Chapter 4 : Separation methods

Separation methods refer to techniques used to divide a heterogeneous or homogeneous mixture into its individual components or elements, based on fundamental chemical and physical principles. These methods can be employed for purification, qualitative identification, or quantitative determination of the components in a sample.

1- Filtration

1-1-Definition

Filtration is the separation process of removing solid particles, microorganisms or droplets from a liquid or a gas by depositing them on a a porous medium, which is essentially permeable to only the fluid phase of the mixture being separated. The suspension of solid and liquid to be filtered is known as the feed or slurry. The porous medium used to retain the solids is described as the filter medium, also called a septum. The accumulation of solids on the filter is referred to as the filter cake, while the clear liquid passing through the filter is the filtrate.

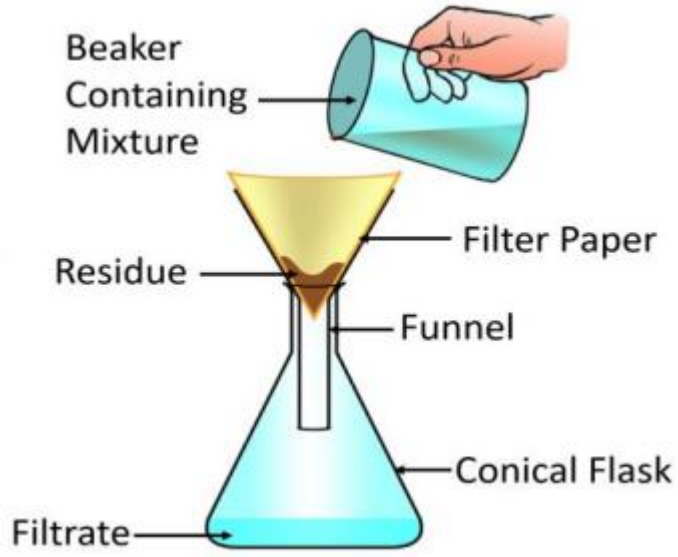

Figure 1: Filtration.

1-2- Types

1-2-1- Gravity filtration : Gravity filtration refers to pouring a solid-liquid mixture through a funnel containing a filter paper, allowing the liquid to seep through while trapping the solid on the paper.

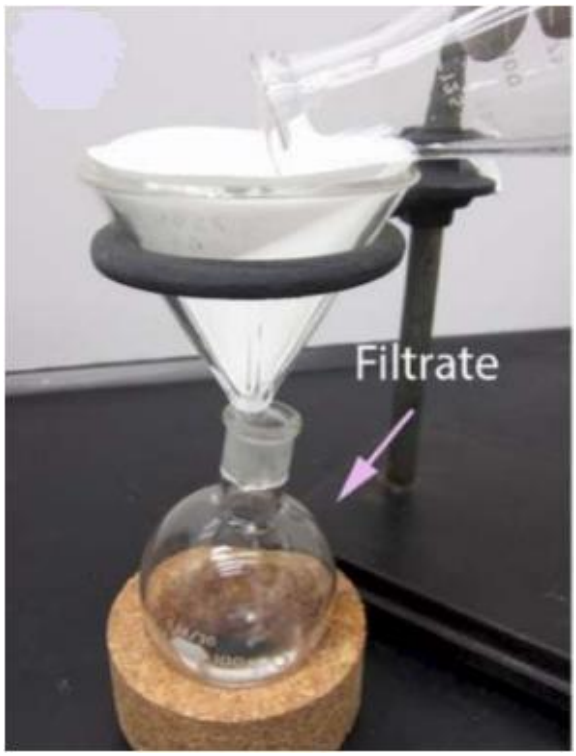

Figure 2 : Gravity filtration.

To gravity filter a mixture, pour the mixture through a quadrant-folded filter paper or fluted filter paper in a funnel and allow the liquid to filter using only the force of gravity. It is best to pour as if attempting to decant, meaning to keep the solid settled in the flask for as long as possible. When solid begins to pour onto the filter paper, it has the possibility of clogging the filter paper pores or slowing filtration. After finished pouring, rinse the solid on the filter paper (and in the flask) with a few portions of fresh solvent to remove residual compound adhering to the solid.

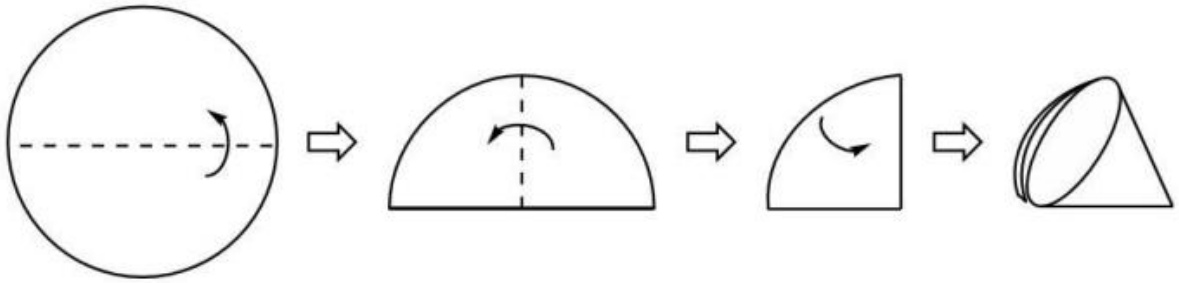

Figure 3 : Creating a quadrant-folded filter paper. The dotted lines represent locations to crease and fold the filter paper. The arrows show the direction of folding.

1-2-2- Suction filtration (vacuum filtration)

Suction filtration (vacuum filtration) is the standard technique used for separating a solid-liquid mixture when the goal is to retain the solid (for example in crystallization). Similar to gravity filtration, a solid-liquid mixture is poured onto a filter paper, with the main difference being that the process is aided by suction beneath the funnel.

Figure 4 : Vacuum filtration.

- **Water Aspirator**

A water aspirator is an inexpensive attachment to a water spigot, and the nub on the aspirator connects with tubing to the vessel to be evacuated. As water flows through the faucet and the aspirator, suction is created in the flask.

Water coming from the faucet is constricted inside the aspirator. As the water flow must be the same going into the aspirator as it is going out, the water speed must increase in the constricted area in the direction of flow. A similar phenomenon can be seen in creeks and rivers where the water flows the fastest at the narrowest portions of streams. When the water increases its velocity in the direction of the water flow, conservation of energy dictates that its velocity in perpendicular directions must decrease. The result is a lowered pressure adjacent to the fast-moving liquid. In other words, the gain in velocity of the constricted liquid is balanced by a reduction in pressure on the surrounding material (the gas).

For this reason, the speed at which the water flows through the faucet is correlated with the amount of suction experienced in the connected flask. A strong flow of water will have the fastest speeds through the aspirator and the greatest reduction in pressure.

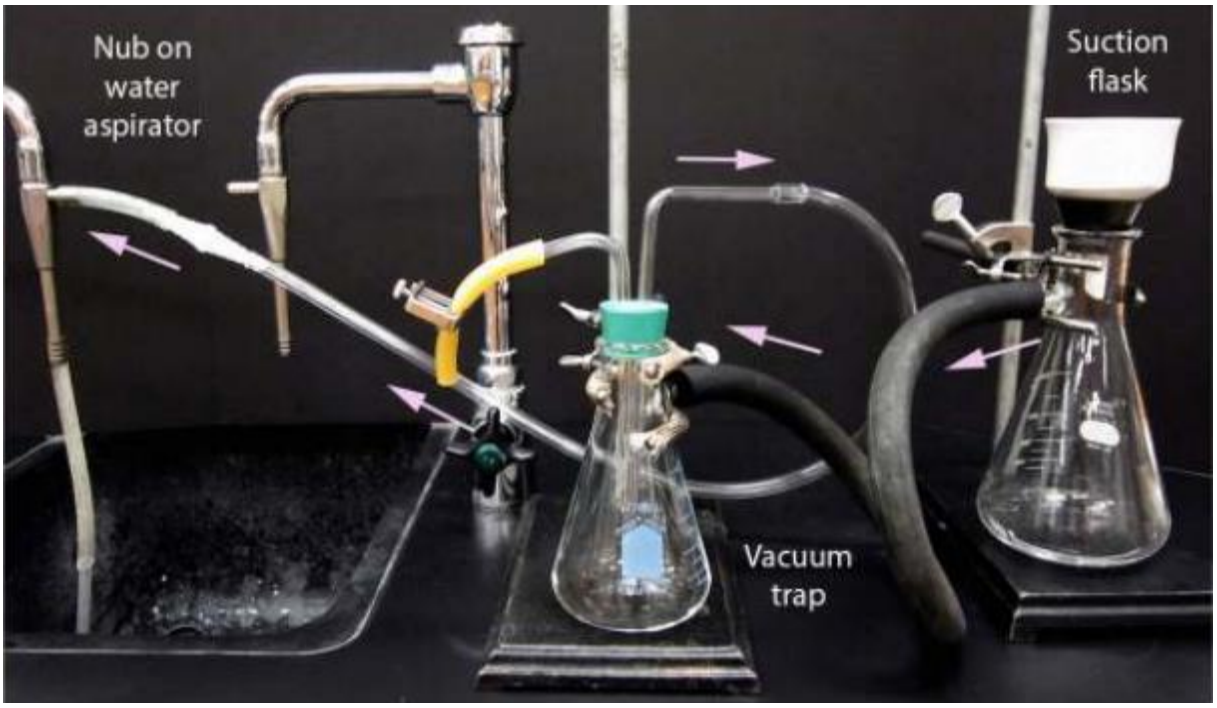

Figure 5 : Suction filtration flask attached to a vacuum trap and water aspirator.

- **Procedures**

1. Clamp a side-arm Erlenmeyer flask to a ring stand or latticework and attach a thick-walled rubber hose to its side arm. Connect this thick tubing to a "vacuum trap" and then to the water aspirator. It is best to not bend or strain the tubing as much as is practical, as this may cause poor suction. A vacuum trap is necessary when connecting apparatuses to a vacuum source as changes in pressure can cause back-suction. When using a water aspirator, back-suction might cause water from the sink to be pulled into the vacuum line and flask (ruining the filtrate), or the filtrate to be pulled into the water stream (contaminating the water supply).
2. Place a rubber sleeve (or filter adapter) and Buchner funnel atop the side-arm Erlenmeyer flask. Alternatively use a Hirsch funnel for small scales.
3. Obtain a filter paper that will fit perfectly into the Buchner or Hirsch funnel. Filter papers are not completely flat and have a subtle arc to their shape. Place the filter paper inside the funnel concave side down. The paper should cover all the holes in the funnel, and with the paper arching downward, solid will be less likely to creep around the edges.
4. Turn on the faucet connected to the water aspirator to create a strong flow of water (the degree of suction is related to the water flow). Wet the filter paper with cold solvent (using the same solvent used in crystallization, if applicable).

5. Suction should drain the liquid and hold the moist filter paper snugly over the holes in the filter. If the solvent does not drain or suction is not occurring, you may need to press down on the funnel to create a good seal between the glass and rubber sleeve.

6. Swirl the mixture to be filtered in order to dislodge solid from the sides of the flask. If the solid is very thick, use a spatula or stirring rod to free it from the glass.

7. With a quick motion, swirl and dump the solid into the funnel in portions. If the solid is very thick, scoop it out of the flask onto the filter paper. It's best if the solid can be directed toward the middle of the filter paper, as solid near the edges may creep around the filter paper.

8. A small amount of chilled solvent (1 -2 mL for macroscale work) can be used to help rinse any residual solid from the flask into the funnel.

9. Rinse the solid on the filter paper to remove contaminants that may remain in the residual liquid.

1-2-3- Hot filtration

Hot filtration is useful to remove the small amount of impurities from crystalline compounds. Dissolve the crystalline compound in a suitable solvent at suitable high temperature, remove the impurities from liquid compound through a medium and slowly cool down to get the clear recrystallized compounds.

Figure 6: Hot filtration.

As it is essential that a solution filters quickly before it has a chance to cool off in the funnel, a "fluted filter paper" is commonly used instead of the quadrant-folded filter paper sometimes used with gravity filtration. The greater number of bends on the fluted filter paper translate into increased surface area and quicker filtration. The folds also create space between the filter paper and glass funnel, allowing for displaced air to more easily exit the flask as liquid drains.

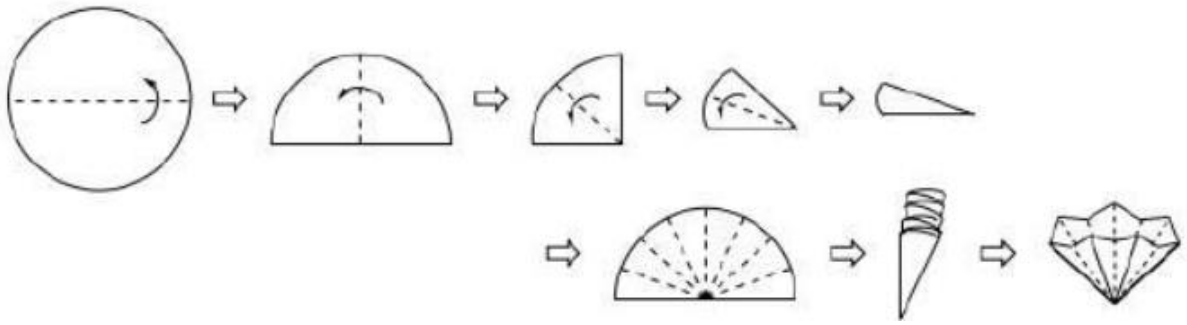

Figure 7 : Creating a fluted filter paper.

1-2-4- Chill / Cold filtration

This filtration process is performed after maintaining a required lower temp. (May be in negative temperature also) of fluid. This filtration is useful to remove available fatty acids, proteins or esters which can be mixed / created in fluid during preceding process. After maintaining a required lower temperature fluid has been passed through a filter medium to remove the chilled suspended particles. This filtration is useful to avoid sedimentation during final use of filtrate at lower temperature.

Figure 8 : Cold filtration.

1-2-5- Pipette filtration (Microscale)

For the separation of small volumes (<10mL) of solid-liquid mixtures, pipette filters are ideal as filter papers absorb a significant amount of material. Pipette filtration may also be used if small amounts of solid are noticed in NMR or GC samples, as both instruments require analysis of liquids without suspended solids.

To create a pipette filter, use a long rod to wedge a small piece of cotton into the bottom of a Pasteur pipette. For very small volumes (<2mL), a GC vial makes a nice receiving flask. These vials are very easy to tip over, but can be held in place by an upside-down large septum. Pipette the solution to be filtered through the top of the filter pipette. It's best to allow the liquid to trickle through the filter on its own, and to at first not use pressure from a dropper bulb, or else solid may be forced through. After the majority of the liquid has filtered, the residual liquid that stays in the pipette, can then be gently pushed through with pressure from a dropper bulb.

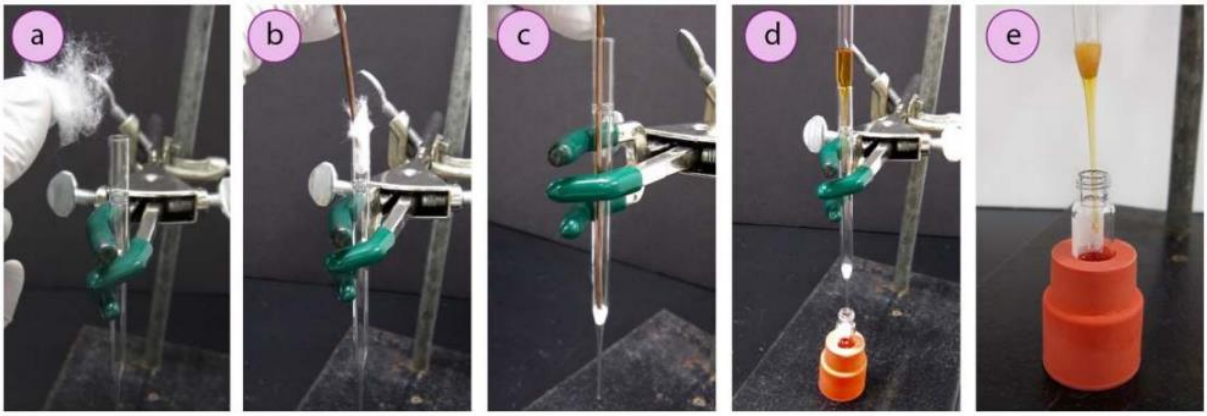

Figure 9 : Creating and using a filter pipette : a) Piece of cotton, b+c) Shoving cotton to the bottom of the pipette, d) Filtering a mixture into a GC vial, e) GC vial held with an upside down large septum.

1-3- Mechanism of filtration

1. Straining : It is similar to sieving, means the particles of larger size cannot pass through the smaller pore size of filter medium.

2. Impingement : Solids move with streamline flow and strike the filter medium.

3. Entanglement : Particle becomes entangled in mass of fiber due to small size of particle than pore size.

4. Attractive force : Solids are retaining due to attractive forces between particles and filter medium.

2- Sedimentation, centrifugation, ultracentrifugation

2-1- Sedimentation

Sedimentation in liquids means particle movement in the direction of gravity or a centrifugal field to concentrate a suspension or separate particles as sediments. It is generally applicable for most solid–liquid separation problems if the solid density is greater than the liquid density. The velocity and phenomenology of particle motion depend essentially on density difference, acceleration, particle size, size distribution, particle shape, concentration in the liquid, and liquid viscosity. In highly diluted and stable slurries, particle settling is unhindered and individual according to their size. A slight increase in concentration leads to hydrodynamically induced formation of particle clusters, which settle faster than the individual particles. If the particle concentration is increased further, the particles increasingly hinder each other, and the settling behavior changes to swarm and finally zone sedimentation. A sharp and self-stabilizing particle-settling front forms at a critical slurry concentration, and all particles settle with more or less the same velocity. Finally, the particles touch each other in the sediment and are only able to move further downward by consolidation.

- **Velocity :** how fast the particle settles.
- **Phenomenology :** how the particle behaves while settling.

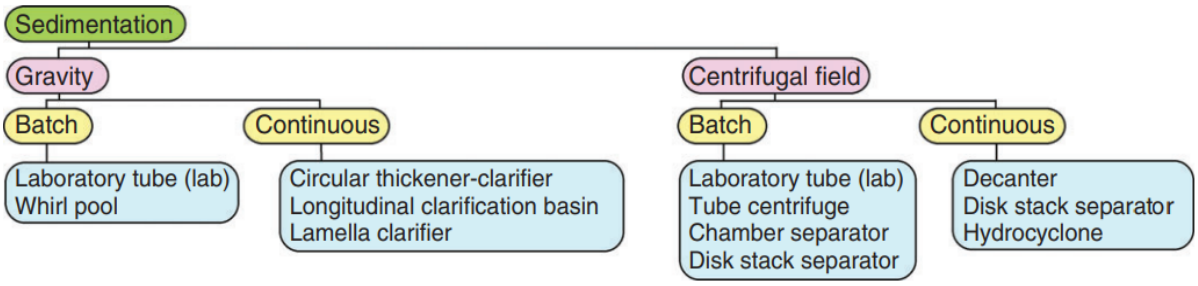

Figure 10 : Sedimentation apparatuses and machines.

2-2- Centrifugation

2-2-1- Definition

Centrifugation is a method of separating molecules having different densities by spinning them in solution around an axis (in a centrifuge rotor) at high speed. It is one of the most useful and frequently employed techniques in the molecular biology laboratory. Centrifugation is used to collect cells, to precipitate DNA, to purify virus particles, and to distinguish subtle differences in the conformation of molecules.

2-2-2- Principle

Particles having a size above 5µm sediment at the bottom due to gravitation force. Such a suspension can be separated by simple filtration techniques. If the size of particles is less than 5µm they undergo Brownian motion. In such suspension a stronger centrifugal force is applied to separate the particles. Considering a body of mass m rotating in a circular path of radius r at a velocity of v. The force acting on the body in a radial direction is given by:

$F = mv^2 /r$

Where F = centrifugal force, m = mass of body, v = velocity of the body, r = radius of circle of rotation. The gravitational force acting upon the same body G = mg Where, G = gravitational force g = acceleration due to gravity The centrifugal effect is the ratio of the centrifugal force and gravitational forces so that

$C = F/G = mv^2 /mgr = v^2 /gr$

Since, $v = 2\pi r n$ where n = speed of rotation (r.p.m.)

$C = F/G = (2\pi r n) 2 /g r = 4\pi 2 r 2 n2$

$= 2\pi 2 /g D n2 = kD n2$

where, $k = 2\pi 2 /g$ = constant D = maximum diameter of the centrifuge

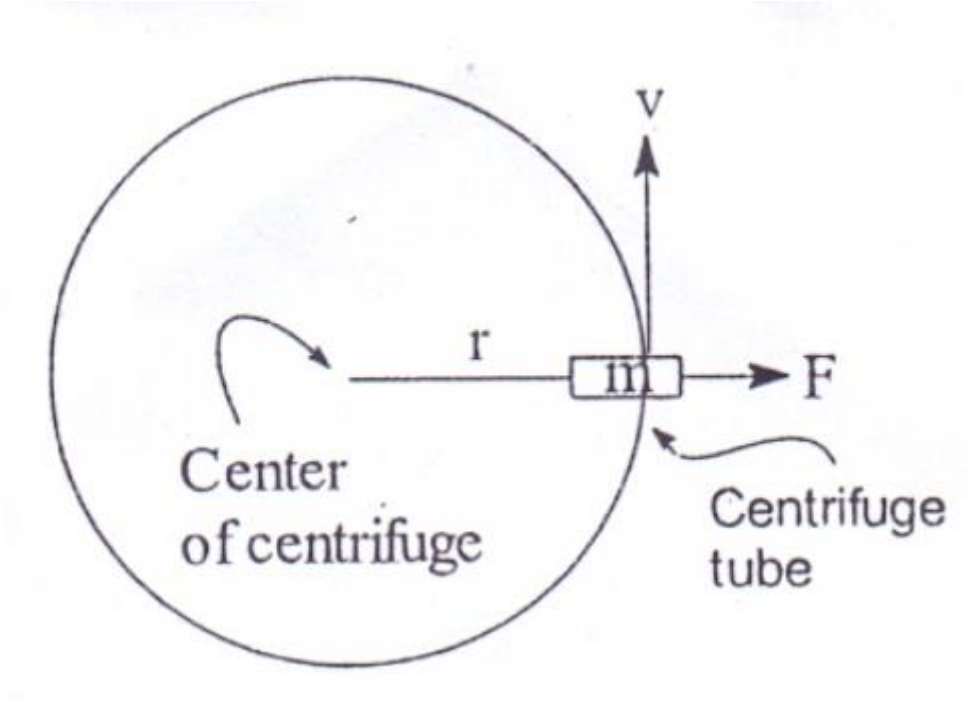

Figure 11 : Illustration of the principle of centrifugation.

2-2-3- Centrifuges

A centrifuge is a device that is used for the separation of fluids, gas or liquid, based on density. Separation is achieved by spinning a vessel (rotor) containing material at high speed; the centrifugal force pushes heavier materials to the outside of the vessel.

- **Classification**

A. Divided by structure

1- Desktop centrifuge: This desktop centrifuge is ideal for spinning down 50 mL samples in breweries and wineries. Ruggedly built, the centrifuge attaches solidly to your bench with suction cups, and offers programmable speeds and times. Centrifuges up to six 50 mL samples simultaneously. With following specification Speed: 4,000 RPM, Capacity: 6 x 50 mL tubes, Voltage: Varies by model

2- Floor-standing centrifuge: Floor standing centrifuges are designed for the centrifugation of large volumes, or for very high speeds. floor standing centrifuges are designed for holders of large sample containers. They are Ideal for use in routine clinical diagnostics, clinical laboratories, blood banks and pharmaceutical laboratories.

B. Divided by speed

1- Low speed centrifuge: A separation method where the components of a sample are separated on the basis of their density in a centrifuge according to the centrifugal force they experience. Samples are spun at <5000 rpm.

2- High speed centrifuge: A High Speed Centrifuge is a device that uses centrifugal force to separate particles of different mass or densities suspended in a liquid. Rotating the solution in the tube at high speeds gives the angular momentum of each particle a centrifugal force proportional to its mass.

C. divided according to temperature control

1- Low temperature centrifuge: Refrigerated centrifuges have been developed to help minimize the effects of temperature on several analytes, including many temperature-sensitive enzymes and hormones. Laboratories should include the required centrifugation temperatures in their sample handling operating procedures.

2- Normal temperature: Most of the work in a refrigerated centrifuge is done at 4 °C. Most centrifuges will claim a lower temperature range of –20 °C, but not all. The rating may pertain to all conditions, or it may be the result of a single rotor being tested in a 70 °C lab in low humidity.

- **Rotors**

A. Fixed Angle Rotor: These hold the tubes in the centrifuge at a fixed inclination (typically about 35 degrees) to the vertical. The most common devices hold eight tubes and they have the advantage of not having moving parts on the rotor. This arrangement means that the solute is forced against the side of the tube. This leads to a faster separation of the solute from fluid, but risks abrasion of the particles as they are forced down the wall of the centrifuge tube. Also the end result is a smear along the side of the tube rather than the precipitate forming a neat pellet.

- **Advantage:**

Sedimenting particles have only short distance to travel before pelleting.

Shorter run time This is the most widely used rotor type.

B. Swinging Bucket Rotor: This form of rotor allows the centrifuge tubes to freely swing out towards the horizontal as the device operates. This gives the longest path of particle movement as the centrifugation proceeds and has the advantage th at the solid forms in a clear pellet at the bottom of the centrifuge tube.

- **Advantage:**

Longer distance of travel may allow better separation eg. Density gradient centrifugation. Easier to withdraw supernatant without disturbing pellet.

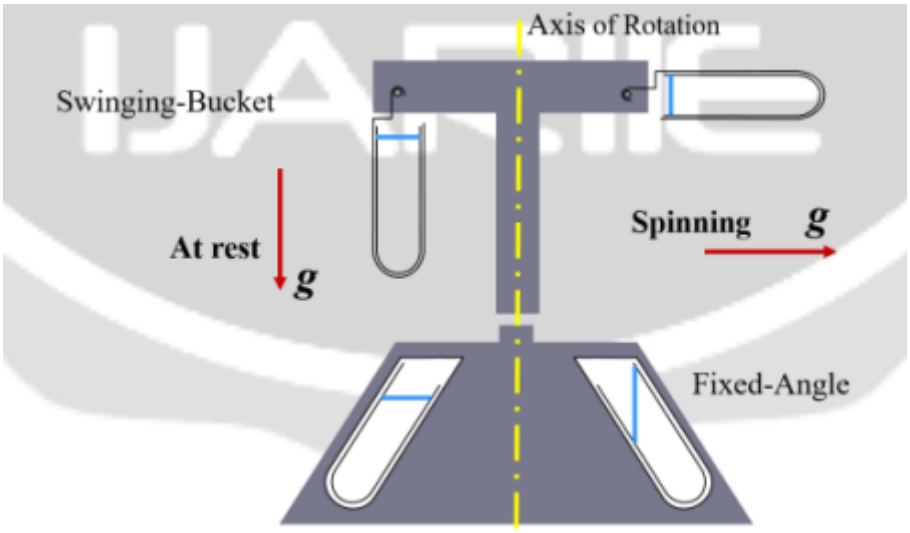

Figure 12 : Types of Rotors

2-2-4- Types of centrifugation

2-2-4-1- Differential centrifugation

Differential centrifugation, also termed pelleting, fractionates particles according to their size and shape. An initially uniform mixture of the particles in sample buffer is separated by

centrifugation into two fractions: a pellet containing the sedimented particles, and a supernatant comprising the unsedimented ones and the buffer.

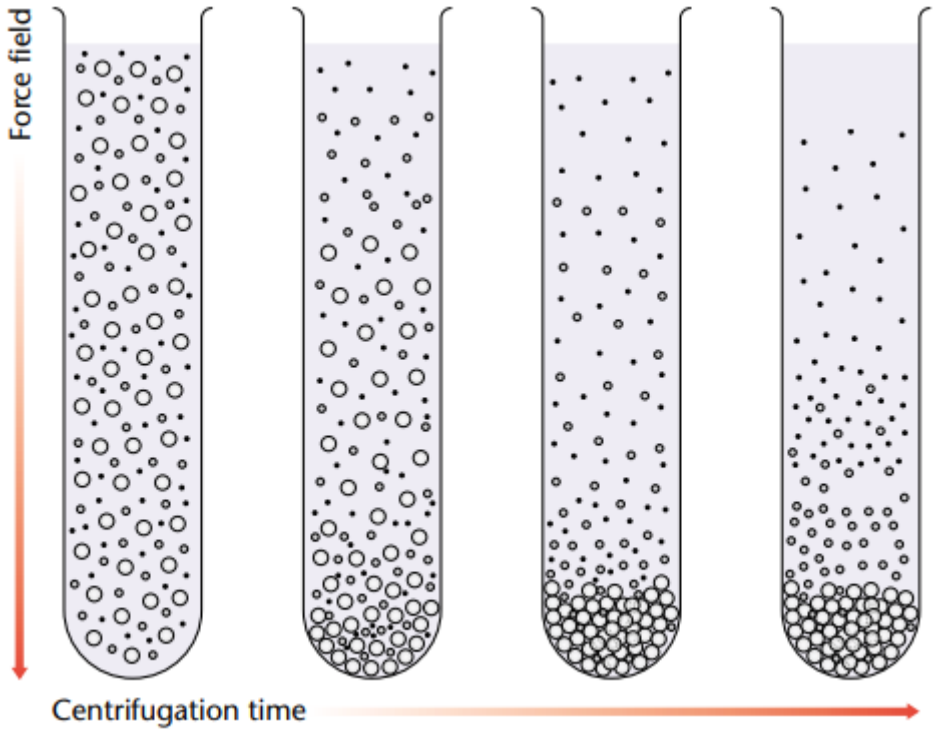

Figure 13 : Differential centrifugation.

2-2-4-2- Density gradient centrifugation

The organelles in a total tissue homogenate can be enriched stepwise by differential fractionation. Yet, their actual purification will not be accomplished in this way because of the cosedimentation of other components. To get a genuinely purified preparation of the organelle of interest, the particular contaminants have to be removed in a subsequent step by the more sophisticated technique of density gradient centrifugation. The rationale of this method is that particles already enriched in a fraction are subjected during centrifugation to a medium that varies in its density along the tube instead of being homogeneous as it is in differential pelleting. In doing this, even slight differences in the particles' physicochemical properties (size, sedimentation velocity, drag force) can be exploited for their resolution.

Two modes of density gradient centrifugation can be distinguished: rate zonal and isopycnic centrifugation. In both methods a supporting column of fluid is used – the gradient – on top of which the sample is layered. This fluid, the gradient medium, consists of a suitable inert solute, usually of low molecular weight, dissolved in a solvent in which the sample particles can also

be suspended. Basically, the density of a gradient may be increased continuously or discontinuously from top to bottom of the centrifuge tube. Discontinuous gradients, also called step gradients, are formed by adding consecutively less dense layers on top of the preceding more dense one. Continuous gradients are prepared by means of a so-called gradient mixer, which is commercially available.

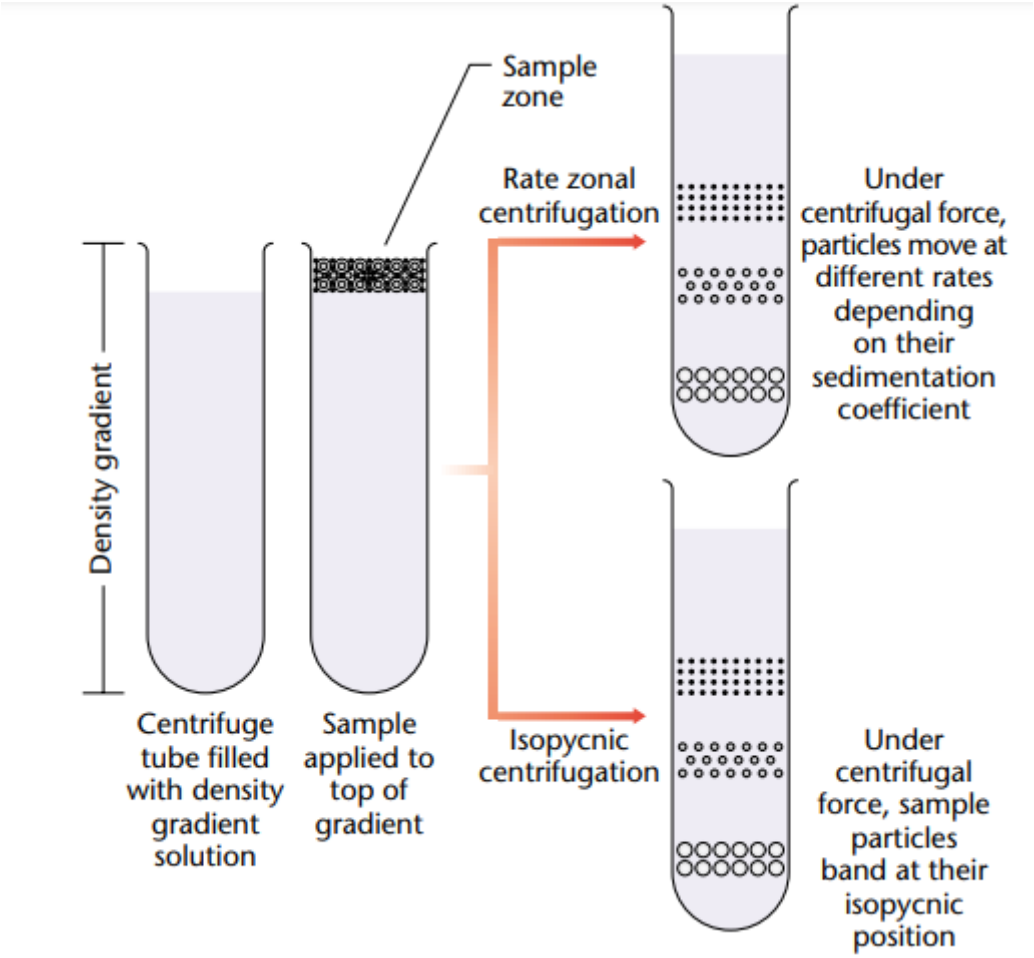

Figure 14 : Modes of density gradient centrifugation: (upper) rate zonal centrifugation; (lower) isopycnic centrifugation.

2-3- Ultracentrifugation

2-3-1- Definition

Ultracentrifugation is a specialized technique used to spin samples at exceptionally high speeds. Current ultracentrifuges can spin to as much as 150 000 rotations per minute (rpm) (equivalent to 1 000 000 g). However, extreme centrifugal forces may cause overheating, so to avoid sample damage, ultracentrifuges are equipped with vacuum systems that keep a constant temperature in the centrifuge's rotor.

2-3-2- Principle

The basis of ultracentrifugation is the same as normal centrifugation: to separate the components of a solution based on their size and density, and the density (viscosity) of the medium (solvent). A general principle, (ultra)centrifugation abides by the following rules:

-The denser a biological structure is, the faster it sediments in a centrifugal field.

-The more massive a biological particle is, the faster it moves in a centrifugal field.

-The denser the biological buffer system is, the slower the particle moves in a centrifugal field.

-The greater the frictional coefficient (i.e., the friction between the component and the neighbouring environment) is, the slower a particle moves.

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

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

2-3-3- Types

2-3-3-1- Preparative ultracentrifugation

Preparative ultracentrifugation is used to separate and isolate components of biological samples, typically through differential and density-gradient methods for tissue and subcellular fractionation.

2-3-3-2-Analytical ultracentrifugation

Analytical ultracentrifugation uses optical systems such as UV absorption or refractive index interference to monitor samples in real time and determine properties like sedimentation, viscosity, and concentration, mainly for macromolecular characterization.

3- Dialysis and electrodialysis.

3-1- Dialysis

3-1-1- Definition

Dialysis is the diffusion based size selective transportation of molecules or particles through a semipermeable membrane from higher concentration to lower concentration through Brownian Motion. Selectivity of dialysis is determined by pore size of semipermeable membranes. The end point of dialysis is the concentration equilibrium. Dialysis is an often-used method to separate bigger molecules like proteins or DNA from accompanying substances such as salt or detergents. It is a very gentle method for sensitive substances. In osmosis, it is the solvent rather than the solute that crosses the membrane.

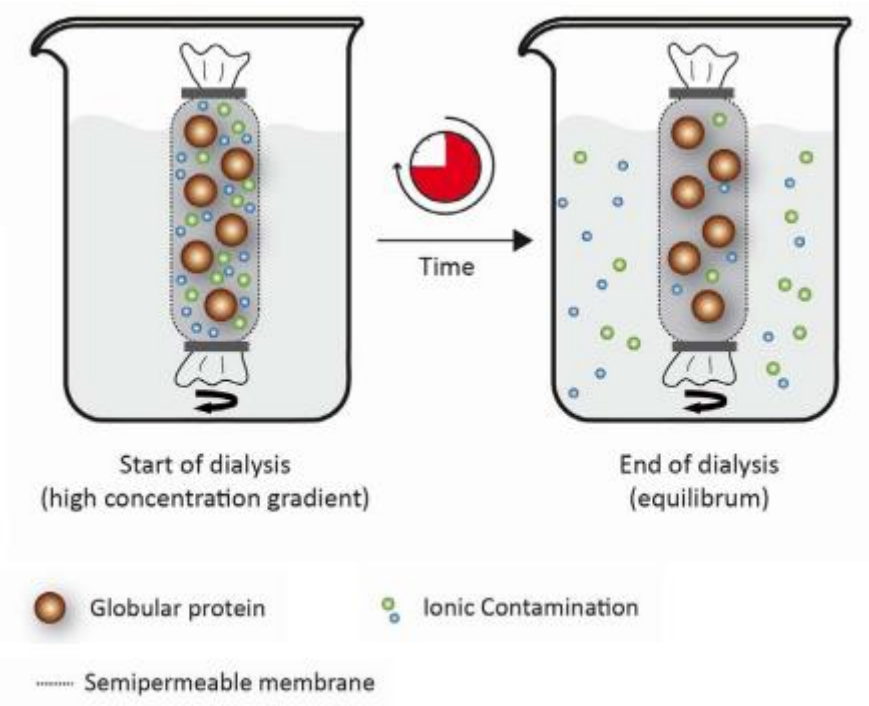

Figure 15 : Principle of protein sample dialysis.

3-1-2- Modes of dialysis

Dialysis can be implemented in four modes, the first and last of which are the most widely used:

1- passive or conventional dialysis;

2- active dialysis in the Donnan mode;

3- active dialysis in the electrodialysis mode; and,

4- microdialysis (MD), which is also a passive mode.

The driving force in dialysis is the analyte-concentration gradient across the membrane (i.e. passive dialysis); if an external electric field is applied, electrodialysis occurs. When the analytes are charged species, they can be separated from the sample matrix and preconcentrated in an acceptor solution by Donnan dialysis. In this case, an appropriate ion-exchange membrane (e.g., Nafion) is used to separate the sample from the acceptor solution, of smaller volume and higher ionic strength. Ions of appropriate charge from the acceptor solution are transported into the sample solution as a result of the existing ionic strength gradient, while co-ions from the sample solution, including analyte ions, diffuse in the opposite direction in order to maintain electroneutrality. Unlike passive dialysis, electrodialysis and Donnan dialysis also afford preconcentration of the analyte. The concept of permselectivity, related to dialysis, refers to the preferential permeation of certain ionic species through ion-exchange membranes.

3-1-3- The dialyzer

Two main types of membrane separation modules for dynamic dialysis: sandwich; and, tubular or hollow-fiber.

1- The sandwich type comprises two blocks made of Perspex, Teflon, aluminum, or some other material having identical internally engraved conduits (usually semi-circular, triangular or rectangular grooves 0.1– 0.5 mm deep and 0.5–2 mm wide) that make up the inner chamber, the geometry of which varies from model to model. The membrane is placed between the two blocks, which must be joined tightly in order to avoid leakage. Each engraved microconduit has two holes on its ends that connect it with the manifold tubing. The best relative position of donor and acceptor chambers is with the acceptor chamber below the donor chamber in order to favor mass transfer.

2- The tubular module comprises two concentric tubes, the inner one being a porous tube of an appropriate polymer through which the donor stream (the sample) is circulated internally while the acceptor stream is circulated externally or vice versa.

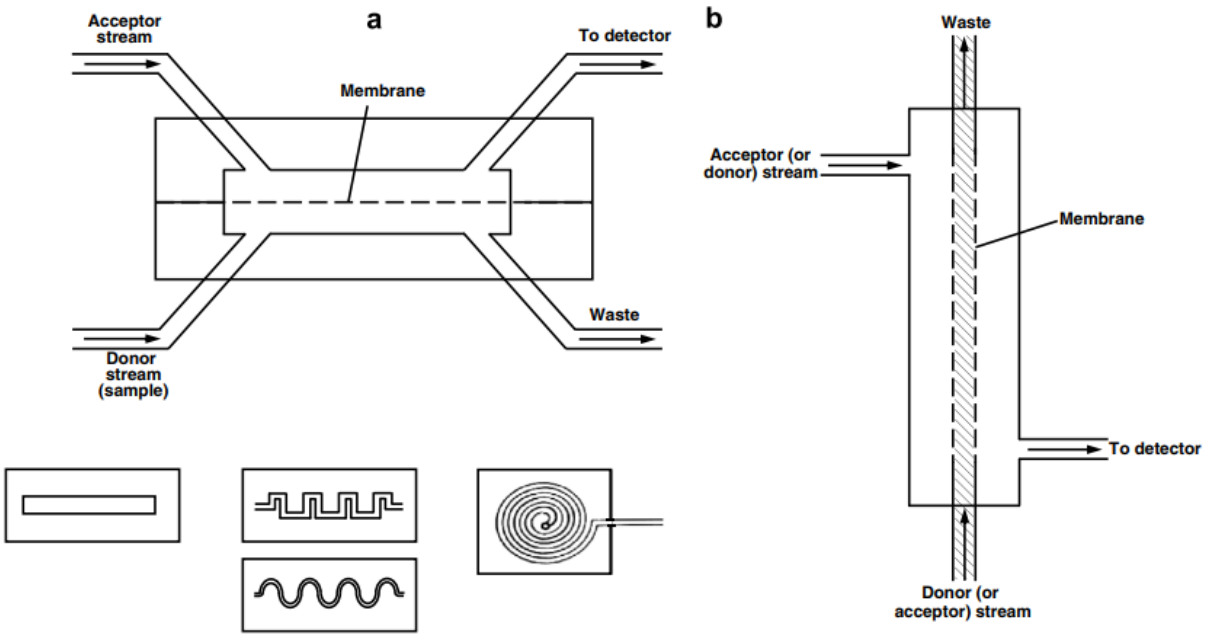

Figure 16 : Different types of dialyzers: (a) Sandwich, with different chamber designs: parallelepipedal, winding and spiral; and, (b) Tubular or hollow-fiber.

3-2- Electrodialysis

3-2-1- Definition and principle

Electro dialysis (ED) is usually defined as an electrochemical separation process in which electrically charged membranes and an electrical potential difference are used to separate ionic species from an aqueous solution and other uncharged components. Electrodialysis uses ion-

exchange membranes arranged alternately: cation-exchange membranes (CEM) and anion-exchange membranes (AEM), separated by a spacer frame that ensures the system's hydrodynamics and tightness.

Under the effect of an electric field, cations migrate toward the cathode: they leave the D compartments by passing through a cationic membrane (CEM), but they cannot leave the C compartments because they are blocked by an anionic membrane (AEM). As a result, the D compartments become depleted in salts and are called "dilution or desalination compartments." The C compartments become enriched in salts and are called "concentrate or brine compartments."

Current distribution is ensured by a pair of electrodes supplied with an electrolyte and separated from the dilute or concentrate streams by an end membrane. The redox chemical reactions at the electrodes are not taken into account in electrodialysis technology, unlike membrane electrolysis.

All these components are held between two plates under high clamping pressure, thus forming a reactor or stack. The unit pattern is called a cell. A single reactor can contain several hundred cells.

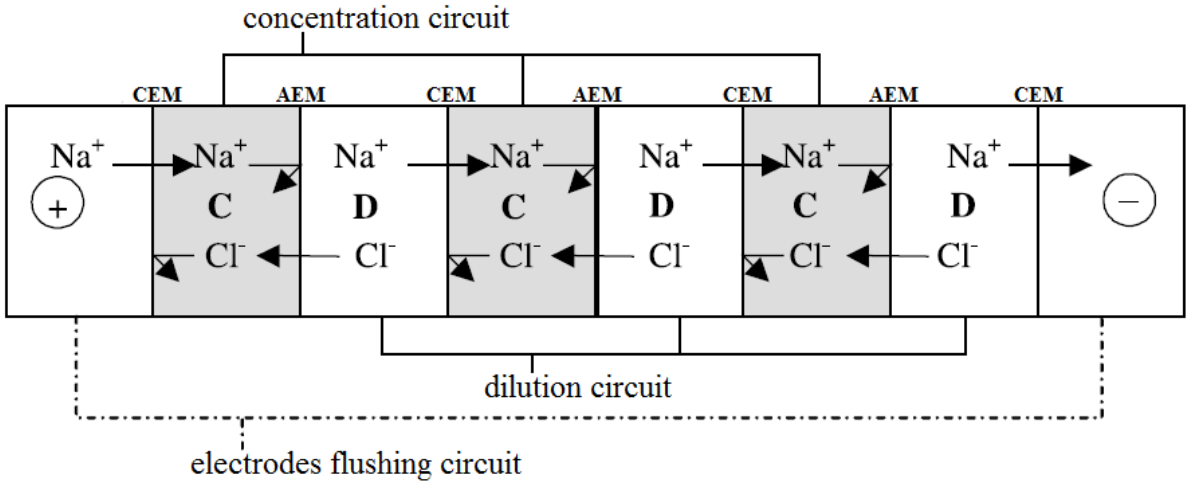

Figure17: Principle of Electro-dialysis.

3-2-2-Homopolar ion-exchange membranes

Ion-exchange membranes consist of a matrix onto which ionized functional groups are grafted: sulfonate groups for cation-exchange membranes and alkylammonium groups for anion-exchange membranes. These membranes can exhibit certain selectivities toward ions and organic anions depending on the selective layer on their surface or the degree of polymer crosslinking.

An ion-exchange membrane must have high permselectivity, high conductivity, and good mechanical and chemical stability. It is the improvement of these various properties that has enabled the development of new applications.

3-2-3- Driving forces and mass transport

The driving forces in electro dialysis are a result of the combined effects of electro migration and electro osmosis:

-**Electro migration:** The primary driving force, electro migration, is a consequence of the electric field applied across the electro dialysis cells. Ions experience an electric force proportional to their charge, leading to their migration towards the respective electrodes. This process is highly efficient and plays a pivotal role in achieving ion separation.

-Electro osmosis: Electro osmotic flow arises due to the movement of solvent molecules in response to the electric field. This flow can influence mass transport by aiding or hindering ion migration. Controlling and minimizing electro osmotic flow is essential to enhance separation efficiency.