Chromatography

For S. Y. B. Sc. Sem-IV

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The technique was invented by Russian botanist Mikhail tswett in 1906. The word Chromatography is derived from latin language and in this word chroma means “colour” and graphein means “to write”.

It can be defined as a method of separating a mixture of components into individual component through equilibrium distribution between two phases. Essentially, it is based upon the differences in rate at which the components of mixture move through a porous medium (stationary phase) under the influence of gas or liquid (mobile phase).

Some basic terminology

Analyte: substance that to be analysed.

Chromatogram: Visual output of chromatography.

Stationary phase: Non-moving phase that provides support for mixture to move.

Mobile phase: Moving phase to specific direction.

Eluent: Solvent that carries analyte.

Retention time: it is specific time required for a particular analyte to pass through the system.

Classification of chromatography

Chromatography can be classified into different way based upon different aspects.

1. Based upon geometry

On the basis of geometry, chromatography can be classified into following.

a. Planar: Paper chromatography and thin layer chromatography (TLC)
b. Column: Gas chromatography (GC), High performance liquid chromatography (HPLC), Ion exchange chromatography (IC), Super critical fluid chromatography (SFC).

2. Based upon mobile phase

On the basis of mobile phase, chromatography can be classified into following.

a. Gas chromatography: Mobile phase is gas.

b. Liquid chromatography: Mobile phase is liquid.

c. SFC: Supercritical fluid chromatography. Mobile phase is CO_2 in liquid form.

3. Based upon stationary phase

On the basis of stationary phase, Chromatography can be classified into following.

a. Adsorption chromatography: Mobile phase liquid, stationary phase solid

b. Partition chromatography: Mobile phase liquid, Stationary phase liquid

c. Ion exchange chromatography: Mobile phase liquid, stationary phase solid

d. Molecular exclusion chromatography: Mobile phase is liquid, Stationary phase is chemically inert material.

e. Affinity chromatography: Mobile phase is buffer solution, stationary phase is gel matrix.

Types of chromatography

- Chromatography offers diverse selection of stationary and mobile phase.
- The separation process is dependent upon stationary and mobile phase and based upon various phenomenons like migration, capillary action, adsorption etc.
- This type of stationary or mobile phase helps us to classify chromatography into various techniques.

Technique	Stationary phase	Mobile phase
Column Chromatography	Solid	Liquid
Partition Chromatography	Liquid	Liquid
Paper Chromatography	Liquid	Liquid
Thin Layer Chromatography	Liquid or Solid	Liquid
Gas-Liquid Chromatography	Liquid	Gas
Gas Solid Chromatography	Solid	Gas
Ion-Exchange Chromatography	Solid	Liquid

In recent times, various hybrid techniques have been evolved. The various techniques are listed into following table according to stationary phase and mobile phase.

Stationary Phase	Mobile Phase	Name
Solid	Liquid	Plane Chromatography
		Paper Chromatography
		Thin Layer Chromatography
		Adsorption Column Chromatography
		High Performance Liquid Chromatography
		Ion Exchange Chromatography
	Gas	Gas Solid Chromatography
Solid Matrix	Liquid	Gas-Permecation Chromatography
Liquid	Gas	Gas Liquid Chromatography
	Liquid	Liquid-Liquid Chromatography

Paper Chromatography

Always keep in mind

- **It is partition chromatography**
- **The stationary phase is liquid**
- **The mobile phase is liquid**

Introduction

- The paper chromatography includes a specially designed filter paper on which solvent flows and the migration of different substance is observed.
- One of the two solvents is immiscible or partially miscible with other solvent.
- The separation is dependent upon differential migration of mixture of substances that occurs due to difference in partition co-efficient.
- The components of mixture to be separated migrate at different rates and appear as spot on filter paper.

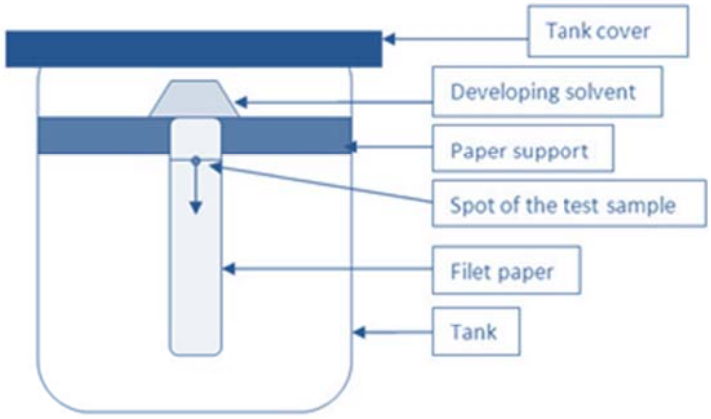
Migration Parameters

- $R_f = \frac{Distance\ travelled\ by\ solute\ from\ the\ origin\ line}{Distance\ travelled\ by\ solvent\ from\ origin\ line}$
- $R_x = \frac{Distance\ travelled\ by\ solute\ from\ the\ origin\ line}{Distance\ travelled\ by\ standard\ substance\ from\ origin\ line}$
- $R_M = \log[{}^{1}/_{R_f - 1}]$
- R is function of partition co-efficient.
- R is constant for a substance for a constant chromatography conditions (Paper, Temperature, duration and direction of development, humidity, size of vessel etc.)
- Rx is used when the solvent runs off the paper. In such cases movement of substance is denoted by Rx instead of Rf.
- R_M is additive term.

Types of Paper Chromatography

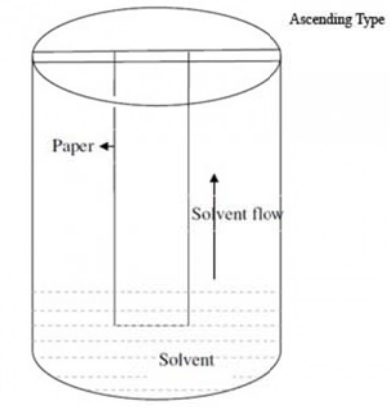
The paper chromatography can be classified into following techniques.

1. Descending Chromatography



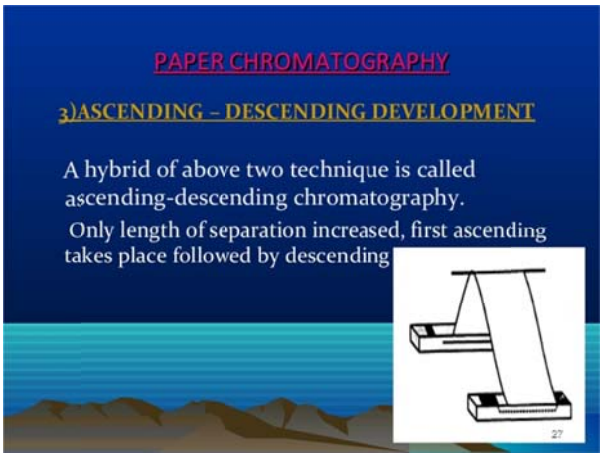
- In such chromatography technique, solvent travels down the filter paper.
- It is advantageous technique as it is continuous development technique.
- It is fast.

2. Ascending Chromatography



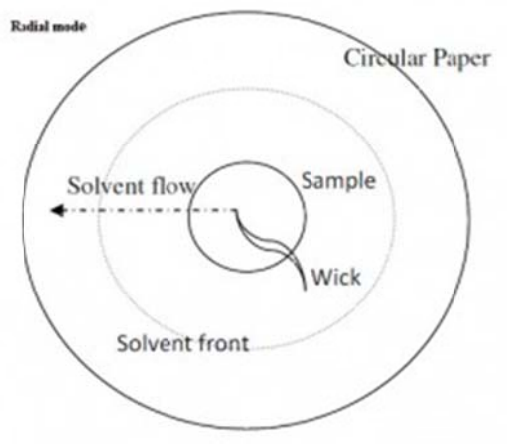
- In such chromatography technique solvent travels up the filter paper.
- It is routine technique and employed when Rf value is quite different for components of mixture.

3. Ascending-Descending Chromatography



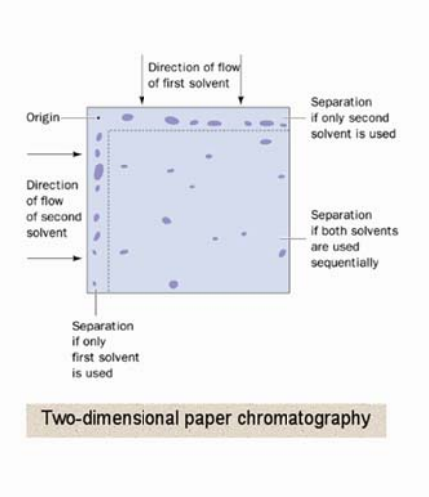
- It is hybrid technique comprise of ascending as well as descending chromatography.
- First, solvent travels up the paper and at edge the paper is bended with the help of support (glass road) and from that point solvent travels down the filter paper.

4. Radial Paper Chromatography



- It is also known as circular paper chromatography.
- In this technique, circular shape paper is used.
- The spots of mixture employed in circular shape.
- The solvent travels through paper via a wick dipped in solvent and attached with paper in the middle.
- The solvent travels horizontally.
- After sufficient travelling, it is allowed to dry and spot can be visualize by appropriate visualizing agent.

5. Two Dimensional Chromatography



- In such technique, square rectangular paper is used.
- The sample is employed in corner and allowed to run with solvent in both directions one by one.

Experimental details of paper chromatography

1. Choice of paper chromatographic technique

- The choice of technique depends upon nature of substance to be separated.
- The type of technique determines the efficiency and speediness of results.

2. Choice of filter paper

- It is dependent upon technique (either qualitative or quantitative)
- Nature of substance (hydrophilic or lipophilic)

3. Proper developing solvent

- The choice of developing solvent is dependent upon the Rf values of substance to be separated.
- A solvent or mixture of solvent, which gives Rf value 0.2 – 0.8 for sample should be selected.

4. Preparation of samples

- It is impossible to decide standard procedure of preparation of sample.
- The sample having trace amount of substance (10-20 Ng) can be identified easily.

5. Spotting

- A horizontal line is drawn on the paper by a pencil.
- The sample solution is spotted on that line (origin line) and allowed to dry.

6. Drying the chromatograms

- The wet chromatograms are allowed to dry in drying cabinet.

7. Visualization

- It can be done by either chemical or physical means.
- Chemical Detection: Various chemicals are used to visualize spots on colourless chromatogram. The visualizing agents are sprayed or the paper is dipped into them.
- Physical Detection: UV lamp is used to visualize spots.

Applications of Paper Chromatography

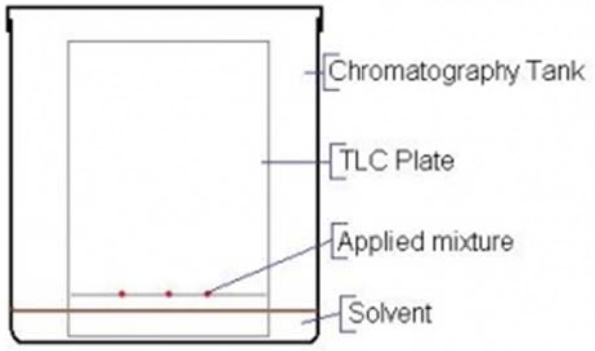
- It is widely used for qualitative and quantitative analysis of organic, inorganic and biochemical substances.
- It is used for separation of amino acids.
- It is also used for separation of sugars.

Thin Layer Chromatography

Always keep in mind

- **It is partition chromatography**
- **The stationary phase is liquid or solid**
- **The mobile phase is liquid**

Introduction



- Thin layer chromatography (TLC) is partition chromatography.
- It is closely resemblance to column and paper chromatography.
- The partition occurs on a thin layer made up of finely divided adsorbent that is supported on glass or tough material.
- It is most commonly used chromatography technique.

Advantages of TLC

- It is simple chromatographic procedure.
- It is applicable to analytical as well as operative to large scale preparation.
- It is almost applicable to all the chemical compounds.
- It is rapid procedure hence useful to check progress of chemical reactions.
- The resolving power is great and can be useful in identification of adulteration in food.

- It can detect compounds readily.
- Only trace amount of any compound can be visible with TLC.
- It has higher sensitivity.

Basic Operations Involved in TLC

(1) Methods for production of thin layer plates

- The thin layer can be achieved by spreading, pouring, spraying or dipping the thin layer plate to adsorbent. Spreading method is highly useful in producing uniform layer.
- Layers can be classified into solid and loose layers.
- Solid layer can be prepared by applying adsorbent on a clean glass plate with the help of applicator.
- Loose layer can be prepared by pouring of suspension plate, dipping plates in suspension, and spraying suspension on clean glass plate.

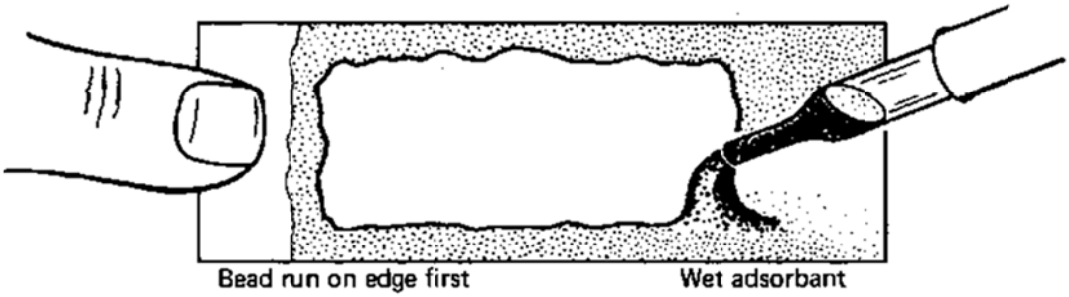
(2) Application of sample on chromatoplates

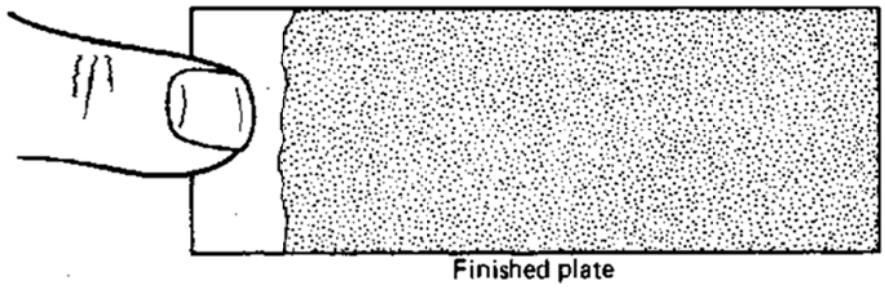
- 0.1 % sample solution is prepared.
- It is spotted on TLC plate with the help of glass capillary or micro pipette.
- The solvent in which the sample is dissolved is allowed to evaporate.
- The sample solution is spotted in a row at one side of TLC plate at about 2 cm from edge.

(3) Choice of adsorbent

- The most common adsorbent used in TLC is silica gel alumina.
- Kieselguhr, powdered cellulose, and several coating materials are also used.
- The choice of adsorbent is dependent upon its acidity or basicity.
- It is also dependent upon activity, separating mechanism, and can be change according to nature of compound.
- Normally, a 0.25 mm thick TLC is prepared by spreading method.

- Thick layer plate can be prepared by mixing silica gel G in water (ratio 25:40), layered on plate then air dried. It can be activated by heating in an oven for 1 to 2 hours.
- Plaster of Paris is incorporated as binding agent.
- A large number of applicators are available commercially.
- The various methods for preparing layers are as follows.
- Pouring: A measured amount of slurry is pouring onto given size of plate. It is put on levelled surface to make layer uniform.
- Dipping: Plate is dipped two times into slurry of adsorbent made in Chloroform.
- Spraying: A sprayer is used for uniform distribution of adsorbent onto glass plate.
- Spreading: Slurry is filled in applicator. Either the plate is kept steady or applicator employ slurry in uniform manner or plate in motion passes beside applicator to make uniform layer.





- Pre-coated plates: pre-coated plates are available in market. These plates are quite expensive. The thickness of pre-coated plastic sheets usually varies from 0.1 to 0.2 mm.

(4) Choice of solvent

- If the nature of substance is unknown then only method for finding out best solvent is trial and error.
- If the substance is known then suitable solvent can be find using original stain's triangle: inter relating adsorbent activity, nature of solute, and nature of solvent.

(5) Detecting reagents

- The chromatogram is generally colourless.
- The spots can be detected by using appropriate detecting reagents.
- Iodine vapour and sulphuric acid (mixed with aromatic aldehyde or oxidation agents like $KMnO_4$ or Chromic acid) are common locating agents used in TLC.
- Iodine forms a number of coloured loose complexes those are visible in day light.
- Sulphuric acid also can form coloured complexes.
- After development the next step is visualization that can be done by using UV light.
- Amino acids can be detected by using spray of ninhydrin.

(6) Development and Detection

- Chromatoplates are developed once with a single solvent either by following methods.
- Ascending or vertical development: The sample is spotted at one of the plate and then developed by ascending technique used in paper chromatography. The plates are placed vertical in container and solvent is allow to run from bottom to top.
- Horizontal development: the sample is placed in the centre of the plated and developed either by slowly dripping solvent on it from micropipette. This procedure is also known as circular TLC.
- Multiple developments: In this technique, the development is carried out number of times in same direction.
- Stepwise development: It is carried out consecutively with two different solvents but in same direction.
- Gradient development: It is useful when substances change the properties of solvents. This technique is also known as gradient elution. In this chromatography technique,

another more polar solvent is added in solvent system to modify the polarity of solvent system.

- Continuous development: It is useful method for separation of substances having close Rf values. In this technique, solvent is forced to run off the edges and collected instead of being left to evaporate.
- Two dimensional development: It is resemble to two dimensional paper chromatography. Square plates are used. The sample is spotted at the corners of the plate and allows to run in both directions with same or different solvents.

Column Chromatography

Always keep in mind

- **It is adsorption chromatography**
- **The stationary phase is solid.**
- **The mobile phase is liquid**

Note: take figures from material for those questions for which figures are not provided herein

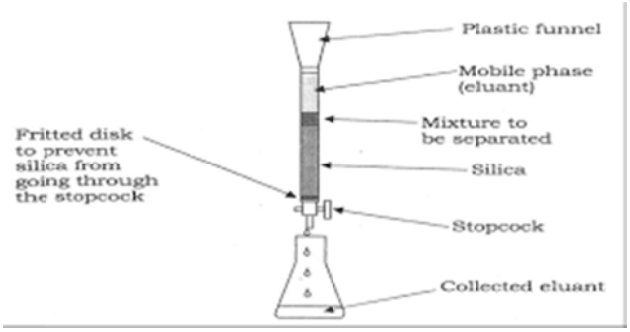
Introduction:

- The basic principle is selective adsorption.
- The long column is used to separate the mixture of substances.
- The solvent system that passes through the column is known as eluent.
- The process is known as elution.
- The mixture to be separated is dissolved in suitable solvent and allows passing through a column.
- The substance having higher adsorbent capacity retains in upper part of column.
- The substance having less adsorbent capacity retains in lower part of column.
- As a result substances retain in different parts of column and separated partially.
- The solvent is passed again to have better separation.
- The various bands in column are visible now.
- The banded column is known as chromatogram.
- The portion of column which is occupied by a particular substance is called its zone.

Apparatus:

- It involves a simple glass tube having length 20-30 cm with diameter of 2-3 cm and stopper at one end.

- The adsorbent is supported by plug or cotton or glass wool.
- Long and narrow tubes are used to carry out difficult or close separation.



Adsorbent Requirement

- Particles should be spherical in shape and uniform in size.
- They should have enough mechanical stability to prevent deposition of dust within them.
- They should be chemically inert hence they do not react with sample or solvent.
- They should contain as small amount of soluble components as possible.
- They should have neutral surface.
- They should be catalytically inactive.

Preparation of Adsorption Column

- Cotton, glass wool or plug is used as a support for the column.
- Then tube is kept vertically.
- Now, adsorbent is filled and column is packed uniformly.
- Glass road is used to press the adsorbent in order to achieve homogenous packing of column.
- The column should be filled two third.
- Now, solvent is passed from top and eluent is collected at bottom with suction or in conical flask.
- The column should never be kept dry and filled with eluent every time.

Solvents

- The choice of solvent is dependent upon the nature of substance to be separated.
- The solvents should be volatile having boiling point between 40 ˚C to 85 ˚C.
- Petroleum ether, cyclohexane, carbon disulphide, ethyl acetate, ethyl alcohol, methylene chloride, chloroform, acetone, ether, carbon tetrachloride, benzene and acetic acid are generally used solvents.
- They serve to introduce the mixture of the column.
- They affect the process of development by which the zone of chromatogram is separated.
- They also used for removing of required content of zone from the column after the development process is completed.

Detectors

There are several detectors those are used to determine the dissolved substances emerging from the column.

- Optical Detectors: These are traditional detectors and of flow analyser type. It is made up of glass and small cells and used for continuous photometric analysis with visible or UV light.
- Differential Refractometer: This method is used for the refraction of the emerging power for detection. It has been improved to diffractional refractometer and has more sensitivity.
- Detector Based Heat Adsorption: These detectors are known as micro adsorption detectors. In this detector, liquid from column is passed through cells.
- Flame Ionisation Detectors: Organic compounds undergo pyrolysis in oxygen flame. As a result, the ions are produced. These ions carry current through flame. The ions are collected at charged electrode. The ionisation of organic compounds in a flame is proportional to the number of reduced carbon atoms. The lower cell is filled up with an adsorbent. Glass covered measuring point of a small thermistor is located in the centre of the packing of each of the cell. The total deflection is proportional to the concentration at least to a range of 102. An endless wire is passed through column and

decomposed products of the substances are transported by the wire and lead to flame ionisation.

- Conductivity Detectors: These are suitable for ionised substance in aqueous solutions. The eluent is passed through the measuring cell of the detectors that contains two or three platinum electrodes within a wheat sore bridge circuit and is operated by AC current.

Factors Affecting Column Efficiency

- Nature of Solvent: High efficiency separation can be achieved by low viscosity solvents. The flow rate is inversely proportional to its viscosity. It is easier for a solvent to travel faster having low viscosity and having higher elution strength.
- Dimension of Column: It is possible to improve column efficiency by increasing the length/width of the column. For common preparative separation (quantitative analysis) column packing rations have found in the range of 1:20 to 1:100.
- Particle Size Column Packing: Decrease in particle size increases column efficiency. Small particles have higher surface area hence improve the separation. The usual particle size ranges from 100 to 200 meshes.
- Pore Diameters of Column Packing: Polar adsorbent possess a pore diameter of less than or equal to 20 Å. A decrease in average pore diameter from 170 to 20 Å does not affect column efficiency.
- Temperature of Column: Substances having low solubility are kept at higher temperature while other samples are separated at room temperature.

Application of Column Chromatography

- For analytical purposes.
- Separation of geometric isomers.
- Separation of diameters and tautameric mixtures.
- Separation of racemates.

Questions:

1. Factors affecting column efficiency
2. Advantages of TLC
3. Write a brief note on chromatography.
4. Why TLC is superior then other chromatographic techniques?
5. Explain detectors used in column chromatography in brief.
6. Types and classification of chromatography.
7. Discuss detecting reagents used in TLC.
8. Write a note on adsorbent requirement in column chromatography.
9. Discuss types of paper chromatography.