What is Chromatography?

In 1901, the Russian botanist, Mikhail Tswett, invented adsorption chromatography during his research on plant pigments. He separated different colored chlorophyll and carotenoid pigments of leaves by passing an extract of the leaves through a column of calcium carbonate, alumina, and sucrose, eluting them with petroleum ether/ethanol mixtures.

In Greek language, *chroma* means "color" and *graphos* means "to write".

According to IUPAC definition: "Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase), while the other (the mobile phase) moves in a definite direction"

Classification of chromatographic method:

The mobile phase can be a gas or a liquid, whereas the stationary phase can only be a liquid or a solid. When the stationary phase is contained in a column, the term **column chromatography** applies. The stationary phase can also occupy a plane surface, such as filter paper. This is called **planar chromatography** and includes thin-layer and paper chromatography and electrophoresis. Column chromatography can be subdivided into **gas chromatography** (GC) and **liquid chromatography** (LC) to reflect the physical state of the mobile phase. If the sample passing through the chromatograph is in the form of a gas, the analytical technique is known as gas chromatography.

Gas chromatography comprises **gas-liquid chromatography** (GLC) and **gas-solid chromatography** (GSC), names that denote the nature of the stationary phase. Liquid-column chromatography embraces several distinct types of interaction between the liquid mobile phase and the various stationary phases. When the separation involves predominantly a simple partition between two immiscible liquid phases, one stationary and one mobile, the process is called **liquid–liquid chromatography** (LLC). In **liquid–solid** (or adsorption) chromatography (LSC) physical surface forces are mainly involved in the retentive ability of the stationary phase. Ionic or charged species are

separated in **ion chromatography** (IC) by selective exchange with counterions of the stationary phase; this may be by **ion-exchange chromatography** (IEC), ion-pair chromatography, or ion exclusion chromatography. In columns filled with porous polymers, components may be separated by **exclusion chromatography** (EC) [also called **gel-permeation chromatography** (GPC)]; separation is based largely on molecular size and geometry.

Mechanism of Separation/ Retention: Chromatographic processes can be classified according to the type of equilibration process or retention mechanism involved, which is governed by the type of stationary phase. Various bases of equilibration are: (1) adsorption, (2) partition, (3) ion exchange, and (4) size dependent pore penetration.

Partition Chromatography: The stationary phase of partition chromatography is usually a liquid supported on a solid or a network of molecules, which functions virtually as a liquid, bonded on the solid support. Again, the mobile phase may be a liquid (liquid–liquid partition chromatography) or a gas (gas–liquid chromatography, GLC). In the normal mode of operations of liquid–liquid partition chromatography, a polar stationary phase (e.g., cyano groups bonded on silica gel) is used, with a nonpolar mobile phase (e.g., hexane). When analytes (dissolved in the mobile phase) are introduced into the system, retention increases with increasing polarity. This is called **normal-phase chromatography**. If a nonpolar stationary phase is used with a polar mobile phase, the retention of solutes decreases with increasing polarity. This mode of operation is termed **reversed-phase chromatography** and is presently the most widely used mode. “Normal-phase” chromatography significantly predates the reversed-phase mode, and was originally called liquid chromatography. Only after “reversed-phase” chromatography came along, the need arose to distinguish between the two, and the older version, still more prevalent then, was termed “normal-phase.”

Adsorption Chromatography: The stationary phase is a solid on which the sample components are adsorbed. The mobile phase may be a liquid (liquid–solid chromatography) or a gas (gas–solid chromatography); the components

distribute between the two phases through a combination of sorption and desorption processes. Thin-layer chromatography (TLC) is a special example of adsorption chromatography in which the stationary phase is planar, in the form of a solid supported on an inert plate, and the mobile phase is a liquid.

Ion-exchange and Size-exclusion Chromatography: Ion exchange chromatography uses supports with ion exchange functionalities as the stationary phase. The mechanism of separation is based on ion exchange equilibria. A reversible exchange of ions is possible betweens ions of liquid phase (mobile phase) and a stationary solid phase (an insoluble substance having ionic sites). Hydrophobic interactions play a strong role in most ion exchange separations nevertheless, particularly in anion exchange chromatography.

In size exclusion chromatography, solvated molecules are separated according to their size by their ability to penetrate into porous pockets and passages in the stationary phase.

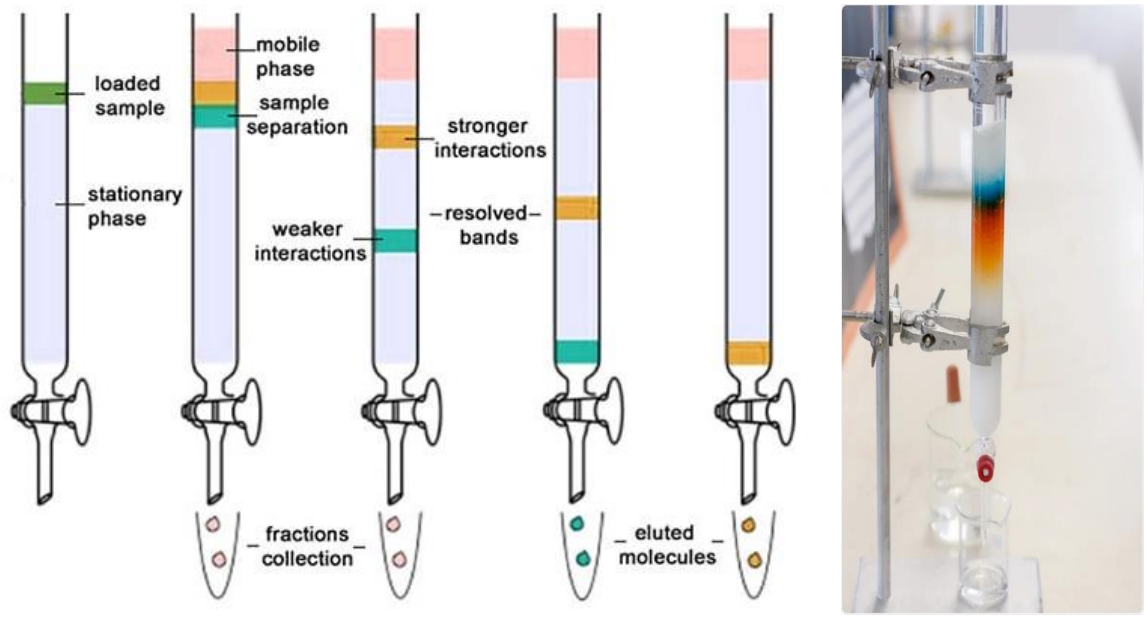
Column Chromatography: This type of chromatography is based on the principle that different compounds get adsorbed to different extent in a particular adsorbent (i.e. the solid stationary phase).

Procedure:

(i) **Filling the column:** The column is filled with the appropriate adsorbent (For example, silica, alumina, activated charcoal, calcium carbonate, magnesium oxide etc.). It is important fill the column uniformly or homogeneously without any air gap. For this the adsorbent is used in the form of slurry of petroleum ether, hexane or any other appropriate solvent.

(ii) **Addition of the mixture:** The analyte is added gently directly from the top of the column if it is a liquid. For solid compound it is normally added as a solution of the eluting solvent.

(iii) **Elution:** The column is then eluted with a mobile phase of desired polarity to affect the separation of the different components as separate bands.



Mechanism: As the solute and the solvent molecules move down the column, a competition sets in between them for adsorption on the adsorption sites of the adsorbent. Ultimately a dynamic equilibrium is set up at the interface where the solute and the solvent molecules get attracted, settle for a moment and leave the solid surface and re-enter the mobile liquid phase. In this competition of desorption and adsorption, the desorbed molecules having lesser affinity for solid surface (stationary phase) flow into the mobile layer faster than the desorbed molecules having greater affinity for solid surface.

The mechanism of adsorption chromatography depends on the difference in the polarity between different molecules. If the molecule is non-polar it will more strongly be adsorbed by the non-polar stationary phase. If the molecule is polar it will be adsorbed most strongly by a polar stationary phase.

Different types of stationary phases used in column chromatography are: silica gel, alumina (neutral, basic and acidic), magnesium oxide, celite etc.

Silica gel is a polar stationary phase. In case of silica gel columns, the low polarity components spend extra time in the mobile phase as compared to the highly polar components, that are retain longer. Hence the components of a sample mixture are eluted in the order of their increasing polarity.

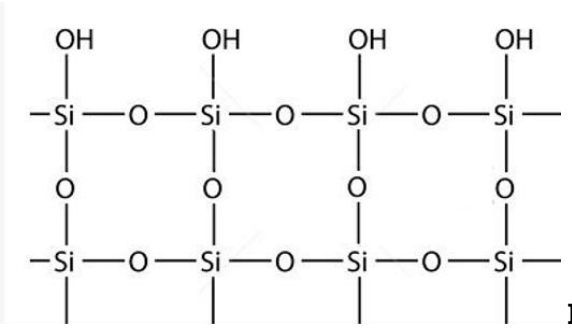


Figure: Structure of silica gel

Function of solvents

In column chromatography, a solvent the following three functions

i) They serve to introduce the mixture to be separated, to the column.

ii) They effect the process of development by which zones of chromatogram are separated to their fullest extent. In such cases, the solvents are called developers.

iii) These are used to remove the necessary content of each zone from the mechanically separated parts of the column or from the column when it is properly developed. Such solvents are called **eluents**.

Characteristics of an adsorbent

And adsorbent should have the following characteristics

i) It should have high surface area and selective adsorption power

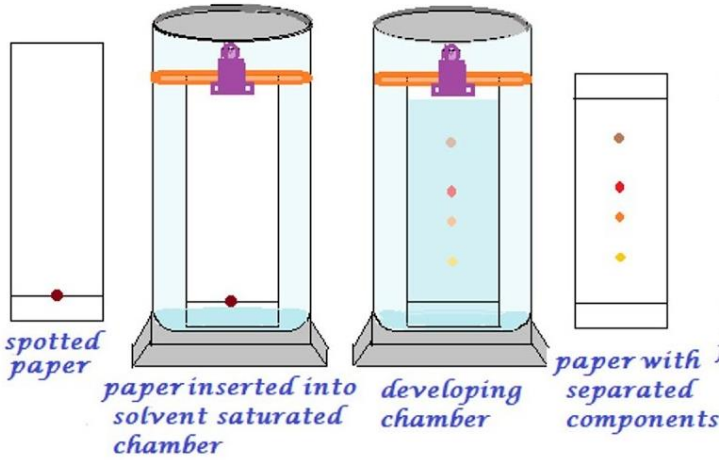
ii) It should be chemical energy inert.

iii) It should be finely divided to provide high surface area per unit mass.

Paper Chromatography:

This type of chromatography is based on the principle of partition. Both the stationary phase and mobile phase are liquid. Stationary phase in the form of a liquid (usually water) is held in the pores of the filter paper (**Whatmann no. 1 or 3**) A straight line is drawn at a distance of 2-5 cm from the bottom of a strip of paper. This is called **base line** or **origin**. A drop of the analyte mixture is placed at the base line. Then the paper is placed in a **chromatography chamber** or **developing chamber** and the solvent is allowed to rise in the paper by capillary action. The mobile phase is usually saturated with water to prevent dehydration of the paper during this phase. When the level of the

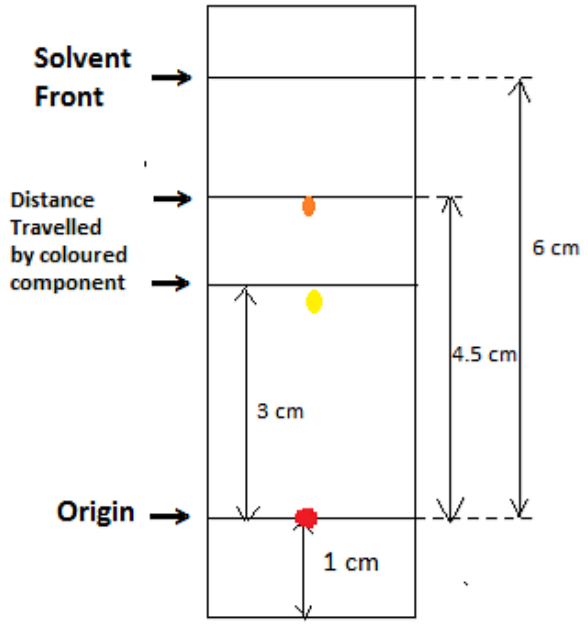
solvent reaches to 2-5 cm from the top of the strip, it is then taken out of the chamber. The level of the solvent is marked, it is called **solvent front**. The paper is then dried, and this dry strip is now called a **chromatogram**.



The Rf value:

Retention Factor also called the Rf value is defined as the ratio of the distance travelled by the solute to the distance travelled by the solvent. As it is the ratio of two similar quantities it is a unitless quantity. It is related to Chromatography. We can find the Rf value of any substance using the formula added below:

Retention factor (Rf) = Distance travelled by a solute / Distance travelled by a solvent



For example, in the above chromatogram

The Rf value of the yellow spot is, 3 cm/6 cm = 0.5

The Rf value of the orange spot is, 4.5 cm/6 cm = 0.75

Under same experimental condition Rf value is constant for a particular compound.

Factors effecting Rf value:

(i) Nature of the solvent used
(ii) Nature of the substance
(iii) Quality of the paper
(iv) Temperature

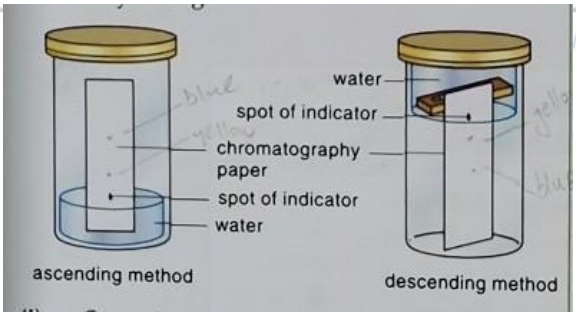
Mechanism of paper chromatography:

A mixture of components separated is generally dissolved in water and is then placed on a strip of paper. The second solvent placed in the vessel is allowed to travel along the strip. The cellulose in the filter paper acts as a support for the stationary phase with water absorbed in it. The mixture of components is thus subjected to partition between the stationary and mobile phase. Due to continuous and repeated partitioning, the rate of migration of each

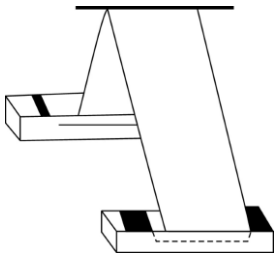
component is different. This property is responsible for the separation of components on the paper.

Types of paper chromatography:

1. **Descending chromatography:**
    - when the development of the paper is done by allowing the solvent to travel down the paper, it is known as descending technique.
2. **Ascending chromatography:**
    - When the development of the paper is done by allowing the solvent to travel up the paper, it is known as ascending technique.
    - Both ascending and descending techniques have been employed for separation of organic and inorganic substances.
    - But the ascending technique is preferred if the R_f values of various constituents are almost same.

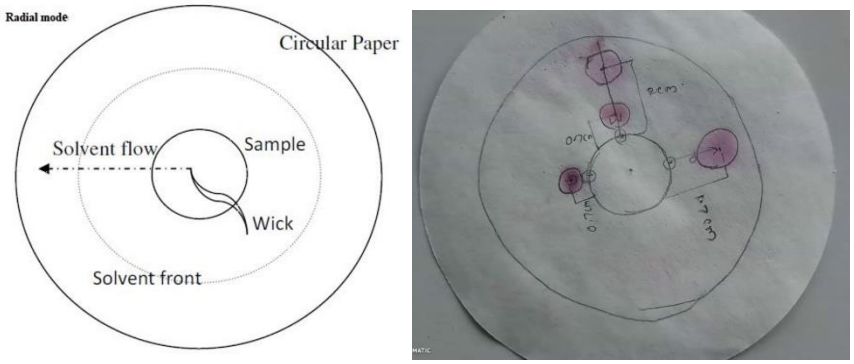


3. **Ascending-Descending Chromatography:**
    - It is the hybrid of the above two techniques.
    - In this technique, the upper part of the ascending chromatography can be folded over a glass rod allowing the ascending development to change over into the descending after crossing the glass rod.



4. **Radial paper Chromatography:**

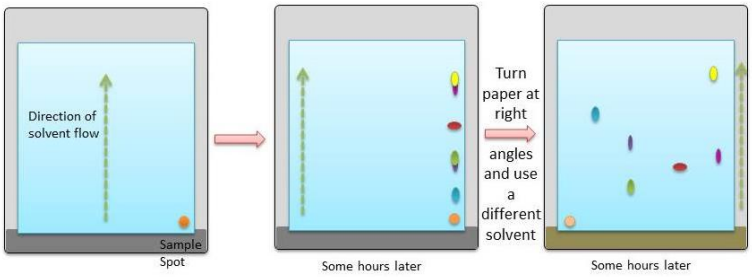
   - This is also known as circular paper chromatography.
   - This makes use of radial development.
   - In this technique a circular filter paper is employed.
   - Then the various materials to be analyzed are placed at the center.
   - After drying the spot the paper is fixed horizontally on the petri-dish possessing the solvent so that the tongue or the wick.
   - When solvent front has moved through a sufficient large distance, the components get separated in the form of concentric circular zones.



5. **Two-dimensional chromatography:**

   - In this, a square or rectangular paper is used.
   - The sample is applied to one of the corners.
   - The second development is performed at right angle to the direction of the first run.

- This type of chromatography can be carried out with identical solvent systems in both the directions or by two solvent systems.



Thin layer chromatography:

It is a type of adsorption chromatography. It is based on difference in adsorption of the components of a given mixture on a given adsorbent.

Types of adsorbent:

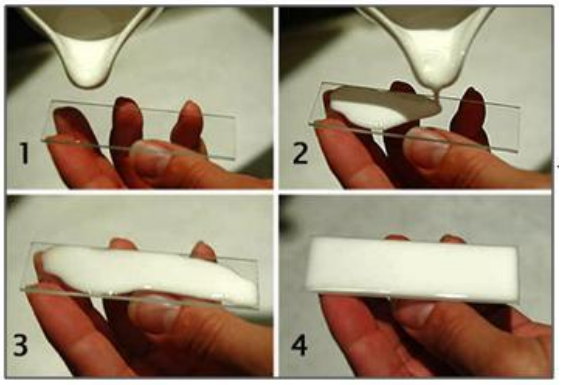
(i) **Silica gel:** It is slightly acidic. Plaster of Paris is used as a binding material for silica gel. Silica gel G: It contains **gypsum** as binder. Silica Gel RP stands for reversed phase, i.e., non-polar stationary phase and polar mobile phase.

(ii) **Alumina:** It is slightly basic. It often contains carbonates and bicarbonates of sodium. However, neutral and acidic alumina are also available.

Procedure: Glass plates of uniform thickness are selected. These are thoroughly cleaned and any greasy matter is removed from its surface. A slurry of the adsorbent in a suitable solvent is prepared (e.g. 25 grams of of silica containing 13% of Plaster of Paris in 50 mL of distilled water; however, for separation of organic compounds slurry is preferably prepared in ethyl acetate with a few drops of methanol). After this a thin coating of the adsorbent is applied on the plates. The thickness of the bed layer should be uniform all over. The ideal thickness of the adsorbent bed is 0.25 mm, but one can change it according to their preference. The plate is then properly dried.

Now-a-days ready-made plates are available in market.

After the preparation of the TLC plate the following procedure is similar to that of paper chromatography.



Application of TLC:

1. In monitoring the progress of reactions
2. Identify compounds present in a given mixture
3. Determine the purity of a substance.

Identification components of unknown mixture:

The mixture is spotted in the plate along with the **probable components in pure form**. After developing the chromatogram, the Rf of the individual spots are calculated and matched.

For example, it the chromatogram bellow **4** is our unknown mixture. After developing it gets separated in to two spots with Rf 0.72 and 0.52.

Spots **1**, **2** and **3** correspond tyrosine, glycine and methionine respectively.

Now Rf of tyrosine: 0.86

Now Rf of glycine: 0.52

Now Rf of methionine: 0.72

So, the unknown mixture contains **glycine** and **methionine**.