Separation techniques (continue)

4- Gel filtration

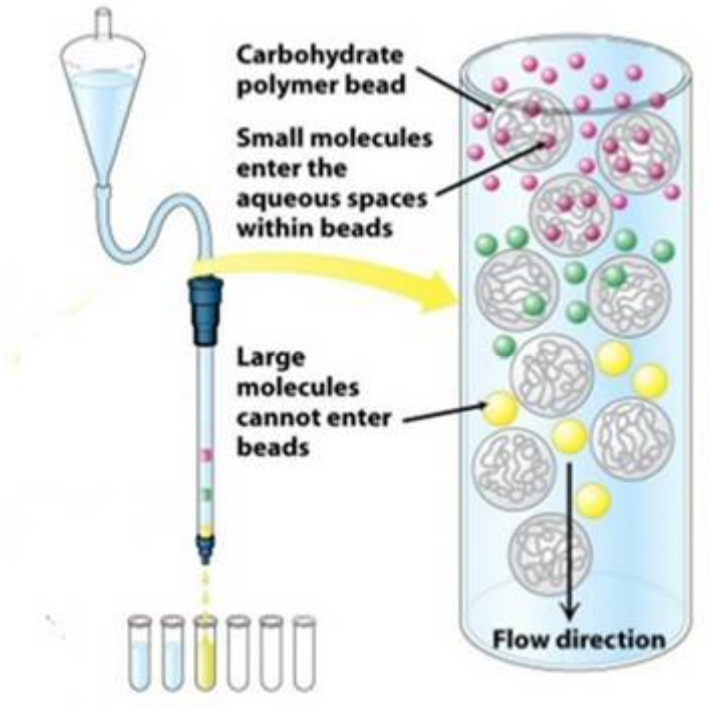
Gel filtration chromatography is also known as **gel permeation, molecular sieve, and size exclusion chromatography**. The molecules are separated based on their size. The column matrix is made up of **small spherical porous beads**. The smaller molecules can enter the pores present in the beads while the molecules larger than the maximum pore size of the bead are completely excluded. The access to the pores is determined by both the shape and the molecular weight of the molecules; the separation, therefore, is based on the ability of the molecules to enter the porous beads.

Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium.

principle

A mixture of molecules (to be separated) dissolved in liquid (the mobile phase) is applied to a chromatography column which contains a solid support in the form of microscopic spheres, or "porous beads" (the stationary phase). The mass of beads within the column is often referred to as the column bed. The beads act as "traps" or "sieves" and function to filter small molecules which become temporarily trapped within the pores

Larger molecules pass around or are "excluded" from the beads . Large sample molecules cannot or can only partially penetrate the pores, whereas smaller molecules can access most or all pores. Thus, large molecules elute first, smaller molecules elute later, while molecules that can access all the pores elute last from the column. Particles of different sizes will elute (filter) through a stationary phase at different rates.



A good stationary phase should have following properties:

- It should be chemically **inert and should not react with component to be separated.**
- It should be **inexpensive.**
- It should be **colorless, uniform in size and shape**.

Advantages

- Short analysis time.
- good separation.
- There is no sample loss.
- Small amount of mobile phase required.

Disadvantages

- Limited number of substances that can be separated.
- dust and other particulates may damage the columns and interfering with the detectors.

Applications:

- separation and Purification (viruses, enzymes, hormones, protein, polysaccharides and nucleic acid.
- Molecular weight determination

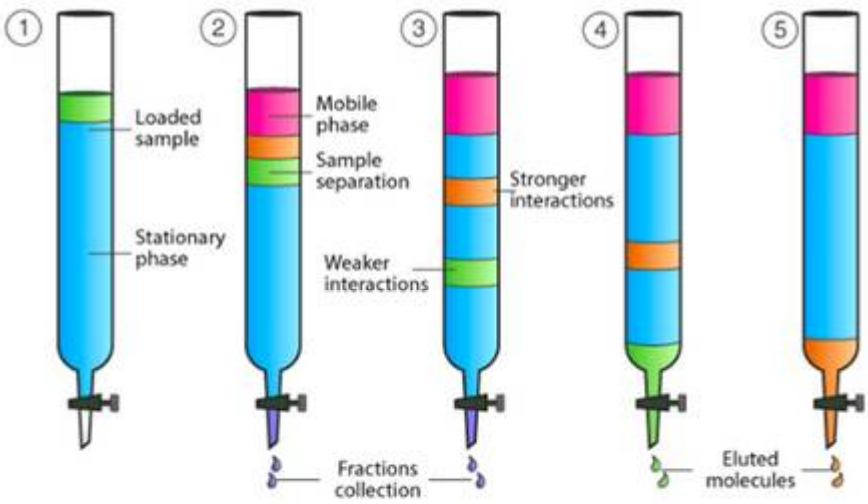
5- Column Chromatography

Column chromatography is a technique which is used to **separate a single chemical compound from a mixture dissolved in a fluid**. It separates substances based on **differential adsorption of compounds to the adsorbent** as the compounds move through the column at different rates which allow them to get separated in fractions.

Column Chromatography Principle

When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slow are eluted out last.

The adsorption of solute molecules to the column occurs in a reversible manner.



Column Chromatography Diagram

Mobile phase – This phase is made up of solvents as ethanol, acetone, water, etc.

and it performs the following functions:

1. It acts as a solvent – sample mixture can be introduced in the column.
2. It acts as a developing agent – helps in the separation of components in the sample
3. It acts as an eluting agent – the components that are separated during the experiment are removed from the column

Stationary phase – It is a solid material which should have good adsorption property and should be:

1. Stable and inert.
2. colorless, inexpensive and readily available.
3. Should allow free flow of mobile phase
4. It should be suitable for the separation of mixtures of various compounds.

Advantages Of Column Chromatography –

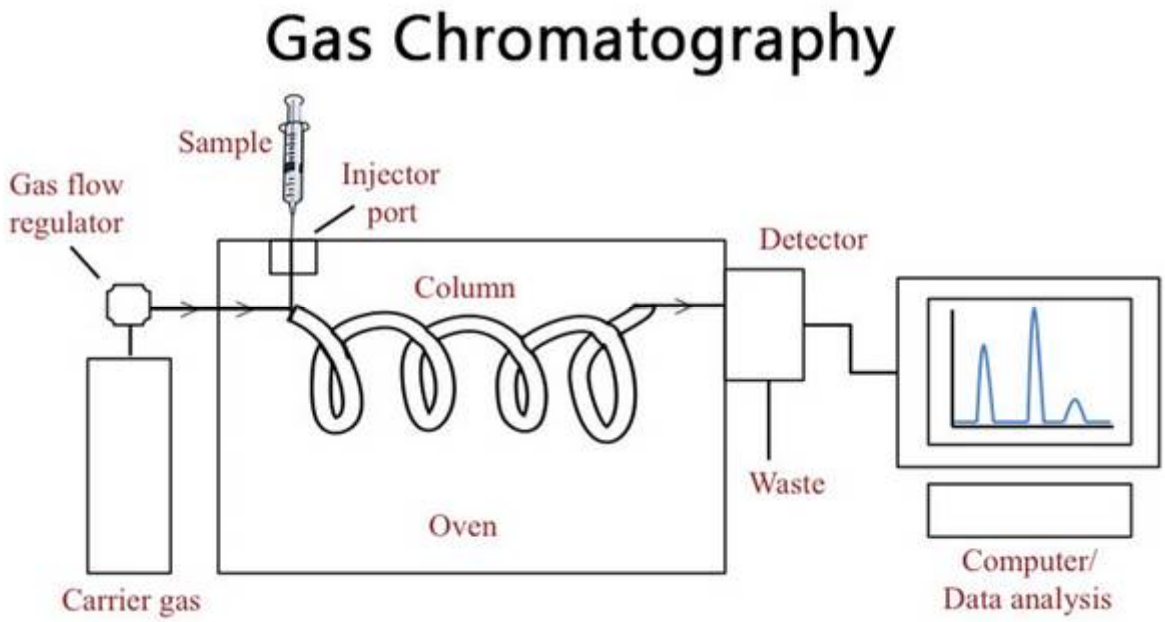
- All different kinds of complex mixtures can be separated by column chromatography.
- No limit for quantity as any amount of mixture can be separated by this technique.
- The separated analytes can be reused.
- This process can be automated.

Disadvantages Of Column Chromatography –

- It is a time-consuming process for the separation of compounds.
- It is expensive as higher quantities of solvents are required.
- The automated process becomes complicated and therefore costy.
- It has a low separation power.

6- Gas chromatography

Gas chromatography is a technique 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.
When the stationary phase is a solid adsorbent, the process is termed **gas–solid chromatography (GSC)**, • When it is a liquid the process is termed **gas–liquid chromatography (GLC)**.



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.

Advantages:

1. This method is accurate with high resolution power
2. This technique gives relatively good accuracy and precision.
3. Separation and analysis of sample very quickly. Sample with less quantity is also separated.

Disadvantages:

1. Only volatile samples or the sample which can be made volatile are separated by this method.
2. The sample of gas which is about to inject must be thermally stable so that it does not get degraded when heated.

7-High-performance liquid chromatography

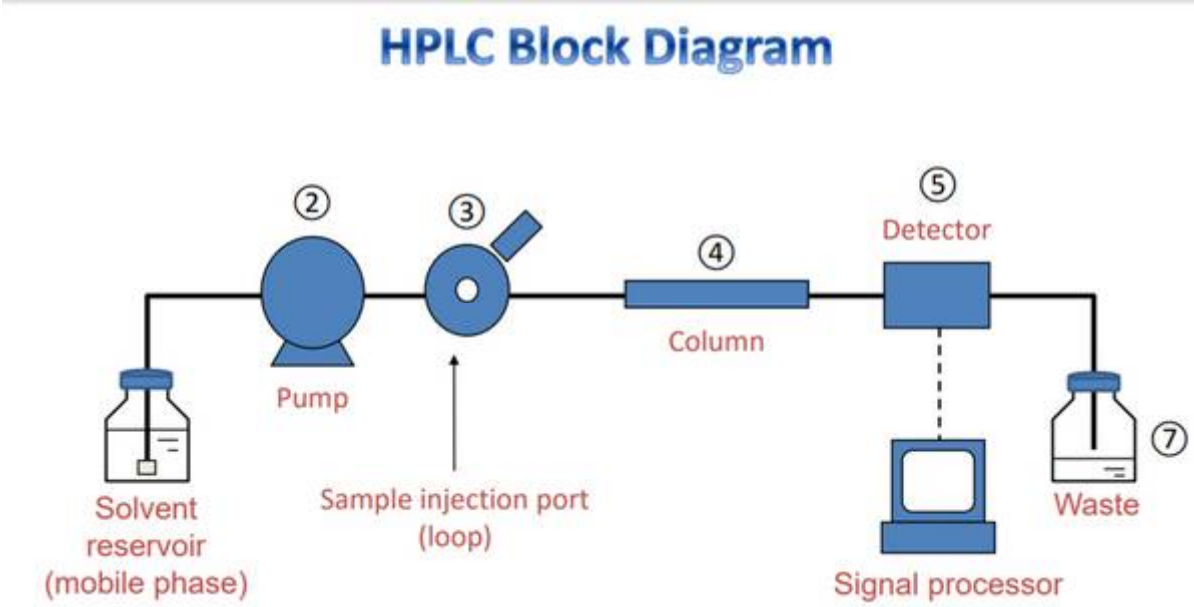
High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a chromatographic technique that can separate a mixture of compounds and is used to identify, quantify and purify the individual components of the mixture.

The technique is based on the pumping of mobile phase through the packed column (containing the stationary phase) under high-pressure. Hence it is also called as high-pressure liquid chromatography.

Principle:

High Performance Liquid Chromatography [HPLC] principle is based on adsorption as well as partition chromatography is depending on the nature of stationary phase, if stationary phase is solid principle is based on adsorption chromatography and if stationary phase is liquid principle is based on partition chromatography. ′ It is important for determination of volatile and non volatile compounds and for qualitative and quantitative analysis.

HPLC utilizes the mobile phase, a pump that provides the higher pressure required to moves the mobile phase and the sample through the column (containing the stationary phase), and a detector that provides a characteristic retention time for the analyte.



Types Of HPLC Techniques

HPLC techniques are classified on the following types:

1- Based on the polarity of stationary and mobile phase:

Normal phase mode: Stationary phase is polar e.g., silica gel and mobile phase is non-polar.

Reverse phase mode: Stationary phase is non-polar and mobile phase is polar.

In normal phase mode, non-polar compounds travel faster and are eluted first. This is because of less affinity between solute and stationary phase. Polar compounds are retained longer time in the column because of more affinity towards stationary phase and take more time to elute.

In reverse phase mode, polar compounds get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature and not retained for a longer time and eluted faster.

2- Based on elution technique:

- Isocratic separation: Same mobile-phase combination is used throughout the process of separation.
- Gradient separation: Mobile-phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.

Advantages:

- It is simple method with high sensitivity.
- Accurate quantitative measurements..
- Rapid process and hence time saving.
- It can be applied to the separation and analysis of very complex mixtures.

8- ELECTROPHORESIS

Electrophoresis is a separation technique based on the movement of charged particles through an electrolyte when subjected to an electric Field. Cations move towards cathode and Anions move towards anode. By this technique solutes are separated by their different rates of travel through an electric field. Commonly used in biological analysis, particularly in the separations of proteins, peptides and nucleic acids, these biological molecules possess ionisable groups and, therefore, at any given pH, exists in solution as electrically charged species either as cations or anions. Under the charge of an electric field these charged particles will migrate either to cathode or to anode, depending on the nature of their net charge.

Factors affecting Electrophoresis

The rate of migration of a solute in an electric field depends on the following factors:

1) Net charge on the particle.

2) Mass and shape of the particles.

3) pH of the medium.

4) Strength of electric field.

5) Temperature.

Migration of the particles is directly proportional to net charge and inversely proportional to molecular size and viscosity of the electrophoresis medium.

Electrophoresis apparatus consists of

1) Buffer tank -to hold the buffer

2) Buffer

3) Electrodes- made of platinum or carbon

4) Power supply

5) Support media on which separation takes place.

Support media for electrophoresis

1) Filter Paper

2) Cellulose acetate membrane

3) Agar or Agarose gel

4) Starch Gel

5) Polyacrylamide gel

Electrophoresis Procedure (where the support medium is filter paper):

1) filter paper is moistened with a suitable buffer solution of the desired pH and the sample is applied transversely across the central part of the strip.

2) Ends are fixed to dip in buffer solutions in two containers fitted with electrodes.

3)Electric field is established.

4)The charged particles of sample migrate along the strip towards respective electrodes of opposite polarity, according to net charges, sizes and interactions with the solid matrix.

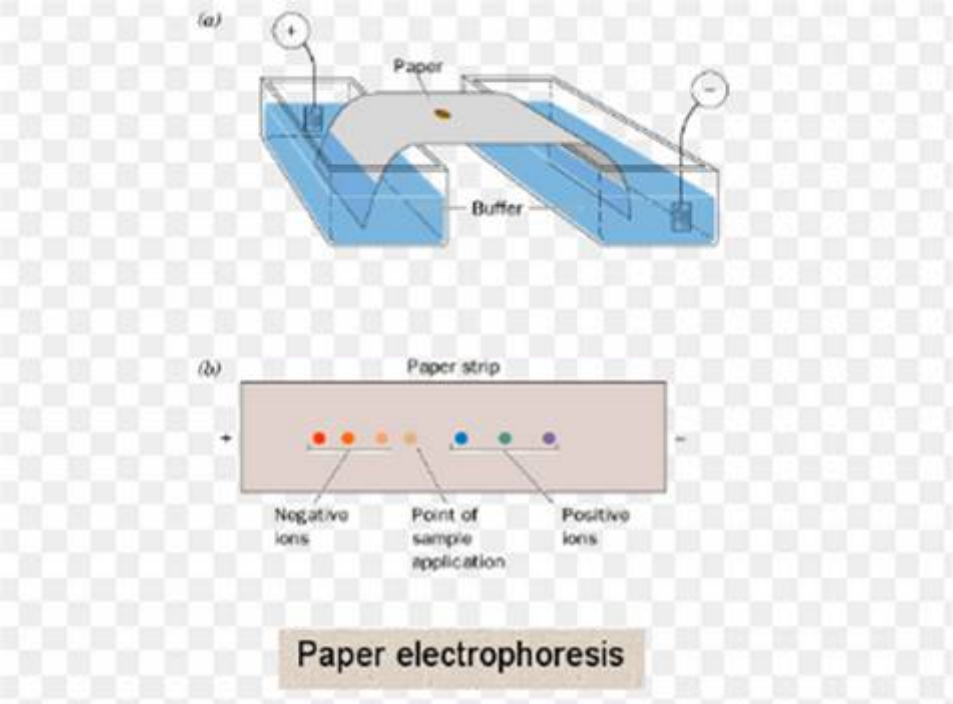
5)Homogeneous group of particles migrate as a separate band.

6)The electrophoresis is carried out for 16-18 hours.

7) Separated Proteins are fixed to a solid support using a fixative such as Acetone or Methanol.

8)Proteins are stained to make them visible

9) The separated proteins appear as distinct bands.



Paper electrophoresis

Advantages of electrophoresis:

1. **High separation efficiency.**
2. **Short analysis time.**
3. **Low sample and electrolyte consumption.**
4. **Ease of operation.**

9- Affinity Chromatography

Affinity chromatography is a type of liquid chromatography used for separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. used primarily for biological molecules such as **proteins and nucleic acids, the separation is achieved by specific interactions of those molecules with a component known as a ligand.**

Principle of Affinity Chromatography

Affinity chromatography is based on highly specific biological interactions between two molecules such as interactions between **enzyme and substrate, receptor and ligand, or antibody and antigen.** These interactions which are typically revesible are used for purification by placing one of the interacting molecules referred to as affinity ligand onto a solid matrix to create a stationary phase while a target molecule is in the mobile phase.

Note: Buffer is used for formation of complex between a matrix and ligand.

In order to for the matrix to be effective it must have certain characters:

1. **It must be insoluble in solvents and buffers employed in the process**
2. **It must be chemically stable..**
3. **It must be easily coupled to the ligand.**
4. **It must exhibit good flow properties and have a relatively large surface area for attachment**

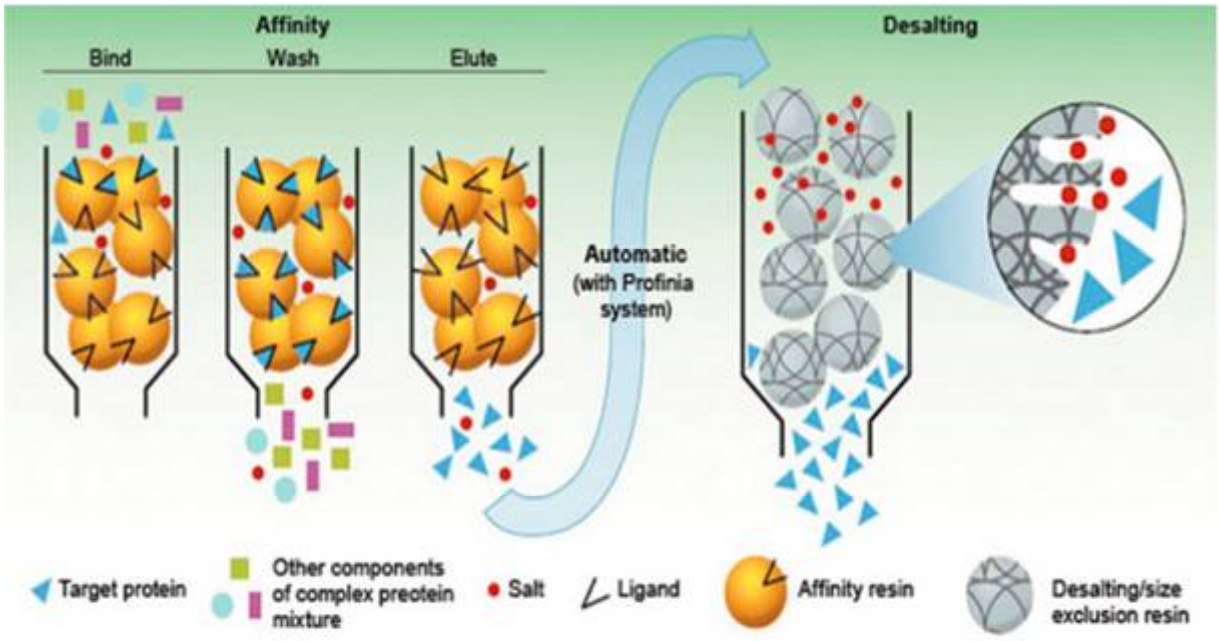
Immobilized Ligand

The ligand can be selected only after the nature of the macromolecule to be isolated is known.

For e.g.

- When a hormone receptor protein is to be purified by affinity chromatography, the hormone itself is an ideal candidate for the ligand.
- For antibody isolation ,an antigen may be used as ligand.

As the mixture of the substances is passed through the chromatography column, substances 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.



Principle of Affinity Chromatography

Application of Affinity chromatography includes:

- **It is used for isolation and purification of all biological macromolecule. E.g. nucleic acid, antibodies, enzymes. etc. .**
- **Removal of impurities.**
- **Detection of substrates**

Advantages of Affinity Chromatography

1. **High specificity**
2. **Target molecules can be obtained in a highly pure state**
3. **Single step purification**
4. **Give purified product with high yield.**

Limitations of Affinity Chromatography

1. **Time consuming method.**
2. **More amounts of solvents are required which may be expensive.**
3. **Non-specific adsorption cannot be totally eliminated, it can only be minimized.**
4. **Proteins get denatured if required pH is not adjusted.**

THANK YOU