-molecules, such as nucleic acids, into their smaller components.

Categories of Electrophoresis

- The supporting medium is a kind of physical support that helps in separating the charged macromolecules.
- This physical support renders **two essential functions** - molecular sieving and adsorption of taken macromolecules for separation.
- Some of the most commonly utilised supporting **mediums include agar, agarose, starch, and polyacrylamide.**
- Based on the **availability or unavailability** of the supporting medium, the electrophoresis procedure is of two categories: **Capillary electrophoresis and Slab electrophoresis.**
- These **two type**s further can be divided into other types of electrophoresis techniques.
- In the **capillary electrophoresis category**, one can find techniques like **paper electrophoresis and gel electrophoresis**.
- **Slab electrophoresis** has additional sub-categories, like **zone electrophoresis, isoelectrofocusing, and immunoelectrophoresis.**

Different Types of Electrophoresis

1. Paper Electrophoresis

- The technique is quite simple. The sample intended to be separated is applied to a strip of paper moisturised using a kind of buffer solution.
- There are separate tanks of this buffer solution, and each end of the paper is dipped in these tanks. Also, there is a different cathode or anode.
- **Next,** an electric current is applied. It forces the sample to move in the direction of the electrode with the opposite polarity.
- Once the procedure is completed, the paper is dried and then viewed using a sound quality detection system.

2. Immunoelectrophoresis

- The process is a blend of **electrophoresis and immune-diffusion**.
- The process involves placing antigen mixture into well cuts in gel without antibodies and separating their components through electrophoresis.

3. Zone Electrophoresis

- ZE or Zone electrophoresis is the process for the analysis of nucleic acids, biopolymers, and proteins.
- This electrophoretic separation procedure involves transporting different species in a buffer system under an electric current.
- Because of differences in mobilities, these species will separate into well-resolved and varied peaks.

4. Isoelectrofocusing

- **IEF or Iso Electrofocusing** is the process of separating charged macromolecules, generally peptides or proteins.
- The separation **depends on their isoelectric point** or the **pH** at which a particular **molecule does not have any charge.**
- This process works mainly because the macromolecules in the pH gradient tend to move towards their **pI** in an electric field.
- IPG strips consisting of acrylamide gel with wide pores for preventing sieving effects are used for the procedure.

Gel Electrophoresis

- It is **one of the most preferred** electrophoresis procedures in the majority of the experimental environments.
- There **are three essential varieties** of gel electrophoresis.
- They are **starch gel electrophoresis**, **polyacrylamide gel electrophoresis, and agarose gel electrophoresis**.
- In the **starch gel electrophoresis procedure**, potato starch granules are used in the form of a supporting medium.
- Things are different in the **agarose gel electrophoresis technique.**
- Here, a wholly purified polysaccharide in large molecular mass is used as the support media.
- **Polyacrylamide gel electrophoresis** is one of the most common techniques due to its high stability.
- Also, it works on a large assortment of molecular concentrations.

Agarose Gel Electrophoresis

- **Agarose gel electrophoresis** is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA , RNA or proteins in a matrix of agarose.
- **Agarose** is a natural linear polymer extracted from seaweed that forms a gel matrix by hydrogen-bonding when heated in a buffer and allowed to cool.
- They are the most popular medium for the separation of **moderate and large-sized nucleic acids and have a wide range of separation**.

Principle of Agarose Gel Electrophoresis

- Gel electrophoresis separates **DNA fragments** by size in a solid support medium such as an agarose gel.
- **Sample (DNA)** are pipetted into the **sample wells**, followed by the application of an **electric current** which causes the negatively-charged DNA to migrate (electrophorese) towards the anodal, positive (+ve) end.
- The rate of migration is **proportional to size**: *smaller fragments move more quickly and wind up at the bottom of the gel.*

- DNA is visualized by including in the gel an intercalating dye, ethidium bromide.
- DNA fragments take up the dye as they migrate through the gel.
- **Illumination** with ultraviolet light causes the intercalated dye to fluoresce.
- The larger fragments fluoresce more intensely.
- Although each of the fragments of a single class of molecule is present in equimolar proportions, the smaller fragments include less mass of DNA, take up less dye, and therefore fluoresce less intensely.
- **A "ladder" set of DNA fragments of known size** can be run simultaneously and used to **estimate the sizes of the other unknown fragments.**

Requirements/ Instrumentation of Agarose Gel Electrophoresis

- The equipment and **Requirements** necessary for conducting agarose gel electrophoresis are relatively simple and include:

1) An **electrophoresis chamber** and **power supply**
2) **Gel casting trays**, which are available in a variety of sizes and composed of UVtransparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
3) **Sample combs**, around which molten medium is poured to form sample wells in the gel.
4) **Electrophoresis buffer**, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).

5) **Loading buffer**, which contains something dense (e.g. glycerol) to allow the sample to “fall” into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.

6) **Staining**: DNA molecules are easily visualized under an **ultraviolet lamp** when electrphoresed in the presence of the extrinsic fluor ethidium bromide. Alternatively, nucleic acids can be stained after electrophoretic separation by soaking the gel in a solution of ethidium bromide. When intercalated into doublestranded DNA, fluorescence of this molecule increases greatly. It is also possible to detect DNA with the extrinsic fluor 1-anilino 8-naphthalene sulphonate.

7) **Transilluminator** (an ultraviolet light box), which is used to visualize stained DNA in gels.

Steps Involved in Agarose Gel Electrophoresis

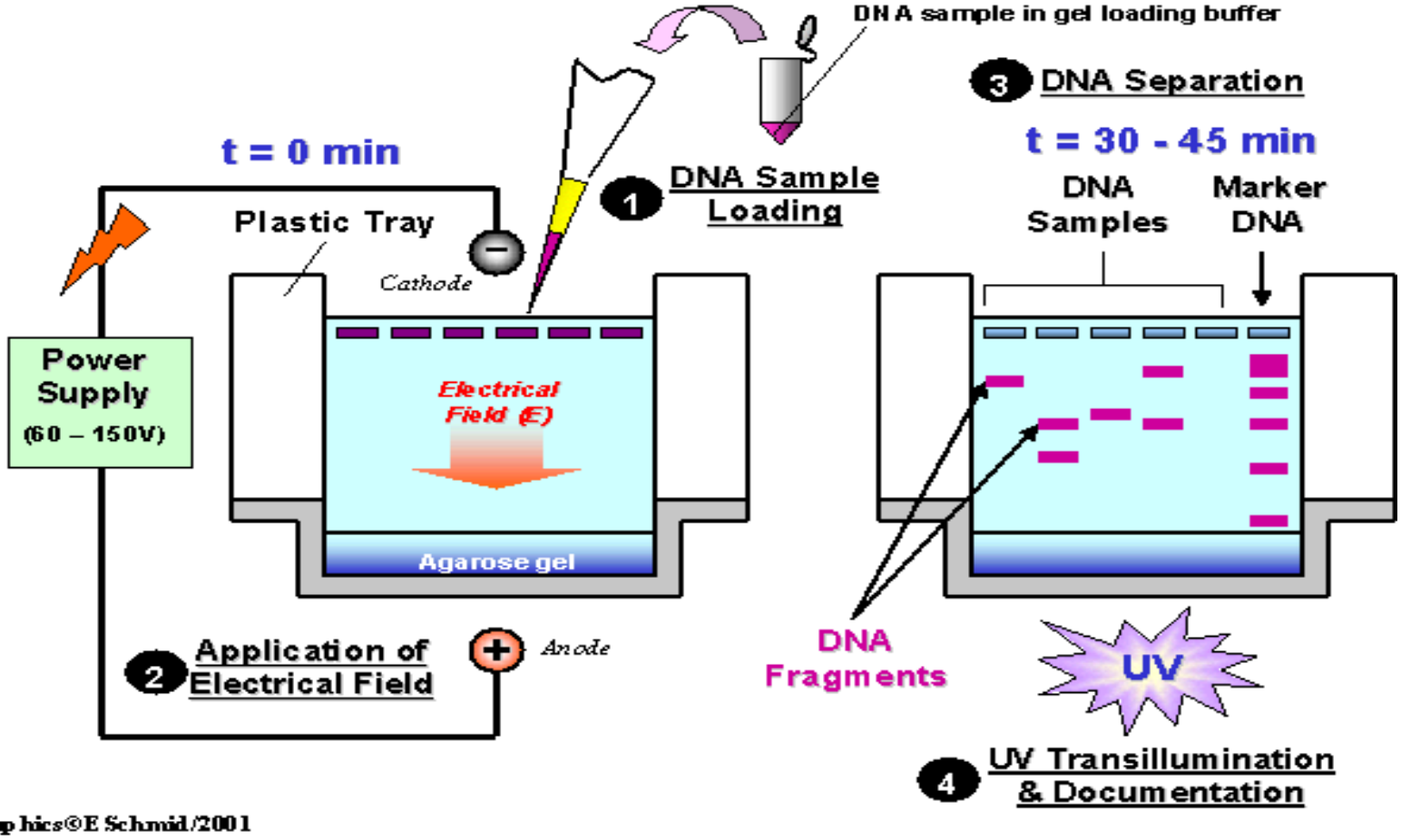
- To prepare gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, and heated in a microwave oven to melt it.

1) The concentration of Agarose Gel

- The percentage of agarose used depends on the size of fragments to be resolved.
- The concentration of agarose is referred to as a percentage of agarose to volume of buffer (w/v), and agarose gels are normally in the range of 0.2% to 3%.
- The lower the concentration of agarose, the faster the DNA fragments migrate.
- In general, if the aim is to separate large DNA fragments, a low concentration of agarose should be used, and if the aim is to separate small DNA fragments, a high concentration of agarose is recommended.

2) Ethidium bromide is added to the gel (final concentration 0.5 ug/ml) to facilitate visualization of DNA after electrophoresis.
3) After cooling the solution to about 60^{o}C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.
4) After the gel has solidified, the comb is removed, taking care not to rip the bottom of the wells.
5) The gel, still in plastic tray, is inserted horizontally into the electrophoresis chamber and is covered with buffer.
6) Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied.
7) The current flow can be confirmed by observing bubbles coming off the electrodes.
8) DNA will migrate towards the positive electrode, which is usually colored red, in view of its negative charge.
9) The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes like bromophenol blue and xylene cyanol dyes.

Schematic Steps Involved in Agarose Gel Electrophoresis



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