Chapter 5 : Chromatographic methods

Chromatography is the collective term for a set of laboratory techniques for the separation of mixtures into their components. All forms of chromatography work on the same principle. They all have a stationary phase (a solid or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The mobile phase flows through the stationary phase and carries the components of the mixture with it. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. There are different types of chromatography such as Column chromatography, Paper chromatography etc.

1- Column chromatography

1-1- Principale

Column chromatography involves packing a column with a stationary phase, typically silica gel or alumina, which interacts with the compounds in the mixture as they are eluted through the column by a solvent (the mobile phase). The separation occurs based on various properties such as polarity, size, and molecular weight, leading to distinct bands of different components.

When a mixture is applied to the top of the column, different components interact with the stationary phase to varying extents. Those that have a stronger affinity for the stationary phase will move more slowly, while those that have a weaker affinity will move faster. This difference in movement leads to the separation of components as they travel through the column.

As the mobile phase continues to flow, the separated components exit the column at different times. This process is called elution, and it can be monitored to collect fractions containing the desired compounds.

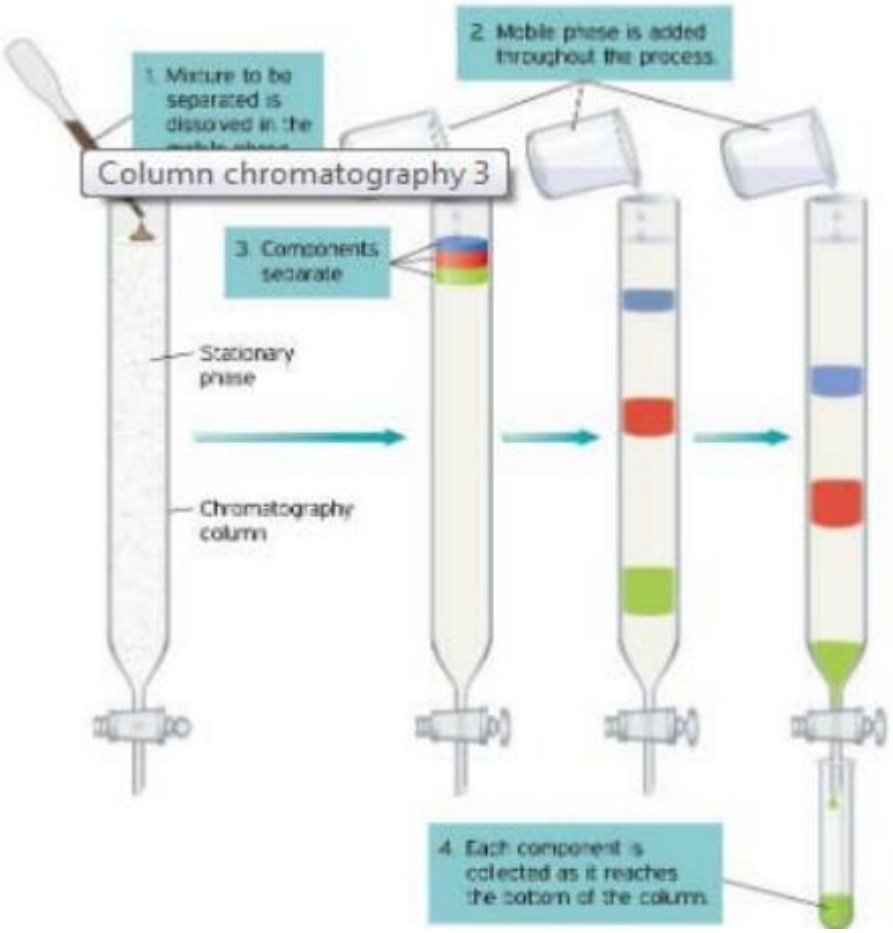

Figure 1 : Column chromatogprahic.

1-2- Types

The choice of chromatography column is highly dependent on the type of chromatography being employed. Columns vary based on the chemical nature of their stationary phase, and each type offers unique advantages for different types of separations. The most common types of chromatography include:

1-2-1- Normal-Phase Chromatography (NPC)

Principle : In normal-phase chromatography, the stationary phase is polar (e.g., silica gel or alumina), and the mobile phase is nonpolar (e.g., hexane or chloroform).

Mechanism: Compounds are separated based on their polarity. Polar compounds have a stronger interaction with the stationary phase and elute more slowly, while nonpolar compounds elute faster.

Applications: Used for separating polar compounds, such as alcohols, amines, and carboxylic acids. Common in the analysis of natural products and organic compounds.

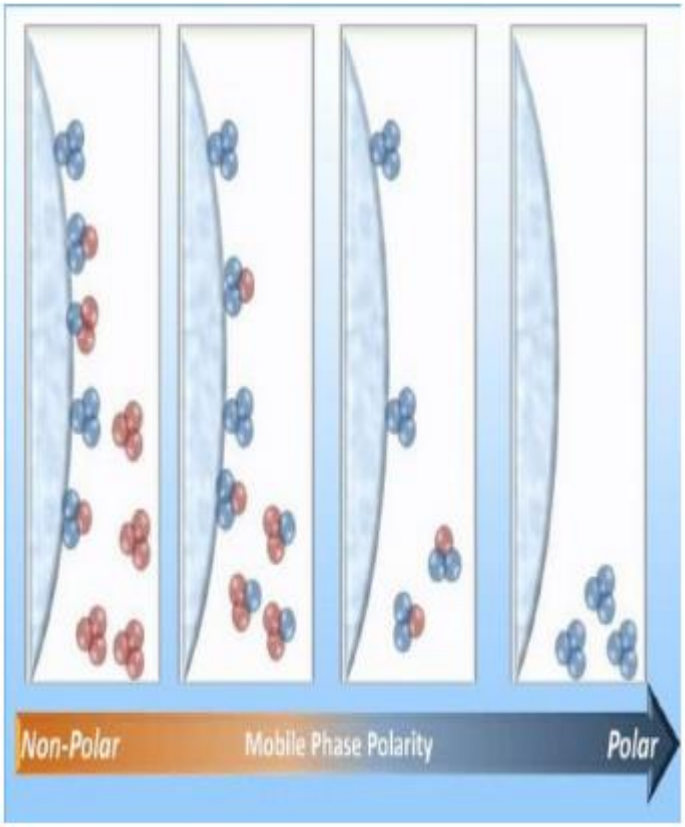

Figure 2 : Normal-Phase Chromatography (NPC)

1-2-2- Reversed-Phase Chromatography (RPC)

Principle: In reversed-phase chromatography, the stationary phase is nonpolar (e.g., C18-bonded silica), while the mobile phase is polar (e.g., water or a water-organic solvent mixture).

Mechanism: Compounds are separated based on their hydrophobicity. Nonpolar compounds interact strongly with the nonpolar stationary phase and elute slower, while polar compounds elute faster.

Applications: Widely used for pharmaceuticals, biomolecules (proteins, peptides, nucleotides), and environmental samples. It is the most common type of chromatography in analytical chemistry.

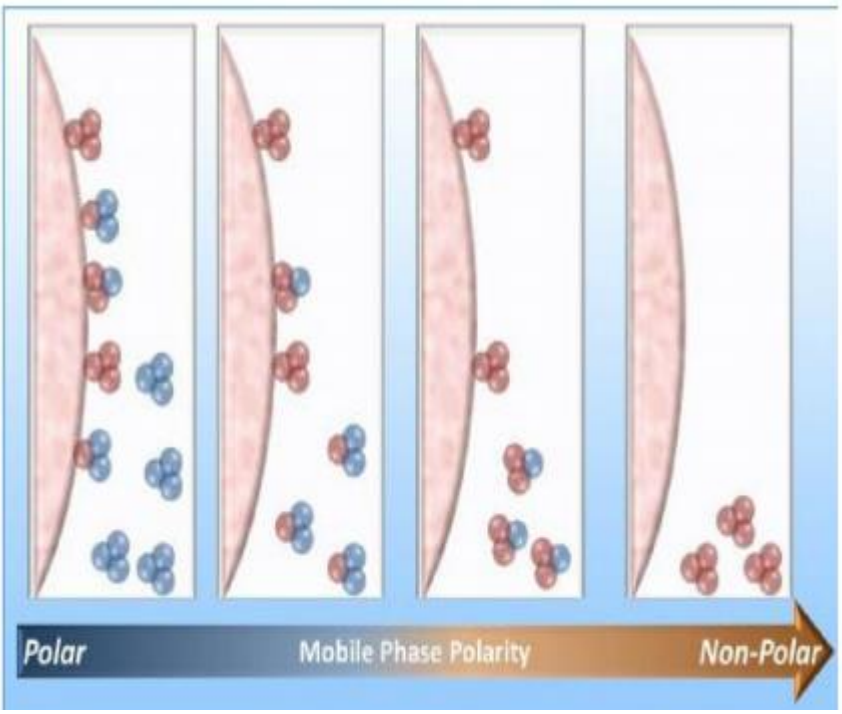

Figure 3 : Reversed-Phase Chromatography (RPC).

1-2-3- Size-Exclusion Chromatography (SEC)

Principle: Also known as gel filtration or gel permeation chromatography, SEC separates molecules based on their size.

Mechanism: The stationary phase consists of porous beads. Larger molecules are excluded from the pores and elute first, while smaller molecules enter the pores and elute later.

Applications: Commonly used for purifying proteins, polymers, and other macromolecules, as well as analyzing molecular weight distributions.

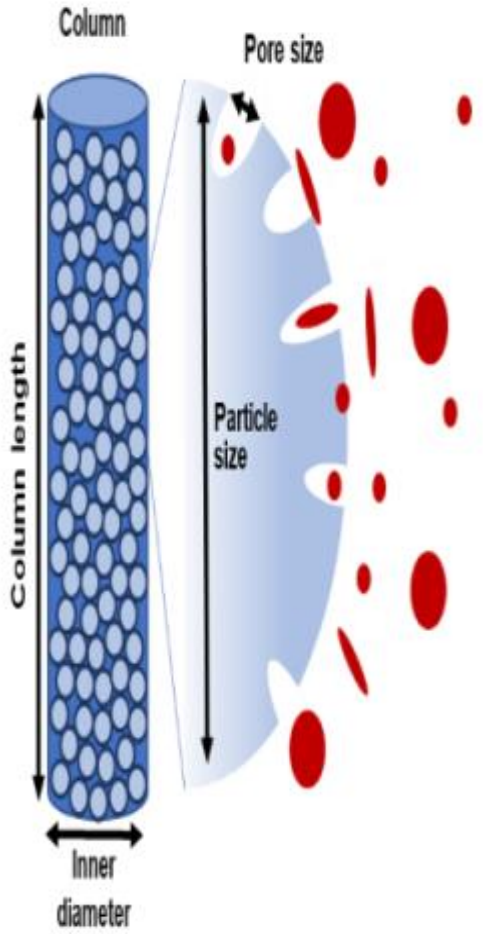

Figure 3 : Size-Exclusion Chromatography (SEC).

1-2-4- Ion-Exchange Chromatography (IEC)

Principle: This type separates charged particles based on their ionic interactions with the stationary phase, which is charged (either cationic or anionic).

Mechanism: Cation-exchange chromatography uses a negatively charged stationary phase to attract and retain positively charged ions (cations), while anion-exchange chromatography uses a positively charged stationary phase for negatively charged ions (anions).

Applications: Widely used for purifying proteins, nucleic acids, and other biomolecules, as well as in water treatment and analysis of ionic species.

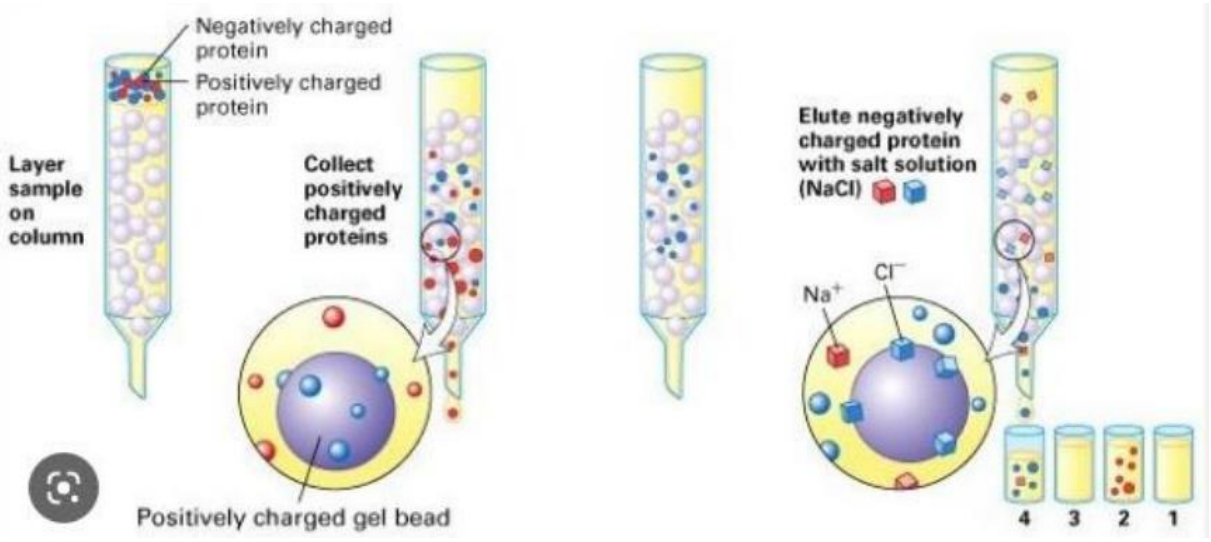

Figure 4 : Ion-Exchange Chromatography (IEC).

1-2-5-Affinity Chromatography

Principle: This technique relies on specific interactions between a target molecule and a ligand immobilized on the stationary phase.

Mechanism: The stationary phase is modified with a ligand that specifically binds to the target molecule (e.g., antibodies, enzymes). Non-target molecules are washed away, and the target is eluted by changing conditions (e.g., pH, salt concentration).

Applications: Commonly used for purifying biomolecules, particularly proteins and antibodies, from complex mixtures. It's highly specific and efficient for isolating target compounds.

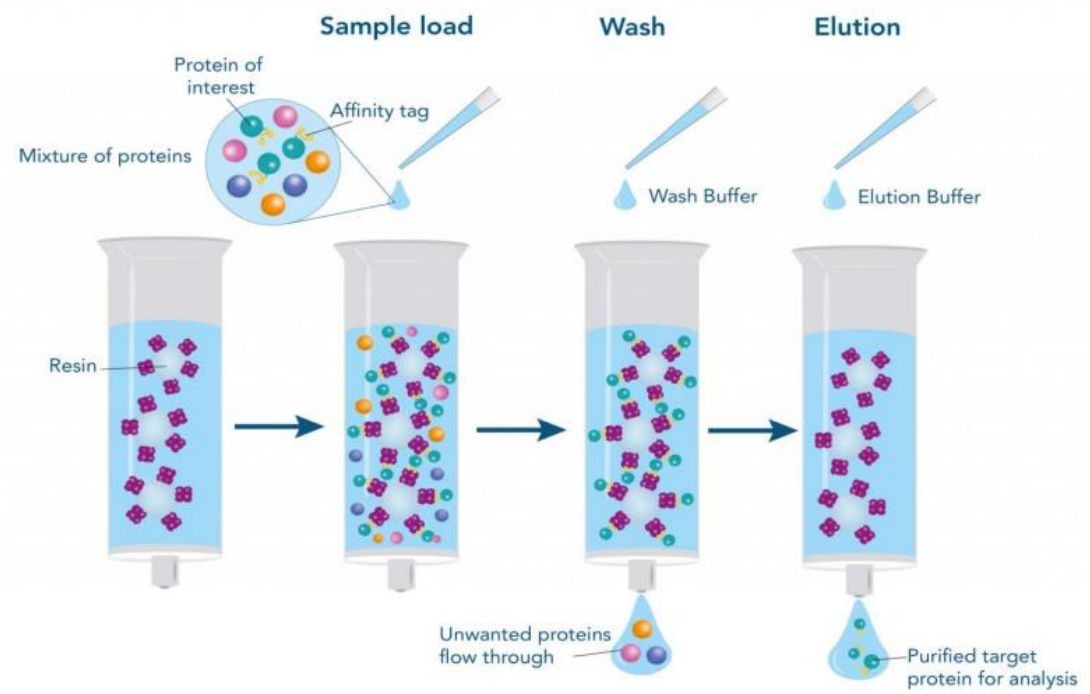

Figure 5 : Affinity Chromatography.

1-2-6- Gas Chromatography (GC)

Columns GC columns are filled with a stationary phase that separates volatile compounds based on their boiling points and interactions with the column material. Typically, GC columns are packed with silica-based materials coated with a liquid stationary phase or consist of capillary columns with walls coated with the stationary phase.

Applications: GC is often used for the analysis of volatile organic compounds (VOCs), environmental pollutants, and petrochemical samples.

1-2-7- High-Performance Liquid Chromatography (HPLC)

Utilizes small particle-packed columns for high-resolution separation of organic and inorganic molecules.

Applications: Widely used in pharmaceuticals, biochemistry, environmental analysis, and food science. It allows for the analysis of a broad range of compounds, including small molecules and large biomolecules.

2- Paper chromatography

2-1- Principale

The basic principle involved in paper chromatography is partition in which the various components get distributed or partitioned between liquid phases. It involves use of aqueous solvent held in pores of filter paper which acts as stationary phase whereas mobile phase travels over the paper. Due to differences in their affinity towards water (in stationary phase) and mobile phase solvents, the compounds in the mixture get separated through capillary action of the pores in the paper. The components may also be separated on the basis of principle of adsorption between solid and liquid phases, where solid surface of paper serves as stationary phase and mobile phase is a liquid solvent.

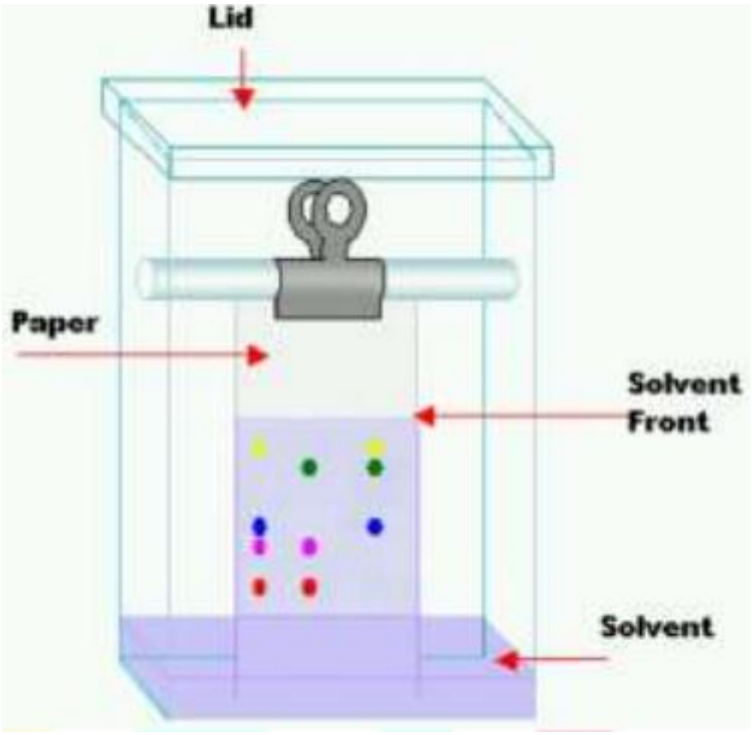

Figure 6 : Paper chromatography.

2-2- Stationary and mobile phase

2-2-1- Stationary phase

Stationary phase Paper chromatography is also known as partition chromatography. In this type of chromatography the usefulness of the mobile and stationary phase has huge applicability. It also focuses on the fact that there does not exist any particular rule for phases to be immiscible. The classification of the types of stationary phase are aqueous, hydrophilic and hydrophobic systems.

2-2-1-1- Aqueous stationary phase

The paper has the property of holding water. Hence, in this technique a paper which is already water-equilibrated is attached to a suspending paper inside a closed chamber whose atmosphere is completely water saturated. When there is a requirement of a salt phase or an aqueous buffer then this paper is moved through the solution and thereafter it is exposed to that part of the chamber which has a water saturated atmosphere. This type of stationary phase is best when the separation is to be done for a polar mixture (moderate to high).

2-2-1-2- Hydrophilic stationary phase

Hydrophilic stationary phase is made up of an organic solvent. Here, the key indicator of separation of any compound is the volatility of organic solvent. However, if the organic solvent is found to be sufficiently volatile then the paper is equilibrated in the solvent saturated

atmosphere of the chamber. In a different method, the solvent of the stationary phase is dissolved in a highly volatile diluent that evaporates which distributes the liquid of the stationary phase uniformly throughout the paper. Some of the most common hydrophilic solvents are methanol, formamide, glycerol and glycols.

2-2-1-3- Hydrophobic stationary phase

For the paper to be hydrophobic it needs to be processed in advance so that it can depict the characteristic of retaining the hydrophobic stationary phase. This technique uses the dripping method in which the equilibration of solvent is done by vapors. Moreover the mixture of solvent and a volatile diluent are chosen to make the hydrophobic solvent react with the paper. Some of the commonly used solvents are, kerosene, dimethylformamide, aromatic and aliphatic hydrocarbons.

2-2-2- Mobile phase

In the process of paper chromatography, mobile phase can be used in multiple combinations. However, the process of selecting the required optimum eluting is only possible through trial and error as there is no specific way of selecting it. Yet, there are particular guidelines that can be referred while making such selection and also for estimating the conditions of elution. For example, in order to make the optimum selection, the component characteristics as well as the stationary phase used in the mixture should be studied well. In this type of chromatography, an organic solvent mixed with water acts like the solvent system. However, in order to control the ionization of analytes, there can be an addition of some acids or bases. Some of the acids used can be HCl, HNO3 and acetic acid, whereas bases like NH3 can also be used to control the ionization. For the purpose of better identification of the compounds, various combinations of solvent systems are used. This is based on the chemical nature of those compounds.

2-3- Modes of Paper Chromatography

2-3-1- Ascending chromatography

The technique of ascending chromatography was introduced by Consden, Gordan and Martin and was later modified by Williams and Kirby. As the name indicates, the chromatogram is allowed to ascend and development of chromatogram takes place due to movement of solvent in upward direction on the paper. In this technique, solvent reservoir is kept at the bottom of the beaker and piece of paper with loaded sample is dipped in solvent. It is always recommended to take care that the spot should remain above the solvent system. Furthermore, it is also important to take care about size of paper to avoid bending and crumpling.

2-3-2- Descending chromatography

In this chromatography, the development of chromatogram is done by allowing the solvent to travel down the paper. The solvent reservoir is kept at the top and process of movement of solvent is assisted against the gravity.

2-3-3- Ascending- descending chromatography

This describes modified form of paper chromatography which involves ascending and descending flow of solvent on the same piece of paper. The advantages of this method over other methods are (i) The run time is reduced, i.e. needs short span of time, (ii) Longer flow distance available which gives better resolution.

2-3-4- Radial Chromatography

Also known as radial or circular chromatography, this method, described by Rutter, uses circular filter paper and results in concentric rings of separated components. The paper is placed horizontally in a petri dish, and the eluant is maintained in a covered dish to facilitate solvent flow from the center to the edges. Radial chromatography offers several benefits, including quick and precise separation, easy and compact apparatus, control over solvent flow rate, reproducibility, and simplicity in handling samples throughout the process.

2-3-5-Two-Dimensional Chromatography

Two-dimensional chromatography, or circular chromatography, is an advanced technique used for the separation of organic and inorganic substances. In this method, the chromatogram is developed in two perpendicular orientations. The sample is spotted at one corner of the circular paper, which is then first developed in one direction before being submerged in the solvent again at a right angle to the initial direction.

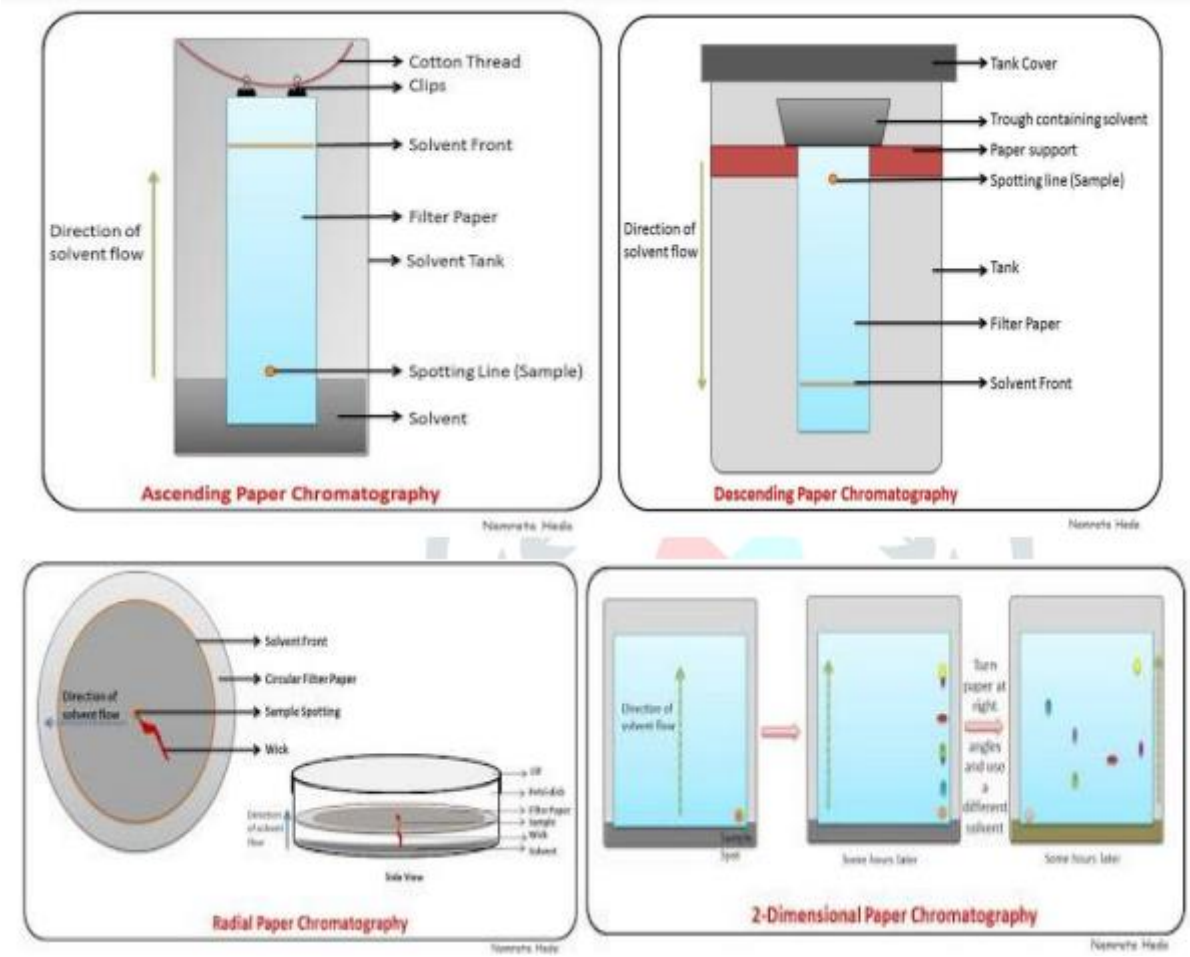

Figure 7 : Different types of paper chromatography.

3- Thin Layer Chromatography (TLC)

3-1- Definition

Thin layer chromatography is an important technique for identification and separation of mixtures of organic compounds. It is useful in:

-Identification of components of a mixture (using appropriate standards).

-Analyzing fractions collected during purification.

-Analyzing the purity of a compound.

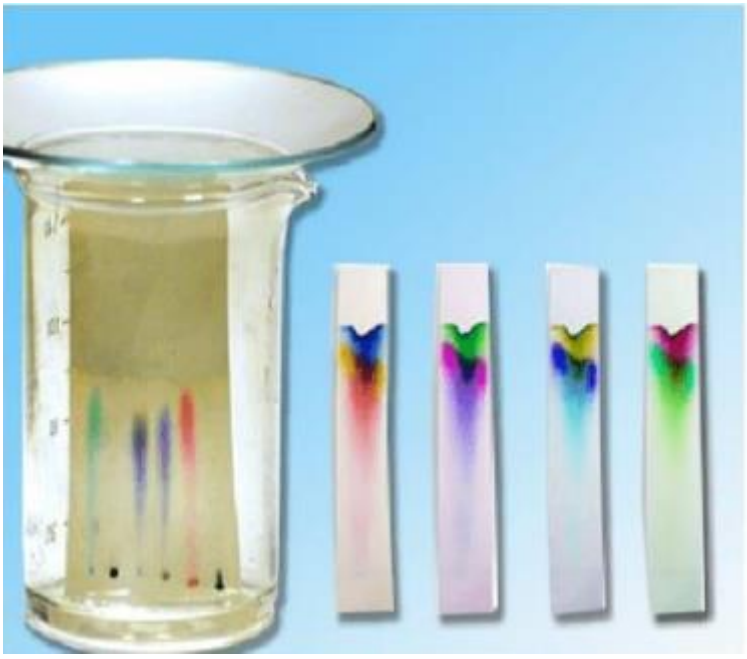

Figure 8 : Thin layer chromatography.

3-2- Principle

In this type of chromatography, for the purpose of stationary phase a glass plate is used which is usually covered with silica gel or aluminum oxide. whereas for the mobile phase, a solvent is used which is chosen based on the mixture properties. Thin layer chromatography works on the principle of distribution of a compound between a solid stationary phase and a liquid mobile phase.

3-3- Nature of adsorbent

The most common TLC sorbents are silica-based. Silica gel (SiO2) is a white porous material made by precipitation from silicate solutions by addition of acid (Wall, 2005). It consists of bonded silicon and oxygen with residual hydroxyl groups. Many sorbents with standardized particle sizes can be obtained commercially bound to plastic, aluminum, or glass plates. Silica gel plates can separate all classes of compounds and can be impregnated with other compounds to increase selectivity. Non-silica-gelbased sorbents include: kieselguhr (composed of the remains of diatoms whose cell wall is composed of silica), cellulose (glucopyranose units joined together by oxygen bridges), polyamide (produced from polycaprolactam, polyhexamethyldiaminoadipate, or polyaminoundecanoic acid), and aluminum oxide (Al2O3: also called alumina, it can be manufactured in three different pH ranges).

3-4- Mobile phase

The mobile phase in TLC refers to the solvent used to separate compounds based on their chemical composition and polarity. It is crucial to choose a solvent that matches the sample's properties to ensure proper separation. Volatile or hygroscopic solvents, like acetone, require

fresh batches for each run. The solvent's composition and polarity significantly influence the movement and separation of compounds on the TLC plate, causing them to travel at different rates. This, in turn, determines how the molecules can be visualized and analyzed.

3-5- Apparatus and working

TLC consists of glass plates, stationary phase, solvent system, aluminum foil, chromatographic chamber.

1. TLC Plate: Coated with a stationary phase (e.g. silica gel, alumina)
2. Solvent System: A mixture of solvents (e.g. hexane, ethyl acetate)
3. Spotting device: For applying samples to the TLC plate
4. Detection methods: UV lamp, fluorescent lamp or chemical staining.

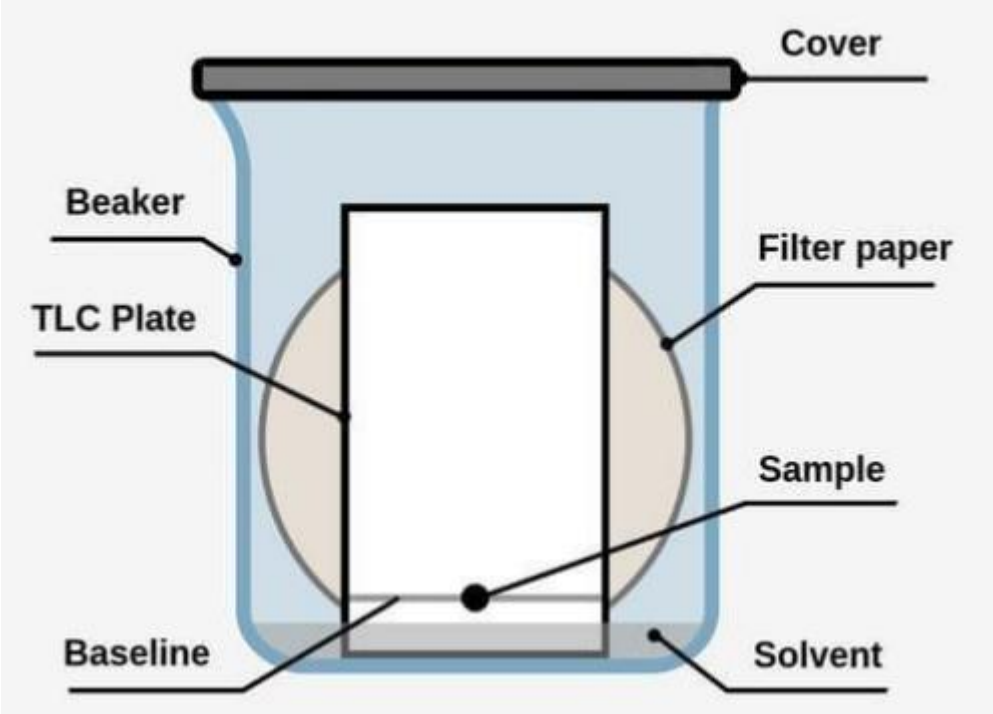

Figure 9 : Thin layer chromatography setup diagram.