Chromatography

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What is Chromatography ?

- The word chromatography originated from two Greek words *Chroma* which means “color” and *graphy* which means “writing”
- the separation of mixtures into their constituents by preferential adsorption by a solid”
- “Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of the phases constituting a Stationary bed of large surface area, the other being a fluid that percolates through or along the stationary bed.”

History of Chromatography

- 1903 - Mikhail Tswett separated plant pigments using paper chromatography **liquid-solid chromatography**
- 1930's - Schuftan & Eucken used vapor as the mobile phase - **gas solid chromatography**
- 1950 - GLC Invented by Martin & Synge
- 1970s - Attachment of Electronic data processing equipment
- 1980s - Use of computer bed automatic control of most of the parameter such as column temperature, flow rate, sample injection & data processing development 1967 - First time used for the measurement of N_2-Fixation

Principle

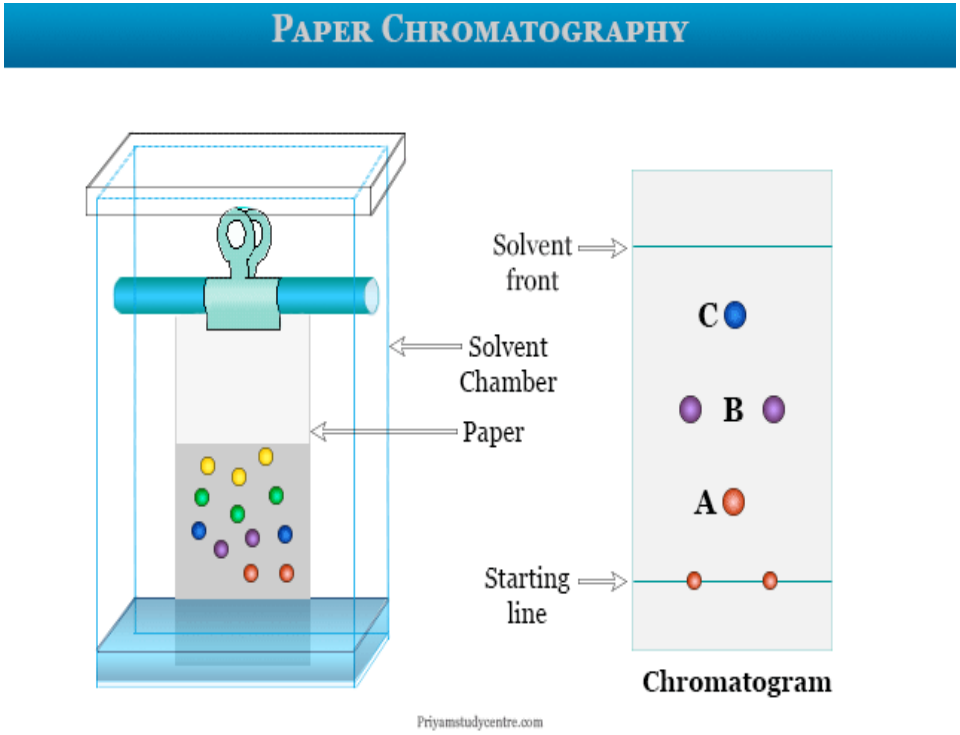
- Chromatography is based on very simple principle
- The sample to be examined called the solute or analyte is allowed to interact with two immiscible phase a mobile phase and a stationary phase.
- Two immiscible phase could be a solid and a liquid or a gas and a liquid or a liquid and another liquid
- The stationary phase is a porous solid matrix through which the sample contained in mobile phase percolates
- The interaction between the mobile phase and the stationary phase results in the separation of the compound from the mixture.

Types of Chromatography

- Paper chromatography
- Thin layer chromatography
- Column chromatography
- Ion exchange chromatography
- Gel Filtration Chromatography

Paper Chromatography (PC)

PC is considered to be the simplest chromatography technique because of its applicability to isolation and identification of organic and inorganic compound .

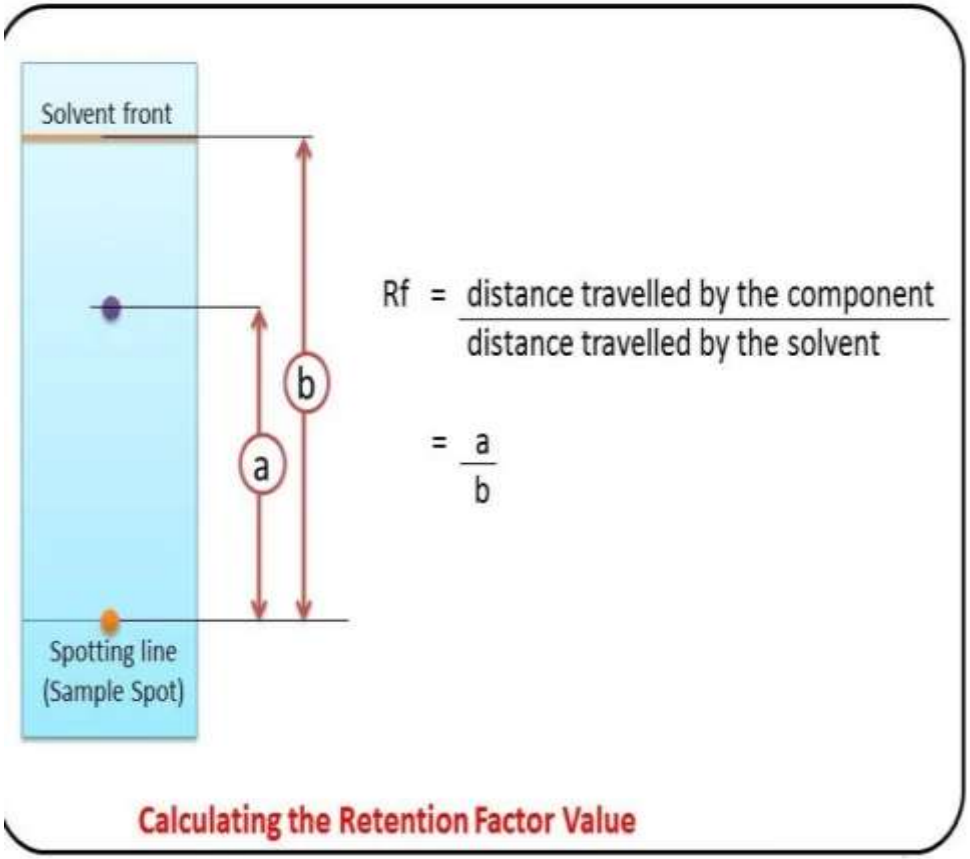


Principle

- The principle of separation is mainly partition rather than adsorption
- Substances are distributed between a stationary phase and mobile phase
- Cellulose layer in filter paper contain moisture which cuts as stationary phase .organic solvents/buffer are used as mobile phase
- Components of the sample will separate readily according to how strongly they adsorb onto the stationary phase versus how readily they dissolve in mobile phase

Rf Value

- Some compounds in a mixture travel almost as far as the solvent does, some stay closer to the base line
- The distance travelled relative to the solvent is a constant for a particular compound as long as other parameters such as type of paper and the exact composition of the solvent are constant
- The distance relative to the solvent is called the Rf value



Application:

- To check the control of purity of pharmaceuticals
- For the detection of drugs and dopes in animals and human
- Analysis of the reaction in biochemical labs

Advantage

- Rapid
- Simple
- Paper Chromatography requires very less quantitative material
- Paper Chromatography is cheaper compare to other chromatography methods

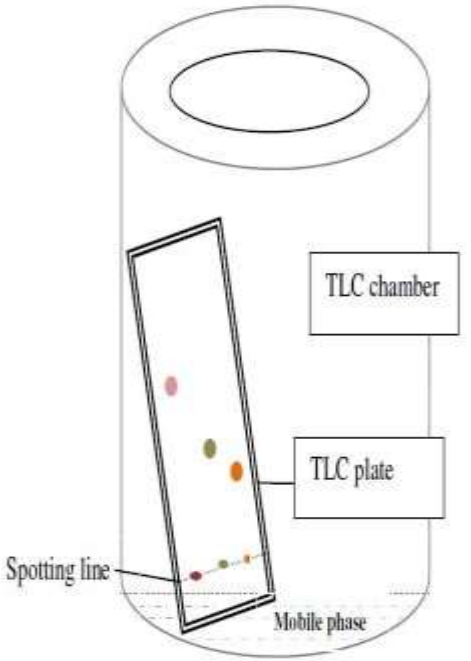
Limitaions

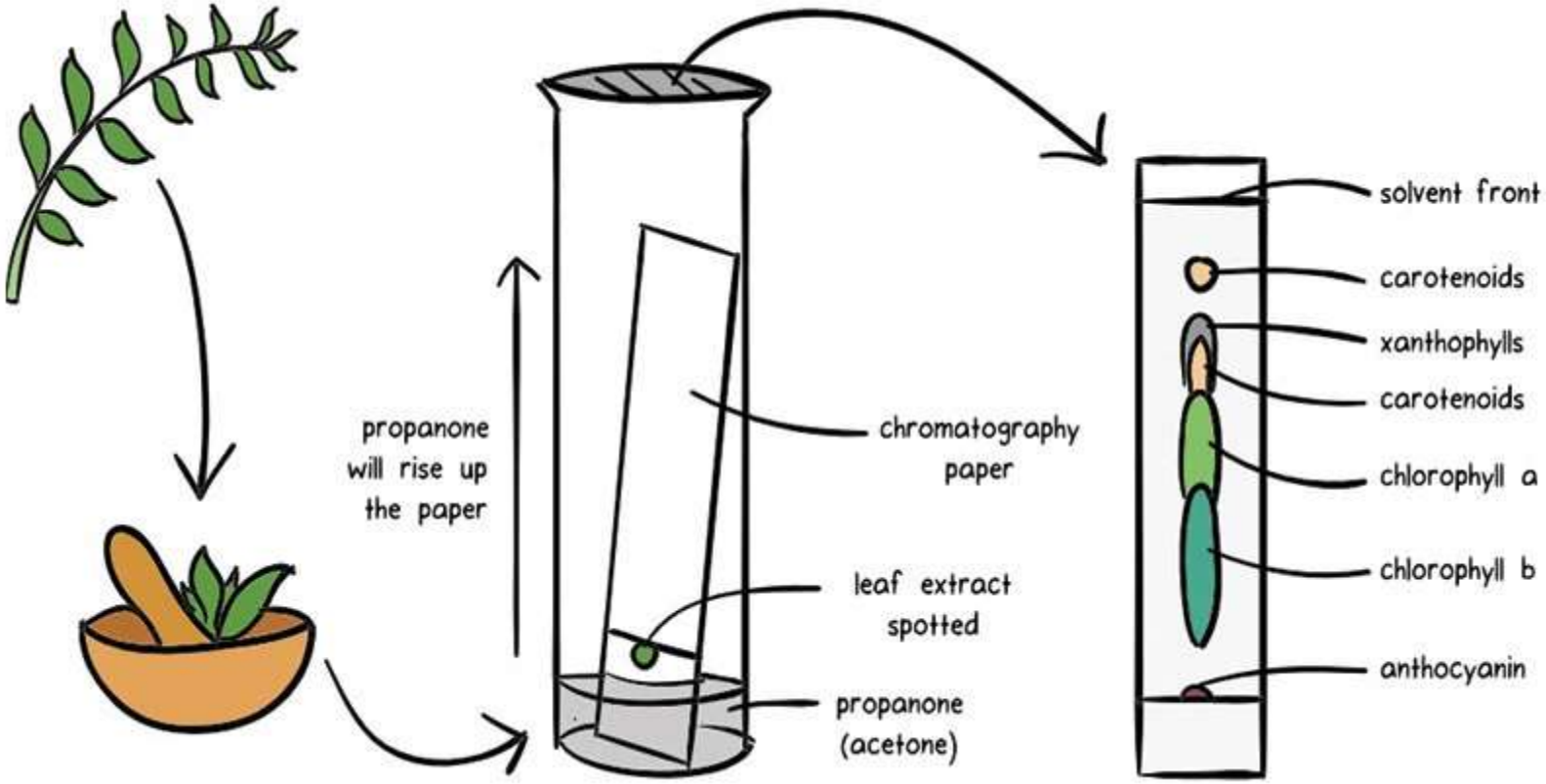
- Large quantity of sample cannot be applied
- In quantitative analysis paper chromatography is not effective
- Complex mixture cannot be separated by paper chromatography
- Less accurate to HPLC

Thin Layer Chromatography (TLC)

Principle

- TLC is performed on a sheet of glass, plastic or aluminum plate which is coated with a thin layer of adsorbent material usually silica gel, aluminum oxide or cellulose. This layer of adsorbent known as stationary phase
- After a sample is applied on the plate or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action because different analysis ascend the TLC plate at different rates
- It is thus based on the adsorption chromatography or partition chromatography or combination of both
- The components with more affinity towards stationary phase travels slower and vice versa.





Preparation of TLC plate

Techniques used for visualizing the TLC plate

- Ultraviolet
- Iodine staining, it is very useful in detecting carbohydrate since it turns black on contact with iodine
- $KMNO_4$ stain (organic molecules)
- Ninhydrin reagent often used to detect amino acids and protein

Applications

- Determine the purity of substance
- Identification of medicinal plants and their constituents
- Detection of pesticide or insecticides in food and water

Limitations

- It cannot tell the difference between enantiomers and some isomers
- TLC plates do not have long stationary phase therefore length of separation is limited

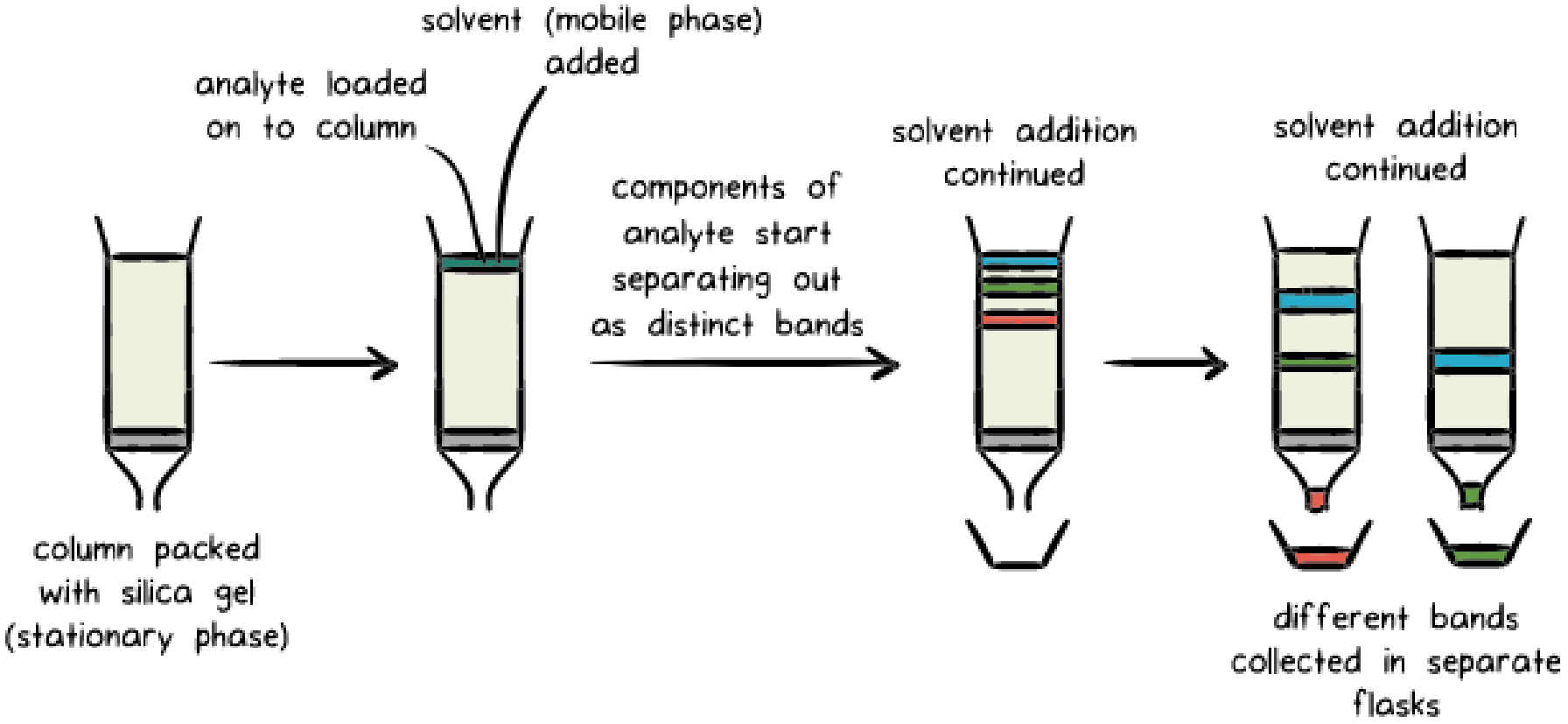
Column Chromatography

- Column chromatography is a separation technique in which the stationary bed is within a tube.

- The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column).

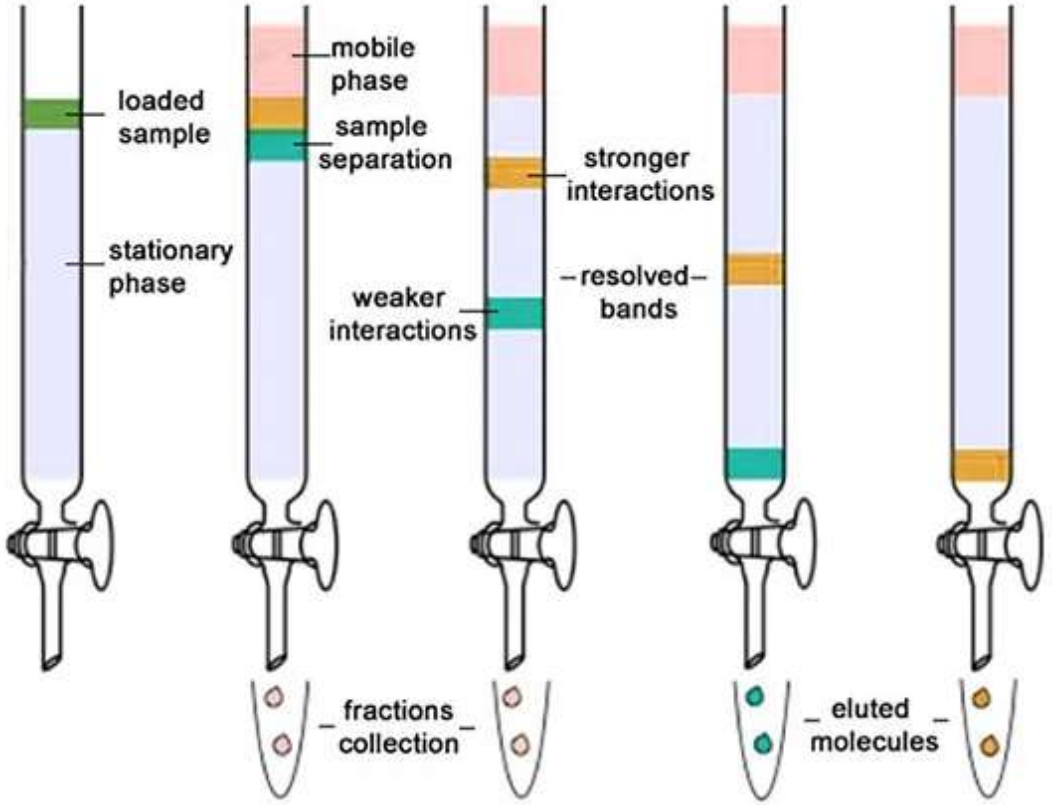
Principle

- The principle involved in column chromatography is adsorption of the solutes of a solution through stationary phase and separates the mixture into individual component.
- This is based on the affinity towards the mobile phase and stationary phase.



Procedure of column chromatography

Column Chromatography



Application

- Separation of mixture of compounds
- Purification process (removal of impurities)
- Isolation of active constituents
- Estimation of drugs in formulation
- Isolation of active constituents

Advantage

- Any type of mixture can be separated
- Any quantity of mixture can be separated
- Wider choice of Mobile Phase

Disadvantage

- Time consuming
- More amount of Mobile Phase are required
- Automation makes the techniques more complicated & expensive

Ion Exchange Chromatography

- Ion Exchange Chromatography is a process that allows the separation of ions and polar molecules based on their affinity to ion exchanger.
- In this process two types of exchangers a) cationic and b) anionic can be used

Principle

- This form of chromatography relies on the attraction between oppositely charged stationary phase, known as an ion exchanger, and analyte.
- There are two types of ion exchanger, namely cation and anion exchangers.
- Cation exchangers possess negatively charged groups and these will attract positively charged cations. These exchangers are also called acidic ion exchangers because their negative charges result from the ionisation of acidic groups.
- Anion exchangers have positively charged groups that will attract negatively charged anions.

- The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.
- The charged groups of the matrix can be positively or negatively charged.
- When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge.
- In this “ion cloud”, ions can be reversibly exchanged without changing the nature and the properties of the matrix**.**
- It is frequently chosen for the separation and purification of proteins, peptides, nucleic acids, polynucleotides and other charged molecules, mainly because of its high resolving power and high capacity.

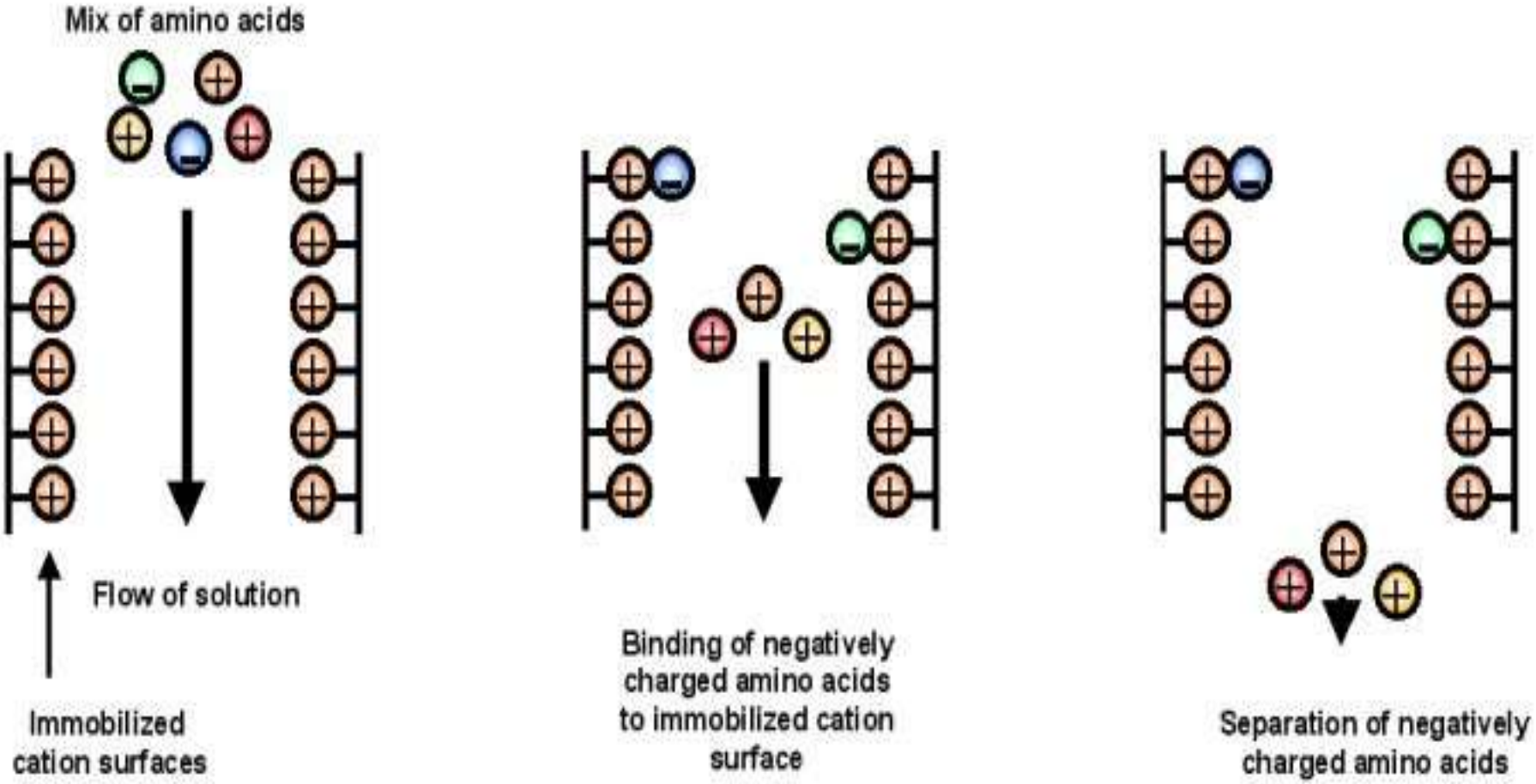
Choice of exchanger

- depends upon the stability of the test analytes, their relative molecular mass and the specific requirements of the separation.
- Many biological analytes, especially proteins, are stable within only a fairly narrow pH range so the exchanger selected must operate within this range. Generally, if an analyte is most stable below its isoionic point (giving it a net positive charge) a cation exchanger should be used, whereas if it is most stable above its isoionic point (giving it a net negative charge) an anion exchanger should be used.
- Either type of exchanger may be used to separate analytes that are stable over a wide range of pH values.

Table 11.3 **Examples of commonly used ion exchangers**

Type	Functional groups	Functional group name	Matrices
Weakly acidic (cation exchanger)	$-COO^-$	Carboxy	Agarose
	$-CH_2COO^-$	Carboxymethyl	Cellulose
			Dextran
			Polyacrylate
Strongly acidic (cation exchanger)	$-SO_3^-$	Sulpho	Cellulose
	$-CH_2SO_3^-$	Sulphomethyl	Dextran
	$-CH_2CH_2CH_2SO_3^-$	Sulphopropyl	Polystyrene
			Polyacrylate
Weakly basic (anion exchanger)	$-CH_2CH_2N^+H_3$	Aminoethyl	Agarose
	$-CH_2CH_2N^+H$ $\vert$ $(CH_2CH_3)_2$	Diethylaminoethyl	Cellulose
			Dextran
			Polystyrene
			Polyacrylate
Strongly basic (anion exchanger)	$-CH_2N^+(CH_3)_3$	Trimethylaminomethyl	Cellulose
	$-CH_2CH_2N^+(CH_2CH_3)_3$	Triethylaminoethyl	Dextran
	$-CH_2N^+(CH_3)_2$ $\vert$ CH_2CH_2OH	Dimethyl-2-hydroxyethyl-aminomethyl	Polystyrene
	$-CH_2CH_2N^+(CH_2CH_3)_2$ $\vert$ $CH_2CH(OH)CH_3$	Diethyl-2-hydroxypropyl-aminoethyl	

Ion-exchange chromatography (anion exchange)



Application

- An important use of ion-exchange chromatography is in the routine analysis of amino acid mixtures.
- The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated and used in clinical diagnosis.
- In the analysis of products of hydrolysis of nucleic acids. In this way, information is gained about the structure of these molecules and how it relates to their biological function as carriers of hereditary information.

Advantage

- It is one of the most efficient methods for the separation of charged particles.
- It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids

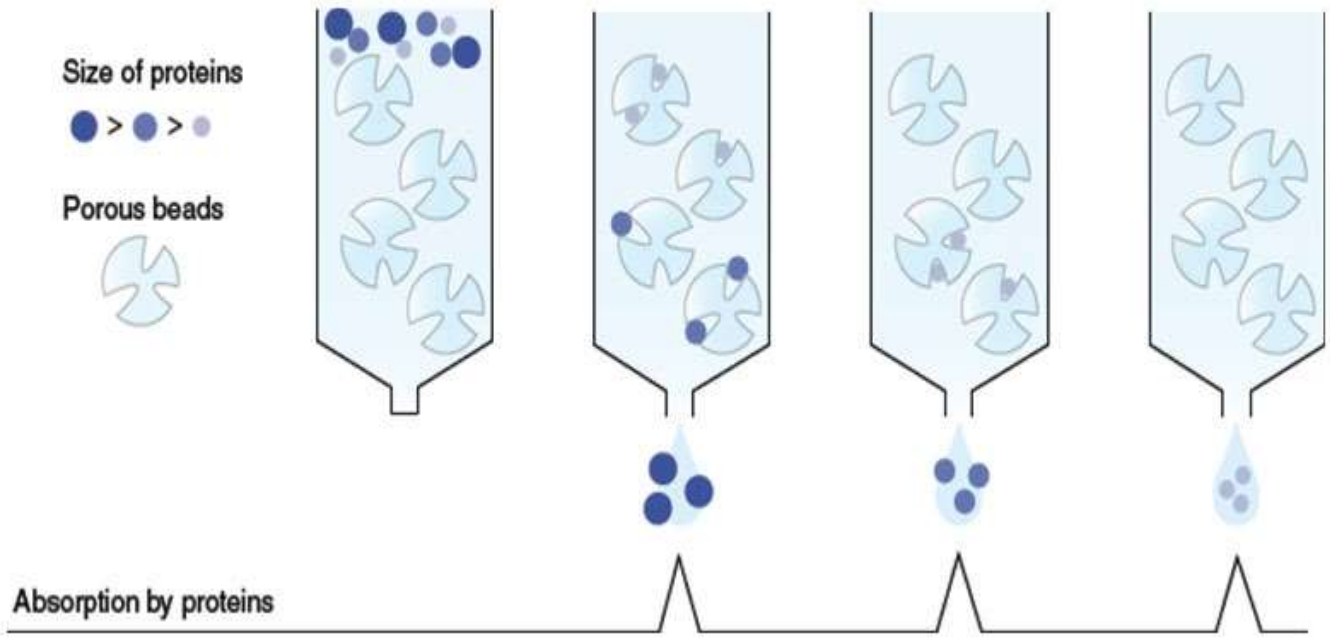
Limitation

- Only charged molecules can be separated.

Gel Filtration Chromatography

- Biomolecules are purified using different techniques that separate them according to the differences in their specific properties such as size, hydrophobicity, biorecognition, charge, etc.
- Also known as Size Exclusion Chromatography.
- Gel filtration is a technique in which the separation of components is based on the difference in molecular weight or size.
- It is the simplest and mildest of all the **chromatography** techniques and separates molecules on the basis of differences in size.

Gel Filtration Chromatography



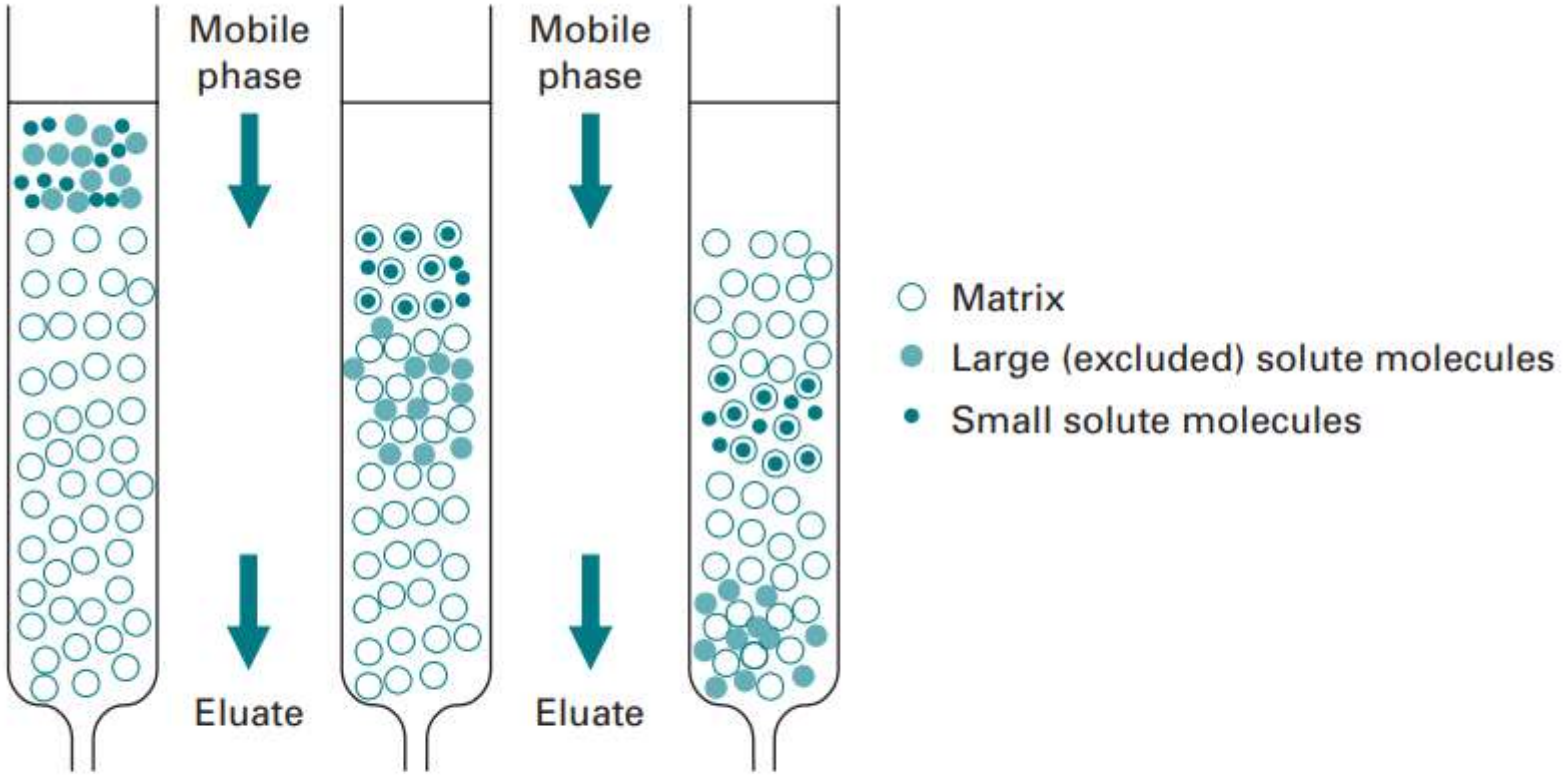


Fig. 11.9 Separation of different size molecules by exclusion chromatography. Large excluded molecules are eluted first in the void volume.

To perform a separation, the gel filtration medium is packed into a column to form a packed bed. The medium is a porous matrix in the form of spherical particles that have been chosen for their chemical and physical stability, and inertness (lack of reactivity and adsorptive properties). The packed bed is equilibrated with a buffer which fills the pores of the matrix and the space in between the particles. The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in equilibrium with the liquid outside the particles, referred to as the mobile phase.

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

Steps in Gel Filtration Chromatography

1. Spherical particles of gel filtration medium are packed into a column.
2. The sample is applied to the column.
3. Buffer (mobile phase) and sample move through the column.
4. Molecules diffuse in and out of the pores of the matrix (also described as the partitioning of the sample between the mobile phase and the stationary phase).
5. Smaller molecules move further into the matrix and so stay longer on the column.
6. As buffer passes continuously through the column, molecules that are larger than the pores of the matrix are unable to diffuse into the pores and pass through the column.
7. Smaller molecules diffuse into the pores and are delayed in their passage down the column.
8. Separation occurs at different intervals which are followed by detection of components.

Applications of Gel Filtration Chromatography

- Gel filtration plays a key role in the purification of enzymes, polysaccharides, nucleic acids, proteins, and other biological macromolecules.
- Gel filtration can also be used to facilitate the refolding of denatured proteins by careful control of changing buffer conditions.
- It is used in protein fractionation experiments.
- Gel filtration technique is also used in molecular weight determination.
- Separation of sugar, proteins, peptides, rubbers, and others on the basis of their size.
- Can be used to determine the quaternary structure of purified proteins.

Advantages of Gel Filtration Chromatography

- Well suited to handling biomolecules that are sensitive to changes in pH, the concentration of metal ions or co-factors and harsh environmental conditions.
- Conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation.
- Separations can be performed in the presence of essential ions or cofactors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37 °C or in the cold room according to the requirements of the experiment.
- Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks).
- Short analysis time.
- Well defined separation.
- Narrow bands and good sensitivity.
- There is no sample loss.
- The small amount of mobile phase required.
- The flow rate can be set


Thank you