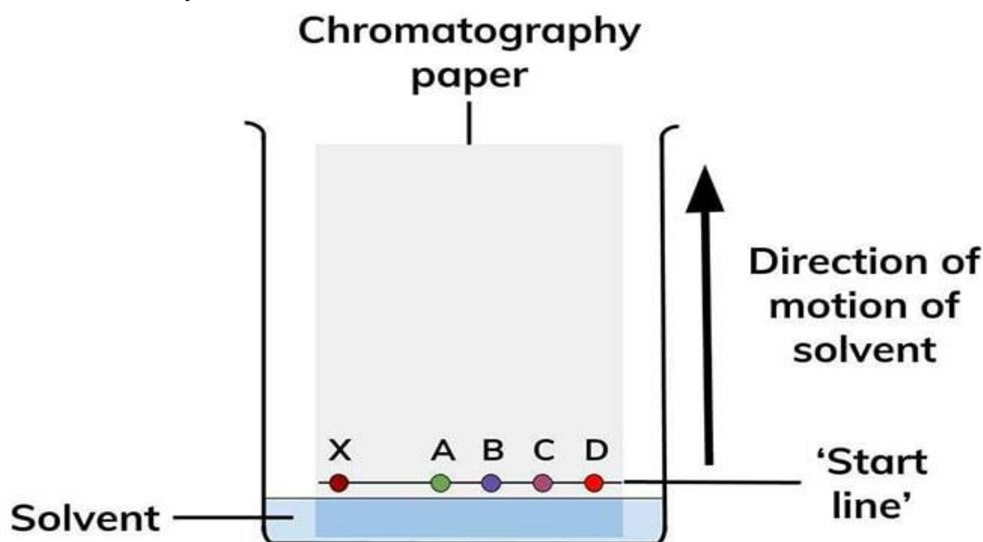
Paper Chromatography

Paper chromatography (PC) is a type of Planar chromatography whereby chromatography procedures are run on a specialized paper.

PC is considered to be the simplest and most widely used of the chromatographic techniques because of its applicability to isolation, identification, and quantitative determination of organic and inorganic compounds.

It was first introduced by German scientist Christian Friedrich Schonbein.



Principle :

The principle of separation is mainly partition rather than adsorption. Substances are distributed between a stationary phase and a mobile phase. Cellulose layers in filter paper contain moisture which acts as a stationary phase. Organic solvents/buffers are used as mobile phase. The developing solution travels up the stationary phase carrying the sample with it. Components of the sample will separate readily according to how strongly they adsorb onto the stationary phase versus how readily they dissolve in the mobile phase.

1. STATIONARY PHASE AND PAPERS

- Whatman filter papers of different grades like No.1, No.2, No.3, No.4, No.20, No.40, No.42 etc
- In general the paper contains 98-99% of α-cellulose, 0.3 – 1% β -cellulose.

Other modified papers

- Acid or base washed filter paper
- Glass fibre type paper.
- Hydrophilic Papers – Papers modified with methanol, formamide, glycol, glycerol etc.
- Hydrophobic papers – acetylation of OH groups lead to hydrophobic nature, hence can be used for reverse phase chromatography.
- Impregnation of silica, alumna, or ion exchange resins can also be made.

2. PAPER CHROMATOGRAPHY MOBILE PHASE

- Pure solvents, buffer solutions or mixture of solvents can be used.

Examples-

Hydrophilic mobile phase

- Isopropanol: ammonia:water 9:1:2
- Methanol : water 4:1
- N-butanol : glacial acetic acid : water 4:1:5

Hydrophobic mobile phases

- dimethyl ether: cyclohexane kerosene : 70% isopropanol

- The commonly employed solvents are the polar solvents, but the choice depends on the nature of the substance to be separated.
- If pure solvents do not give satisfactory separation, a mixture of solvents of suitable polarity may be applied.

3. CHROMATOGRAPHIC CHAMBER

- The chromatographic chambers are made up of many materials like glass, plastic or stainless steel. Glass tanks are preferred most.
- They are available in various dimensional size depending upon paper length and development type.
- The chamber atmosphere should be saturated with solvent vapor.

Steps in Paper Chromatography

In paper chromatography, the sample mixture is applied to a piece of filter paper, the edge of the paper is immersed in a solvent, and the solvent moves up the paper by capillary action. The basic steps include:

1. **Selection of Solid Support**

Fine quality cellulose paper with defined porosity, high resolution, negligible diffusion of the sample, and favoring good rate of movement of solvent.

2. **Selection of Mobile Phase**

Different combinations of organic and inorganic solvents may be used depending on the analyte.
Example. Butanol: Acetic acid: Water (12:3:5) is a suitable solvent for separating amino acids.

3. **Saturation of Tank**

The inner wall of the tank is wrapped with filter paper before the solvent is placed in the tank to achieve better resolution.

4. **Sample Preparation and Loading**

If the solid sample is used, it is dissolved in a suitable solvent. Sample (2-20ul) is added on the baseline as a spot using a micropipette and air dried to prevent the diffusion.

5. **Development of the Chromatogram**

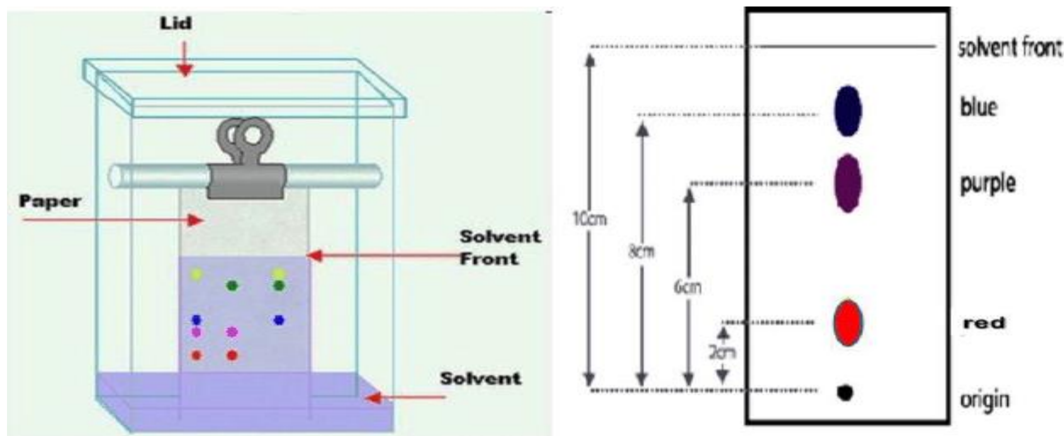
Sample loaded filter paper is dipped carefully into the solvent not more than a height of 1 cm and waited until the solvent front reaches near the edge of the paper.

Types of Paper Chromatography

1. Ascending paper chromatography:

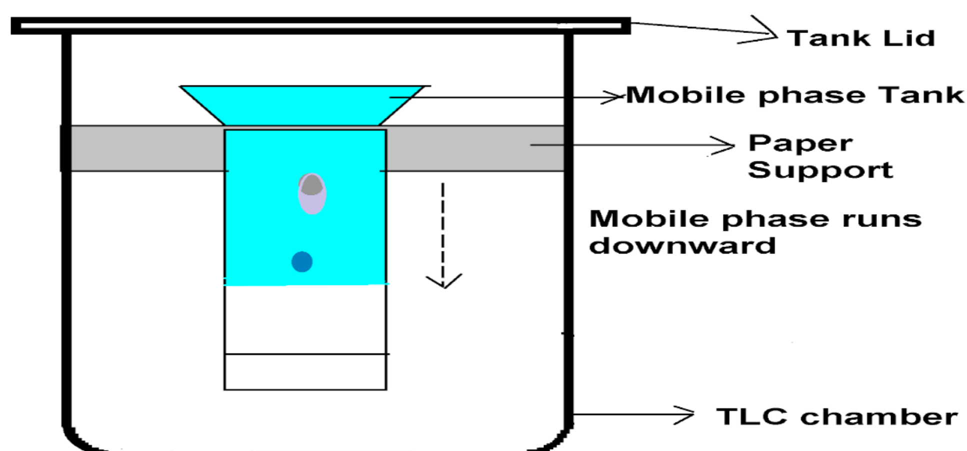
In this case, the separation of the sample is against gravity in upward direction. This is why this is termed as ascending technique. The mobile phase is at the bottom of the tank. The point of application of sample is just above the solvent. The mobile phase will gradually rise upwards and carry the components of the mixture. The most polar substance will be at the bottom with respect to the tank where as the least polar will be on top end of the tank. This is relatively a slow process as compared to descending technique.

$$Rf = \frac{\text{Distance travelled by the solute from the origin}}{\text{Distance travelled by the solvent from the origin}}$$



2)Descending paper chromatography:

Here, the development of chromatogram is done by allowing the solvent to travel down the paper under the influence of gravity. The mobile phase is placed in solvent holder at the top. The sample application spot is kept at the top and the solvent flow down the paper from above.

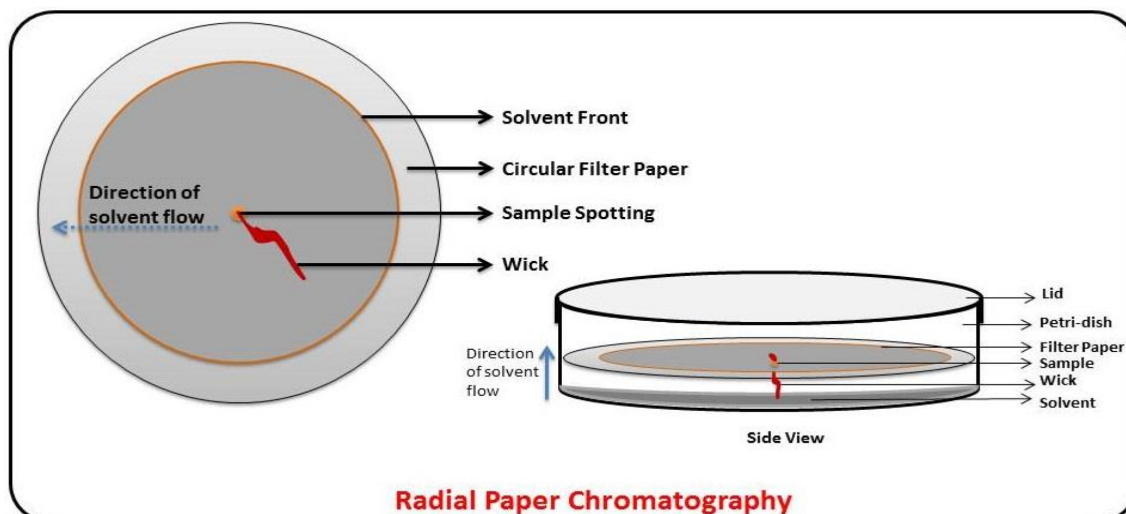


Both descending and ascending chromatography are used for the separation of organic and inorganic substances. For the detection of the separated compounds on the chromatogram, the compounds should be coloured. Several methods can be used to locate the spots, including fluorescence, radioactivity and treatment with chemicals that develop colours. Universal reagents like concentrated sulphuric acid or Iodine can be used to colour the organic molecules black or brown respectively. Specific reagents react with a particular class of compounds.

For example, Rhodamine B is often used for visualization of lipids; Ninhydrin for visualizing amino acids and Aniline phthalate for carbohydrates.

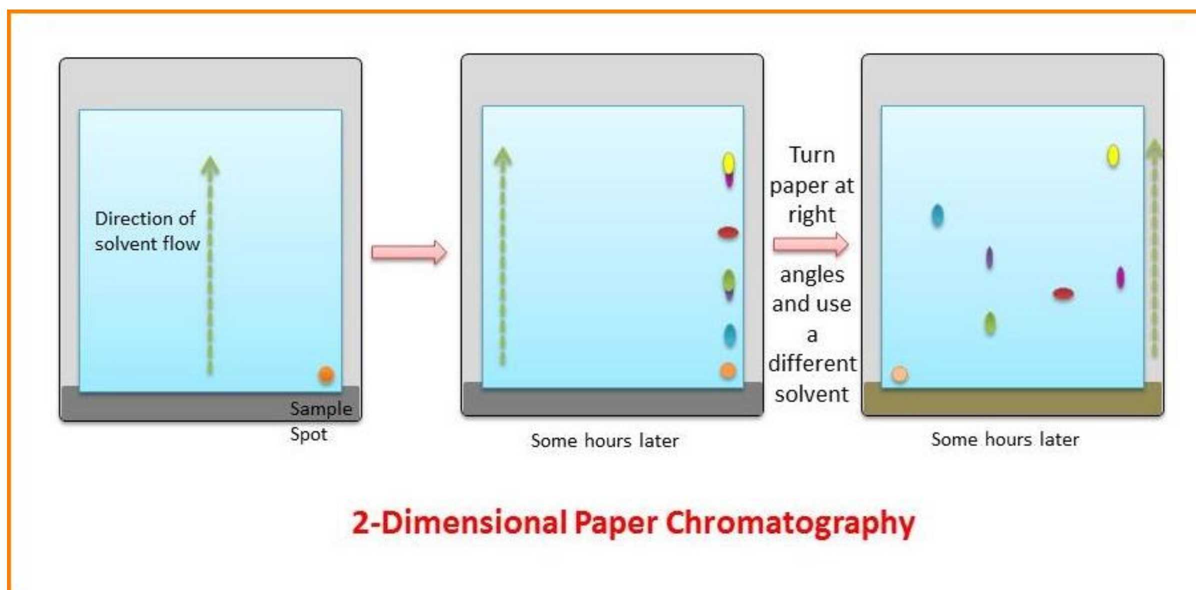
3)Radial paper chromatography:

It is also called circular chromatography. Here a circular filter paper is taken and the sample is deposited at the centre of the paper. After drying the spot, the filter paper is tied horizontally on a Petri dish containing solvent, so that the wick of the paper is dipped in the solvent. The solvent rises through the wick and the components are separated into concentric circles.



4)Two dimensional paper chromatography:

The sample is applied to one of the corners and after the development is performed in the first run, the filter paper is turned 90°clockwise; another solvent is used and then performed a second chromatographic run. This will lead to a satisfactory separation.



6. **Drying of Chromatogram**

After the development, the solvent front is marked and left to dry in a dry cabinet or oven.

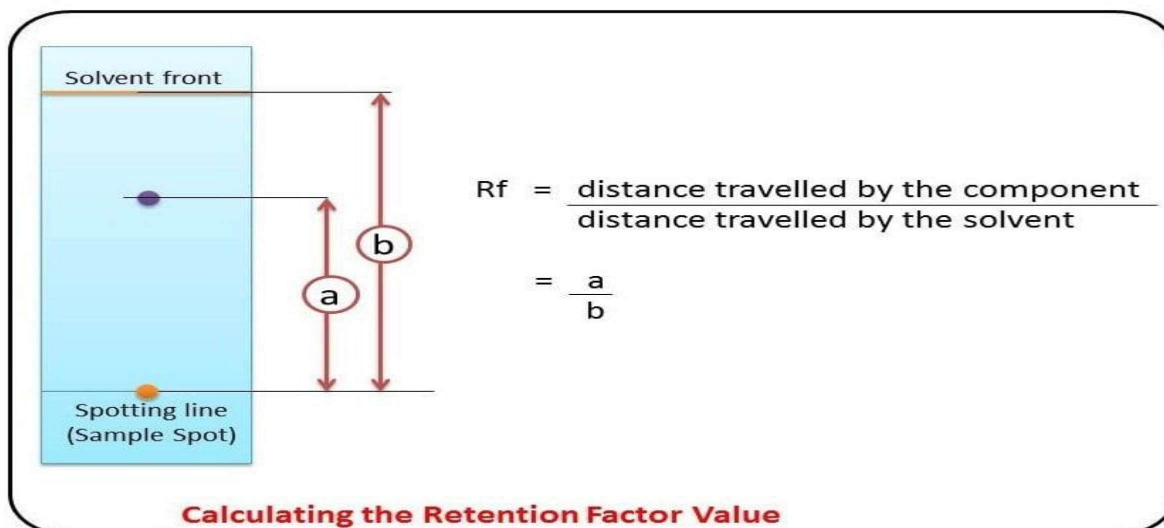
7. **Detection**

Colorless analytes were detected by staining with reagents such as iodine vapor, ninhydrin, etc.

Radio labled and fluorescently labled analytes were detected by measuring radioactivity and fluorescence respectively.

R_f values

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



Thus, in order to obtain a measure of the extent of movement of a component in a paper chromatography experiment, "Rf value" is calculated for each separated component in the developed chromatogram.

Applications of Paper Chromatography

- To check the control of purity of pharmaceuticals,
- For detection of adulterants,
- Detect the contaminants in foods and drinks,
- In the study of ripening and fermentation,
- For the detection of drugs and dopes in animals & humans
- In analysis of cosmetics
- Analysis of the reaction mixtures in biochemical labs.

Advantages of Paper Chromatography

- Simple technique.
- Paper Chromatography requires very less quantitative material.
- Paper Chromatography is cheaper compared to other chromatography methods.
- Both unknown inorganic as well as organic compounds can be identified by paper chromatography method.
- Paper chromatography does not occupy much space compared to other analytical methods or equipments.
- Excellent resolving power

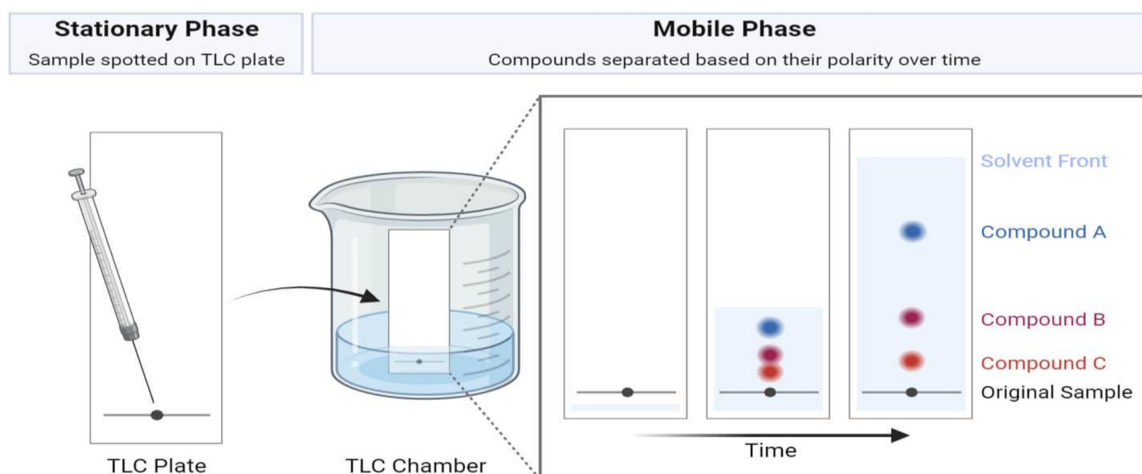
Thin Layer Chromatography (TLC)

Thin Layer Chromatography can be defined as a method of separation or identification of a mixture of components into individual components by using finely divided adsorbent solid / (liquid) spread over a plate and liquid as a mobile phase.

Principle :

- Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase.
- After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

- It is thus based on the principle of adsorption chromatography depending on adsorbent, its treatment and nature of solvents employed. The components with more affinity towards stationary phase travels slower. Components with less affinity towards stationary phase travels faster.
- Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character is identified by means of suitable detection techniques.



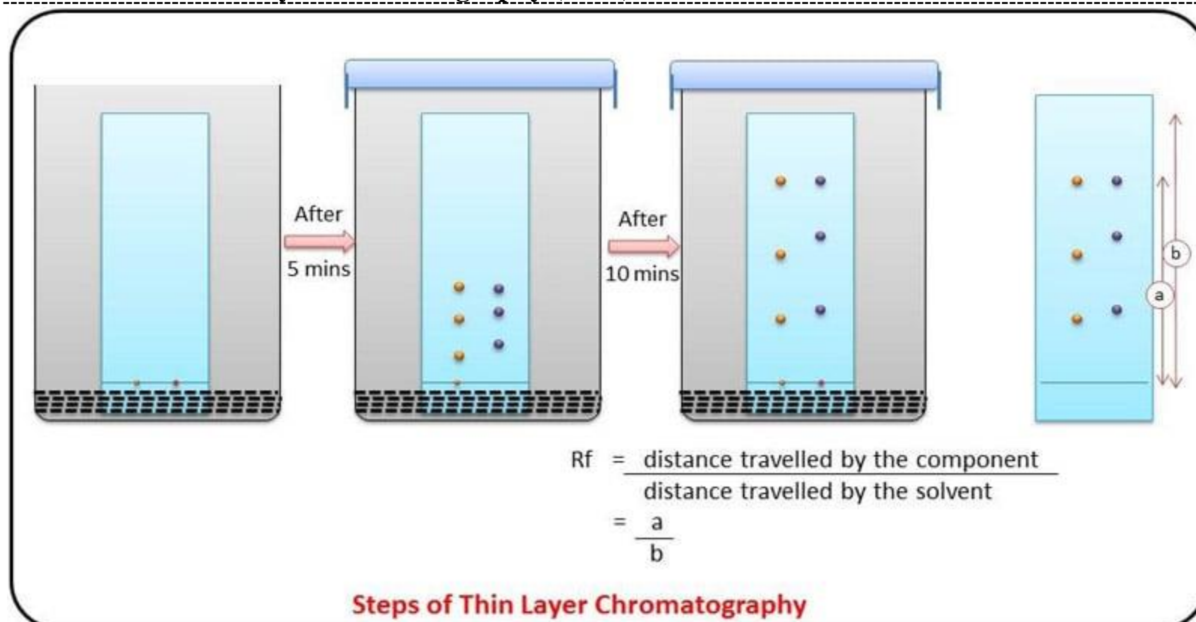
Components of Thin Layer Chromatography (TLC)

TLC system components consists of:

1. **TLC plates,** preferably ready made with a stationary phase. These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.
   A solvent system consisting of $CHCl_3$ and CH_3OH in a 2:1 ratio is taken. 35 grams of silica gel - G is weighed and taken in a bottle and transferred the solvent system into it and gypsum is added as a binder. The bottle is stirred well to ensure uniform silica slurry is obtained.
   Most of the methods use the coating material in the form of slurry or suspension in some liquids.
   a. Pouring: - In this technique, a measured amount of slurry is put on a given size plate which is kept at a level of the surface. For the slurry to spread uniformly over the surface, the plate is moved back and forth. This method of application of stationary phase is easy and commonly used in daily TLC analysis.
   b. Dipping: - This technique was developed by Piefer in 1962. The plates are arranged by dipping them at a time, back to back, in chloroform slurry of adsorbent. This method is generally not used due to its disadvantages.
   c. Spraying: - This technique was first proposed by Reitsema and employs a small point sprayer for distribution of the slurry on the glass plates.
   Activation of chromatographic plates involves the removal of liquid content from stationary phase. For this purpose the TLC plate is permitted to dry at room temperature followed by drying in oven at 110 degree Celsius for 30 minutes. The activation period of TLC plate varies with different adsorbing materials.
2. **TLC chamber-** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.
3. **Mobile phase-** This comprises of a solvent or solvent mixture The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.

4. **A filter paper-** This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.

Procedure of Thin Layer Chromatography (TLC)



Steps of Thin Layer Chromatography

The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are more commonly used.

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a level few centimeters above the chamber bottom.
4. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect).
5. Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
6. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent) for development.
7. Sufficient time is given for the development of spots.
8. The plates are then removed and allowed to dry.

Some common techniques for visualizing the results of a TLC plate include

1. UV light
2. Iodine Staining: is very useful in detecting carbohydrates since it turns black on contact with Iodine
3. KMnO4 stain (organic molecules)
4. Ninhydrin Reagent: often used to detect amino acids and protein

Retention Factor (R_f) Value

- The behaviour of a compound on a TLC is usually described in terms of its relative mobility or Rf value.
- Rf or Retention factor is a unique value for each compound under the same conditions.
- The Rf for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant:
- solvent system
- adsorbent
- thickness of the adsorbent
- amount of material spotted
- temperature
- Since these factors are difficult to keep constant from experiment to experiment, relative Rf values are generally considered.
- Relative Rf" means that the values are reported relative to a standard.

Applications of Thin Layer Chromatography (TLC)

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

- Analyzing ceramides and fatty acids
- Detection of pesticides or insecticides in food and water
- Analyzing the dye composition of fibers in forensics
- Assaying the radiochemical purity of radiopharmaceuticals
- Identification of medicinal plants and their constituents

Advantages of Thin Layer Chromatography (TLC)

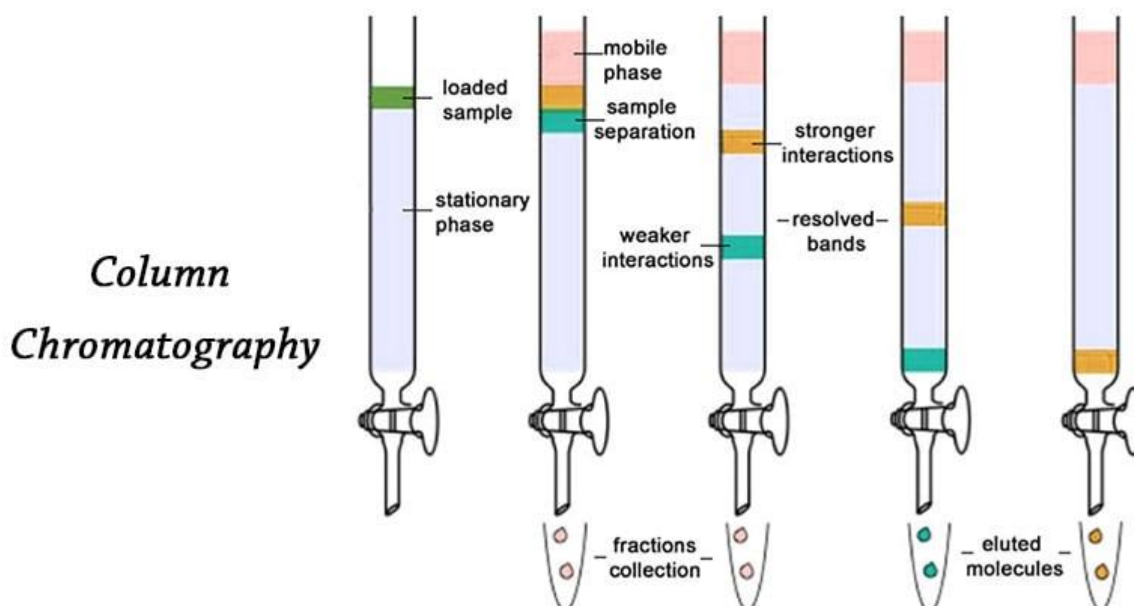
- It is a simple process with a short development time.
- It helps with the visualization of separated compound spots easily.
- It helps in isolating of most of the compounds.
- The separation process is faster and the selectivity for compounds is higher (even small differences in chemistry is enough for clear separation).
- The purity standards of the given sample can be assessed easily.
- It is a cheaper chromatographic technique.

Column Chromatography

Column chromatography is a technique in which the substances to be separated are introduced onto the top of a column packed with an adsorbent, passed through the column at different rates that depend on the affinity of each substance for the adsorbent and for the solvent or solvent mixture, and are usually collected in solution as they pass from the column at different times.

It is a solid-liquid technique in which the stationary phase is a solid & the mobile phase is a liquid or gas.

It was developed by the American chemist D.T Day in 1900 while M.S. Tswett, the Polish botanist, 1906 used adsorption columns in his investigations of plant pigments.



Forms of Column Chromatography

There are two forms of column chromatography.

1. Liquid chromatography (LC)
2. Gas chromatography (GC)

The most widely used forms of column chromatography are:

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Gel chromatography

Principle of Column Chromatography

- In column chromatography the stationary phase is packed into a glass or metal column.
- The mixture of analytes is then applied and the mobile phase, commonly referred to as the eluent, is passed through the column either by use of a pumping system or applied gas pressure.
- The stationary phase is either coated onto discrete small particles (the matrix) and packed into the column or applied as a thin film to the inside wall of the column.
- As the eluent flows through the column the analytes separate on the basis of their distribution coefficients and emerge individually in the eluate as it leaves the column.

Instrumentation of Column Chromatography

A typical column chromatographic system using a gas or liquid mobile phase consists of the following components:

A stationary phase:

- Chosen to be appropriate for the analytes to be separated.

A column:

- In liquid chromatography these are generally 25- 50 cm long and 4mm internal diameter and made of stainless steel whereas in gas chromatography they are 1-3m long and 2- 4mm internal diameter and made of either glass or stainless steel.
- They may be either of the conventional type filled with the stationary phase, or of the microbore type in which the stationary phase is coated directly on the inside wall of the column.

A mobile phase and delivery system:

- Chosen to complement the stationary phase and hence to discriminate between the sample analytes and to deliver a constant rate of flow into the column.

An injector system:

- To deliver test samples to the top of the column in a reproducible manner.

A detector and chart recorder:

- To give a continuous record of the presence of the analytes in the eluate as it emerges from the column.
- Detection is usually based on the measurement of a physical parameter such as visible or ultraviolet absorption or fluorescence.
- A peak on the chart recorder represents each separated analyte.

A fraction collector: For collecting the separated analytes for further biochemical studies.

Steps in Column Chromatography

A. Preparation of the Column

- The column mostly consists of a glass tube packed with a suitable stationary phase.
- A glass wool/cotton wool or an asbestos pad is placed at the botton of the column before packing the stationary phase.
- After packing, a paper disc kept on the top, so that the stationary layer is not disturbed during the introduction of sample or mobile phase.

There are two types of preparing the column, they are:

1. Dry packing / dry filling

In this the required quantity of adsorbent is poured as fine dry powder in the column and the solvent is allowed to flow through the column till equilibrium is reached.

2. Wet packing / wet filling

In this, the slurry of adsorbent with the mobile phase is prepared and is poured into the column. It is considered as the ideal technique for packing.

- Before using column, it should be washed properly and dried.
- The column should also be free from impurity and uniformly filled with the stationary phase.

B. Introduction of the Sample

- The sample which is usually a mixture of components is dissolved in minimum quantity of the mobile phase.

- The entire sample is introduced into the column at once and get adsorbed on the top portion of the column.
- From this zone, individual sample can be separated by a process of elution.

C. Elution

- By elution technique, the individual components are separated out from the column.
- It can be achieved by two techniques:
- **Isocratic elution technique:** Same solvent composition or solvent of same polarity is used throughout the process of separation.

Eg. Use of chloroform alone.

- **Gradient elution technique:** Solvents of gradually ↑ polarity or ↑ elution strength are used during the process of separation.

E.g. initially benzene, then chloroform, then ethyl acetate then chloroform

D. Detection of Components

- If the compounds separated in a column chromatography procedure are colored, the progress of the separation can simply be monitored visually.
- If the compounds to be isolated from column chromatography are colorless.
- In this case, small fractions of the eluent are collected sequentially in labelled tubes and the composition of each fraction is analyzed by TLC.

Factors Affecting Column Efficiency

- Dimensions of the column
- Particle size of the adsorbent
- Nature of the solvent
- Temperature of the column
- Pressure

Applications

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. Its major application includes:

- Separation of mixture of compounds.
- Removal of impurities or purification process.
- Isolation of active constituents.
- Isolation of metabolites from biological fluids.
- Estimation of drugs in formulation or crude extracts.

INTRODUCTION:

In 1906 Tswett used to chromatography to separate plant pigments.

He called the new technique chromatography because the result of the analysis was 'written in color' along the length of the adsorbent column.

Chroma means "color" and graphein means "to write".

Chromatography is defined as "A physical method of separation in which the components to be separated are, distributed between two phases, one of which is stationary phase while the other is mobile phase, moves in a definite direction".

The stationary phase can be the solid/gel/liquid/solid-liquid mixture that is immobilised and it may have the ability to bind some type of solutes on to it.

Mobile phase can be liquid or gas which passes over the stationary phase.

GENERAL PRINCIPLE OF CHROMATOGRAPHY:

All forms of chromatography work on the same principle. During the process, the sample containing many molecular components comes into contact with the stationary phase and the components distribute

themselves between the stationary and the mobile phase. If some components in the sample are bound to the stationary phase then they will spend more time in stationary phase and hence their movement in

the chromatographic system will be retarded. On the other hand, the molecules that show weak affinity/interaction with the stationary phase spends more time with the mobile phase and are rapidly removed and eluted from the system. Thus the rate of migration of the solute depends on the rate of interaction of it with the stationary and mobile phase.

Distribution or Partition coefficient (Kd) describes the way an analyte distributes between the two immiscible phases.

Thus the difference in Kd value of the components results in their separation.

And the general process of moving a solute mixture through a chromatographic system is called development.

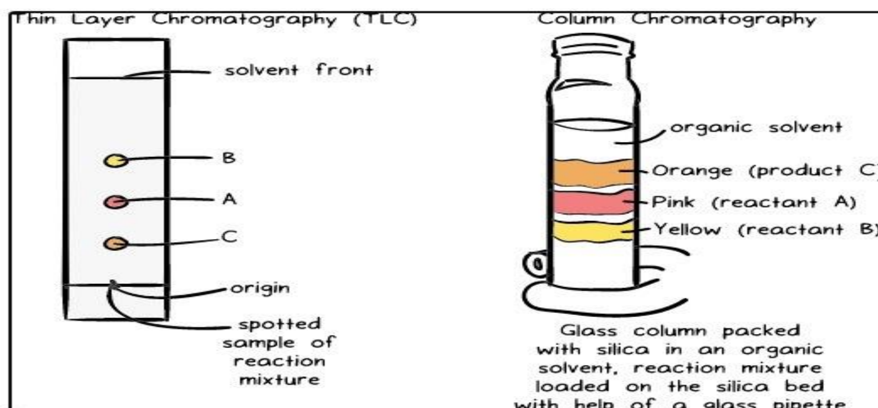
Eluent:
The substance which separates the component of the mixture in chromatographic techniques. Eluent is that part which brings separation when the solution is passed either from the column or from solid support.
Eluate:
The substance which is separated as an individual component of the mixture is eluate.

CLASSIFICATION OF CHROMATOGRAPHY:

- Chromatography technique can be explained into three fundamental ways:
    I. Based on the shape of chromatographic bed.
    II. Based on the physical nature of the stationary and mobile phases.
    III. Based on the mechanism of the separation.

Based on the shape of chromatographic bed:

- On the basis of the chromatographic bed, there are following two types of chromatography-
    I. Planar chromatography.
    II. Column chromatography.
- In planar chromatography, the stationary phase is spread on a flat, planar surfaces. The plane can be paper acting as a stationary phase (paper chromatography), or stationary phase spread on glass, metal or plastic plate (thin layer chromatography). Planar chromatography also known as open-bed chromatography.
- In column chromatography, the stationary phase is within a tube. Then the mobile phase containing the mixture to be separated is added to the top of the column and allowed to flow through the unrestricted, open path down the middle of the tube. This flow can be allowed to occur naturally due to the force of gravity or induced using pressure. The different components of the mixture interact with the stationary phase material and separate as they move through the column.



Based on the physical nature of the mobile and stationary phase:

- On the basis of physical nature of mobile phase, there are two types of chromatography:
  I. Gas chromatography
  II. Liquid chromatography
- Further, on the basis of stationary phase, physical nature there are the following types:
  I. Gas-solid chromatography
  II. Gas-liquid chromatography
  III. Liquid-solid chromatography
  IV. Liquid-liquid chromatography

Based on the mechanism of separation:

- On the basis of mechanism of separation, it is of following types:

I. Adsorption Chromatography
Separation is based mainly on differences between the adsorption affinities of the sample components for the surface of an active solid.

II. Partition Chromatography
Separation is based mainly on differences between the solubilities of the sample components in the stationary phase (gas chromatography), or on differences between the solubilities of the components in the mobile and stationary phases (liquid chromatography).

III. Ion-Exchange Chromatography
Separation is based mainly on differences in the ion exchange affinities of the sample components. Note: Present day ion-exchange chromatography on small particle high efficiency columns and usually utilizing conductometric or spectroscopic detectors is often referred to as Ion Chromatography (IC).

IV. Exclusion chromatography
Separation is based mainly on exclusion effects, such as differences in molecular size and/or shape or in charge. The term Size-Exclusion Chromatography may also be used when separation is based on molecular size. The terms Gel Filtration and Gel-Permeation Chromatography (GPC) were used earlier to describe this process when the stationary phase is a swollen gel. The term Ion-Exclusion Chromatography is specifically used for the separation of ions in an aqueous phase.

V. Affinity Chromatography
This expression characterizes the particular variant of chromatography in which the unique biological specificity of the analyte and ligand interaction is utilized for the separation.