# UNIT-III CHROMATOGRAPHY

**Points to be covered in this topic**

- ❖ **Introduction**
- ❖ **Definition**
- ❖ **Classification of Chromatographic methods**

**CHROMATOGRAPHY**

## ❖ INTRODUCTION

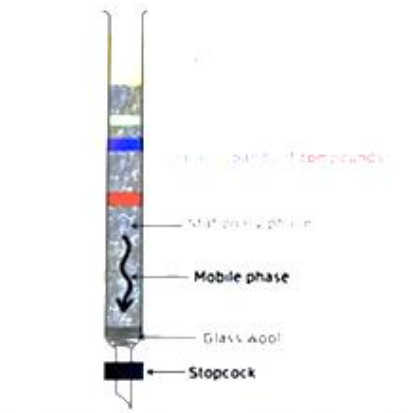
• Chromatography is a **combination of laboratory techniques** that are used for **separating the mixture components**.

• Chromatography deals with a sample (or sample extract) dissolved in a **mobile phase** (a gas, liquid, or a supercritical fluid), which is allowed to **move through an immobile and immiscible stationary phase**.

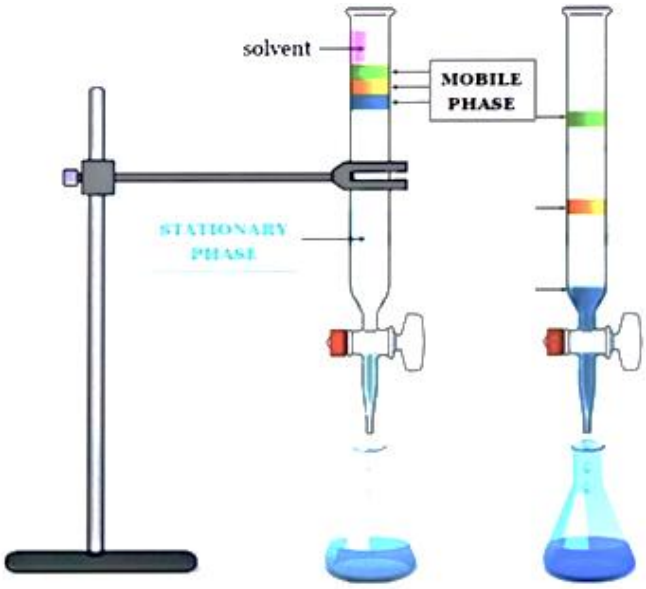
• Selection of phases is done on the basis of **solubility of components of the sample in each phase**.

## ❖ DEFINITION

• Chromatography is a **physical method of separation** in which the components to be **separated or distributed between two phases**, one of which is stationary (**stationary phase**), while the other, the **mobile phase**, moves in a definite direction.



Column Chromatography Set-Up. The mobile phase flows downwards through the solid stationary phase.



## ❖ CHROMATOGRAPHY

• Chromatography is a **combination of laboratory techniques** that are used for **separating the** mixture components.

## ❖ PREPARATIVE CHROMATOGRAPHY

• Preparative chromatography **separates the mixture components for further use**.

## ❖ ANALYTICAL CHROMATOGRAPHY

• Analytical chromatography requires small amounts of material and **determines the relative proportions of mixture analytes**.

## ❖ ANALYTE

• Analyte is a component that gets **separated during** chromatography.

## ❖ CHROMATOGRAM

• Chromatogram is a **visual interpretation of chromatograph**.

## ❖ CHROMATOGRAPH

• Chromatograph is equipment used for sophisticated **separation**.

## ❖ STATIONARY PHASE

• Stationary phase is a phase bonded **covalently on supporting particles or on the inside wall of column tubing**.

## ❖ MOBILE PHASE

• Mobile phase **moves in a specific direction**.

## ❖ RETENTION TIME

• Retention Time is the **time taken by an analyte to pass** through the system (from the column inlet to the detector) under definite conditions.

## ❖ PRINCIPLE OF CHROMATOGRAPHY

• Principle of chromatography involves **separation of components into varied bands (or colour graphs) and their identification**.

## ❖ ADSORPTION

• Adsorption is based on the **polarity of components with respect to the stationary phase**.

## ❖ ELUENT

• The solvent used in the **column as mobile phase** is called **eluent**.

## ❖ DISTRIBUTION COEFFICIENT (Kd)

• The distribution of compounds among immiscible phases is termed **partition or distribution coefficient (Kd)**.

## ❖ CAPACITY FACTOR (K')

• Capacity factor (K') is the **velocity of the analyte relative** to the velocity of the mobile phase.

## ❖ SELECTIVITY FACTOR (α)

• Selectivity factor (α) is the **relative velocities of the analytes with respect to each other**.

## ❖ GRADIENT ELUTION

• In **liquid chromatography**, the mobile phase composition can be **changed during the separation**, which is called gradient elution.

## ❖ RETARDATION FACTOR RF VALUE

• The ratio of **migration velocity of a solute** to the **migration velocity of the mobile phase** is termed the **retardation factor (RF value)**.

## ❖ COLUMN CAPACITY (K)

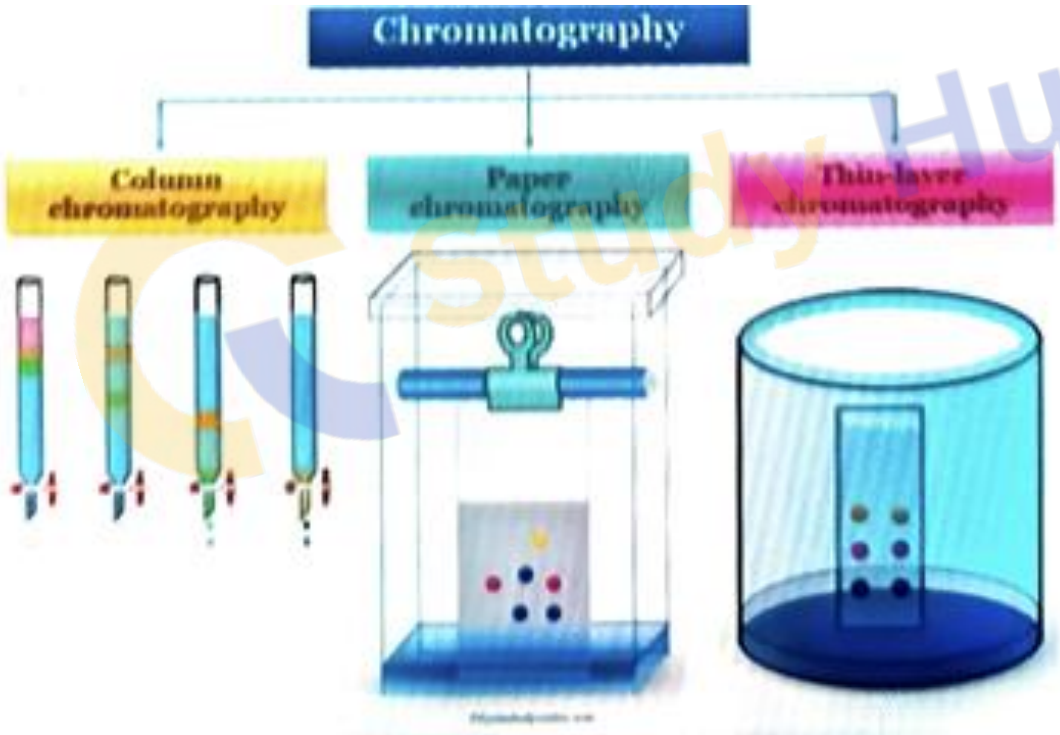
• Column capacity (k) is the ratio of the **amount of solute in the mobile and stationary phases**.

## ❖ ELUTION ANALYSIS

• Elution analysis is the method which is **widely used for developing** chromatograms.

## ❖ GRADIENT ELUTION

• Gradient elution is a **technique of separating the components by changing the eluent composition**.



## ❖ CLASSIFICATION OF CHROMATOGRAPHIC METHODS:

**Liquid Chromatography (LC)**

| General Classification | Type of Method | Stationary Phase | Mobile Phase | Type of Equilibration Process | Name of the Technique |
|---|---|---|---|---|---|
| Liquid Chromatography (LC) | Liquid–Liquid or Partition | Liquid supported on a solid surface | Liquid | Partition between the immiscible liquids | Paper Chromatography (PC), Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) |
| Liquid–Solid or Adsorption | Solid | Liquid | Adsorption | Adsorption Column Chromatography (ACC) | |
| Liquid–Solid or Adsorption | Very finely divided solid packed in a column | Liquid | Adsorption (using very much higher pressures for the flow of mobile phase) | High Performance Liquid Chromatography (HPLC) | |
| Ion-Exchange | Solid (ion-exchange resin) | Liquid | Partition/Sieving | Ion-exchange Chromatography (IEC) | |

| General Classification | Type of Method | Stationary Phase | Mobile Phase | Type of Equilibration Process | Name of the Technique |
|---|---|---|---|---|---|
| Affinity (usually uses enzymes or antigen–antibody highly specific interactions) | Group-specific liquid bonded to a solid surface (an antibody immobilized on a stationary phase by covalently binding to it – an affinity ligand) | Liquid | Partition between surface liquid (immobilized) and mobile phase | Affinity Chromatography | |

| General Classification | Type of Method | Stationary Phase | Mobile Phase | Type of Equilibration Process | Name of Technique |
|---|---|---|---|---|---|
| Gas Chromatography | Gas–Solid (or adsorption) | Solid | Gas | Adsorption | Gas–solid Chromatography (GSC) |

| General Classification | Type of Method | Stationary Phase | Mobile Phase | Type of Equilibration Process | Name of Technique |
|---|---|---|---|---|---|
| Gas–Liquid (or Partition) | Liquid adsorbed on a solid | Gas | Partition between Gas and Liquid | Gas–Liquid Chromatography (GLC) | |

**Supercritical Fluid Chromatography (SFC)**

| General Classification | Type of Method | Stationary Phase | Mobile Phase | Type of Equilibration Process | Name of Technique |
|---|---|---|---|---|---|
| Supercritical Fluid Chromatography (SFC) | Bio-specific adsorption or bio-affinity | Organic species bonded to a solid surface | Supercritical fluid | Partition between supercritical fluid and bonded species | Supercritical Fluid Chromatography (SFC) or Bioaffinity Chromatography (BC) |

# UNIT–III ADSORPTION AND PARTITION COLUMN CHROMATOGRAPHY (PART - 2)

**Points to be covered in this topic**

➤ **Methodology**

➤ **Advantage**

➤ **Disadvantage**

➤ **Application**

## ADSORPTION AND PARTITION COLUMN CHROMATOGRAPHY

### ❖ INTRODUCTION

• When a **column of stationary phase** is used, the technique is called **column chromatography**.

• Based on the nature of the stationary phase i.e., whether it is solid or liquid, it is called as **column** adsorption chromatography or **column partition chromatography**. Column partition chromatography is **not widely used**.

### ❖ PRINCIPLE

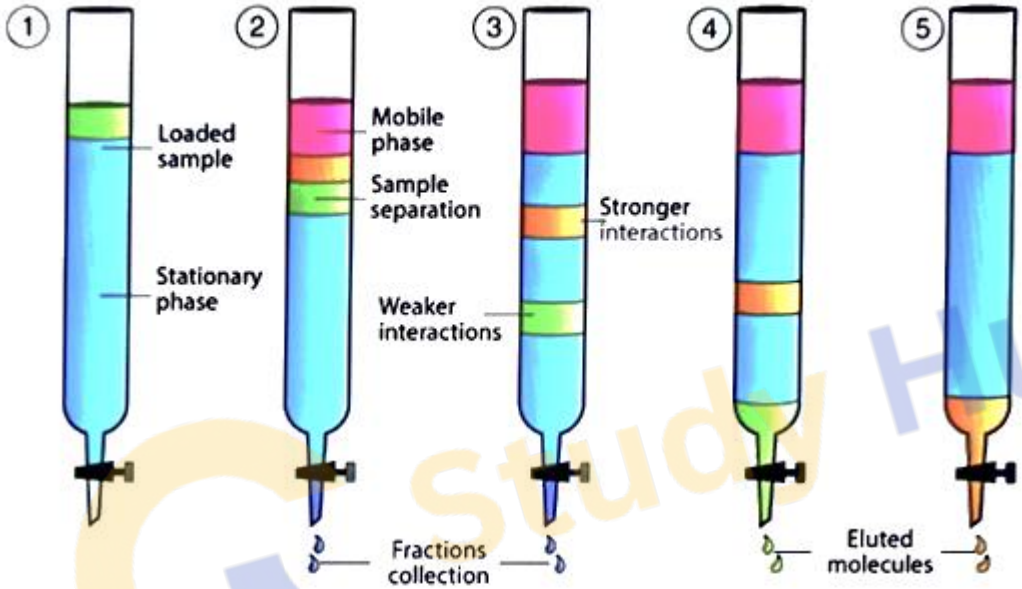
1. This technique is based on the principle of **differential adsorption**, where different molecules in a mixture have different affinities for the adsorbent present in the stationary phase.
2. Molecules having **higher affinity remain adsorbed for a longer time**, decreasing their speed of movement through the column.
3. Molecules with lower affinity move faster, thus allowing the molecules to be separated into different fractions.
4. In column chromatography:
    - Stationary phase = solid (mostly silica)
    - Mobile phase = liquid
    - The mobile phase allows molecules to move through the **column smoothly**.
    - The interaction between stationary phase (adsorbent) & solute is **reversible**.

• The rate of movement of a component (R) is given as follows:

• The equation can be simplified as follows:

$$R = \frac{\text{Distance moved by the solute}}{\text{Distance moved by the solvent}}$$

## COLUMN CHROMATOGRAPHY




• When a liquid mobile phase is used, the equation is written as

$$R = \frac{A_m}{A_m + \alpha A_s}$$

$$\text{Where } \alpha \text{ is the Partition coefficient} = \frac{\text{Conc. in stationary phase}}{\text{Conc. in mobile phase}}$$

● $A_m$ is the average cross section of mobile phase

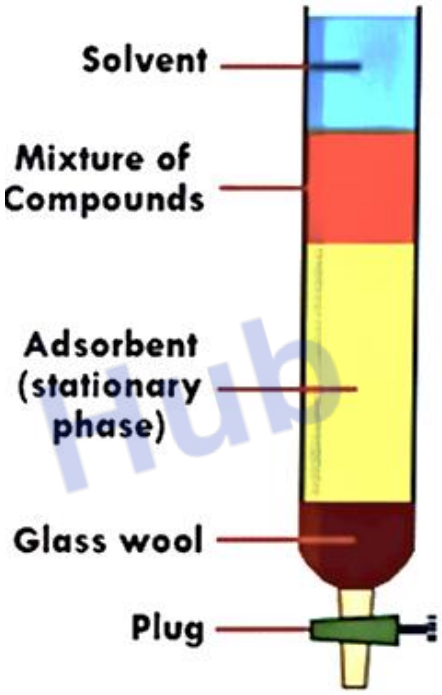
$$R = \frac{\text{Rate of movement of component}}{\text{Rate of movement of mobile phase}}$$

- $A_S$ is the average cross section of stationary phase

## ❖ TYPES OF COLUMN CHROMATOGRAPHY

✓ Column chromatography is of the following four types:

1. **Adsorption column chromatography**
2. **Partition column chromatography**
3. **Ion exchange chromatography**
4. **Gel chromatography**




### Adsorption column chromatography

- In adsorption column chromatography, the **mixture components get selectively adsorbed on the surface of the** material packed in column (i.e., the adsorbent).

- In this technique, the mixture components travel towards the **stationary phase** at different rates due to differences in their affinity.

- **Adsorption indicates a physical attachment** between a compound and the stationary phase particles.

- The **polar compounds** being separated adsorb to the **polar stationary phase with more intensity**, while the non-polar compounds adsorb to the non-polar stationary phase.

- Therefore, when a polar stationary phase is used, the polar components due to greater adsorption **elute out later**, while the non-polar one elute out first.

- On the contrary, when a **non-polar stationary phase** is used, **the reverse happens.**

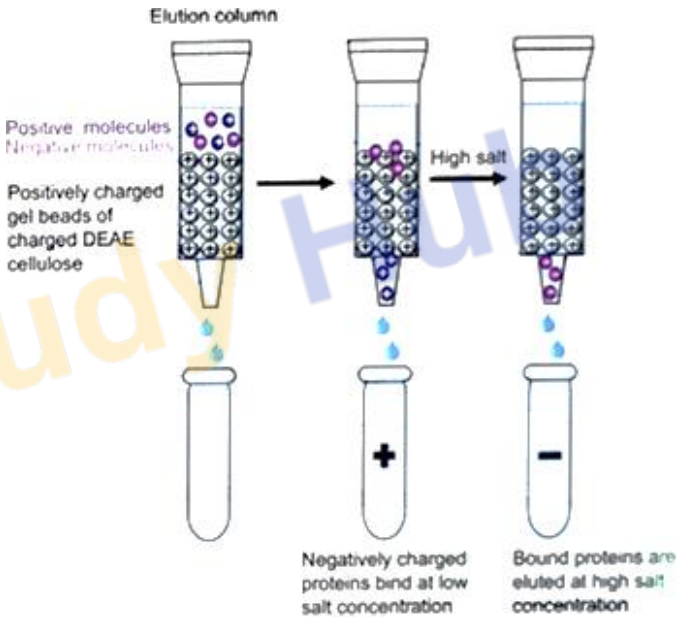
### Partition column chromatography

- In partition column chromatography, the **mixture components distribute between the liquid stationary and mobile phases** held on an inert solid support.

- In this technique, the mixture components distribute between the **two liquid phases due to differences in their** partition coefficients during the flow of the mobile phase in the chromatography column. Since both the stationary and mobile phases are liquid in nature, the molecules disperse into either of the phases.

- The **polar molecules get** partitioned into the polar phase, while the **non-polar ones get partitioned into the non-polar phase**.

- In partition chromatography, the commonly used stationary phases are diatomaceous earth **(kieselguhr, celite, etc.), cellulose, and silica gel**.
However, **glass beads, brick powder, starch, or** purified sand can also be used limitedly.




## Ion Exchange Chromatography

- In ion-exchange chromatography, the **ions and polar molecules get separated based on their charge.**

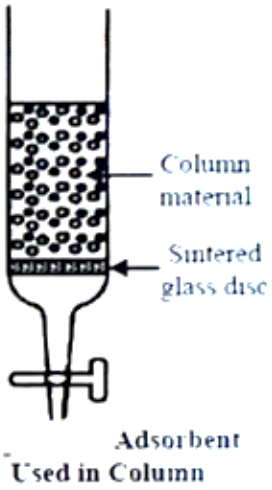
- This technique can be used for **any** type of charged molecules like large proteins, small nucleotides, and amino acids.

- The solution to be injected is termed **sample**, and each separated component is termed an **analyte**.

- Ion-exchange chromatographic technique is **used in protein purification, water analysis, and** quality control**.**

## Gel Chromatography

● In gel chromatography, the column is packed with a **permeable gel**, which helps in **separating the substances by molecular filtration or sieving action.**




## ❖ METHODOLOGY

**• The column chromatography following steps procedure includes:**

**1) Selection of column,**
**2) Selection of adsorbents and solvents,**
**3) Preparation of column,**
**4) Application of sample,**
**5) Elution procedures, and**
**6) Detectors.**

### ❑ Selection of column

• Column is mostly best quality of neutral glass since **it should not be affected by solvents, acids or alkalies.**

An ordinary burette can be used as column for separation.

Length/diameter ratio is **10–15:1.**

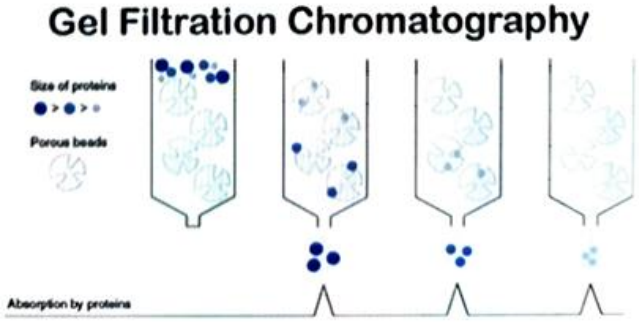
For more efficiency, the length/diameter ratio is **100:1.**

**Column length**

a. Multi-component system long column
b. Components with similar affinities for adsorbent long column
c. Components with different affinities for adsorbent short column

### ❑ Adsorbents and Solvents

#### ➤ Adsorbents



• **Silica gel** and **alumina** are commonly used as adsorbents (**stationary phase**). Sometimes florisil is also used as an adsorbent.

**An ideal adsorbent should possess the following characteristics:**

**1) It should have an appropriate size and shape.**
**2) It should be mechanically stable.**
**3) It should be chemically inert.**
**4) It should have neutral surfaces.**

## ✓ Adsorbents Used in Column Chromatography

| Adsorbents | Used to Separate |
|---|---|
| Alumina and magnesia | Sterols, vitamins, esters, and alkaloids |
| Silica gel | Sterols and amino acids |
| Carbon | Amino acids, peptides, and carbohydrates |
| Magnesium carbonate | Alkaloids, esters, glycerides, and sterols |
| Calcium carbonate | Xanthophylls and carotenoids |
| Aluminium silicate | Sterols |
| Starch | Enzymes |

• The amount of sample that gets adsorbed on an adsorbent is determined by mixing **the known amount of adsorbent with the known volume of solvent at a constant temperature till equilibrium is achieved.**

• Thereafter, the above solution is filtered and the amount of **sample adsorbed per gram** of adsorbent is plotted against the concentration.

**The following curves can be seen in the plot:**

1. **Linear Adsorption Isotherm:**
   Curve appears when the *adsorbed substance* and the *solution concentration* are directly proportional.
2. **Convex Adsorption Isotherm:**
   Curve appears when the *weak concentration* solution gets adsorbed.
3. **Concave Adsorption Isotherm:**
   Curve appears when the *strong concentration* solution gets adsorbed.

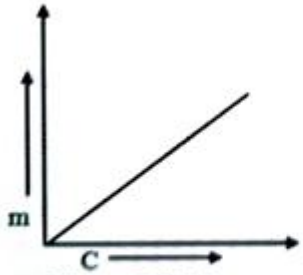


**Figure 8.4: Linear Adsorption Isotherm Curve**

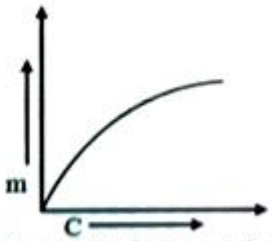


**Figure 8.5: Convex Adsorption Isotherm Curve**

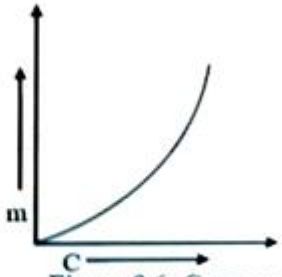


**Figure 8.6: Concave Adsorption Isotherm Curve**

## ➤ Solvents

• The solvents are used to **elute the sample by mixing with it.**

• Commonly used solvents: petroleum ether, cyclohexane, carbon tetrachloride, benzene, chloroform, pyridine, acetone, ethanol, methanol, water, formamide.
They are based on solvent polarity.

**An ideal solvent should possess the following functions:**

**1) It should be able to introduce the sample into the column.**
**2) It should be used as developers.**
**3) It should be removed after each step.**

## ❑ Preparation of the Column

• The column mostly consists of a glass tube packed **with a suitable stationary phase.**

• **Glass wool/cotton wool or an asbestos pad** is placed at the bottom of the column before packing the stationary phase.

• After packing, a paper disc kept on the top ensures that the stationary layer **is not disturbed** during the introduction of sample or mobile phase.

**Two types of preparing the column:**

1. **Dry packing / dry filling:**

- Required quantity of adsorbent is poured as fine dry powder.
- Solvent allowed to flow till equilibrium.

2. **Wet packing / wet filling:**

- A **slurry** of adsorbent with mobile phase is prepared and poured into column.
- Considered ideal for packing.

• Before using column, **wash properly and dry**.

## ❑ Application of Sample

• Sample should be applied uniformly from the top **of the column using a small pipette.**

• The tip of this pipette is **placed just above the adsorbent surface** and opposite to column wall.

• Sample can also be applied by method in which **one or** two small filter paper **discs** (present at column top), saturated with sample material dissolved in volatile solvent, are used.

## ❑ Elution (Development technique)

• By elution technique, the individual components are separated out from the column.

**It can be achieved by two techniques:**

1. **Isocratic elution technique:**

Same solvent composition or solvent of same polarity used throughout separation.

**Example:**
Chloroform alone OR petroleum ether : benzene = 1:1 only, etc.

**2. Gradient elution technique:**

Solvents of gradually **increasing polarity** or **increasing elution strength** used during separation.

**Example:**

Initially benzene ⟶ chloroform ⟶ ethyl acetate ⟶ chloroform.

Other techniques include frontal analysis, displacement analysis, and concentration vs. eluate volume plots.

## ❑ Detectors

• Detectors are used for determining and monitoring the dissolved substances from the column.

**Types of detectors:**

**1) Optical Detectors:**

• Small cells of **glass** or **quartz**.
• Used as **flow analyser** for stable photometric analysis with visible/UV light.
• If absorption is inadequate, reagents are added to form measurable colour reactions.

**2) Differential Refractometers:**

• Based on **refraction of emerging power**.

• Formed as a sensitive differential refractometer with detection limit of $10^{6}$ **gm**.

**3) Detectors Based on Heat of Adsorption (Micro-Adsorption Detectors):**

• A separating column releases liquid that is passed via **two cells** situated above each other…

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**4) Flame Ionisation Detectors:**

**Continuous metal wire passes through column exit.** Decomposition products ⟶ argon or flame ionisation signal.

**5) Conductivity Detectors:**

Suitable for **ionised materials in aqueous solution**. Effluent passes between **platinum electrodes**; works on alternating current.

- Other detectors: microwave detector, ultrasonic detector, mass detector, vapour pressure detector, solvent front detector.

## ❖ ADVANTAGES

- Column chromatography has the following advantages:

1. It can be used to separate **any type of mixture**.
2. It can be used to separate **any quantity** of mixture.
3. Wide range of **mobile phases** can be used.
4. Sample can be **separated and reused**.
5. **Automation** is possible.

## ❖ DISADVANTAGES

- Column chromatography has the following disadvantages:

1. It is a **time-consuming method**.
2. It is an **expensive method** (requires large amount of solvent).
3. Automation is possible but makes the technique **complicated and expensive**.

## ❖ APPLICATIONS

Column chromatography has limited applications in analytical field.

**1) Analytical Uses:**

Glass/copper capillaries (0.05–2 mm internal diameter; 1–20 m length).
Used for amino acids separation using pyridine : butanone : dilute acetic acid (5:5:1).

**2) Separation of Geometrical Isomers:**

Silica gel & charcoal separate **cis/trans isomers of carboxylic acids**.
More strongly adsorbing isomer elutes slower.

**3) Separation of Diastereomers:**

7-chloro-azibicycl-heptane separated by silica gel using **pentane/diethyl ether**.

**4) Separation of Tautomeric Mixtures:**

Done at **high temperature** only by column chromatography.

**5) Separation of Racemates:**

Racemates isolated using **organic solvents on lactose**, e.g., Troger's base enantiomers.

# UNIT-III – THIN LAYER CHROMATOGRAPHY (PART - 3)

**Points to be covered in this topic**

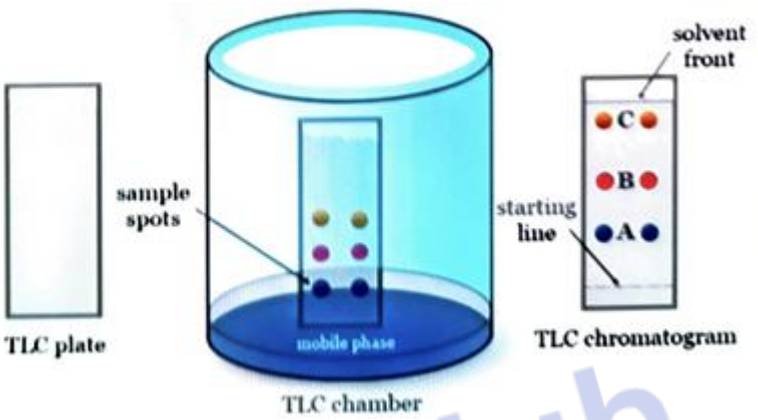
- ❖ Introduction
- ❖ Principle
- ❖ Methodology
- ❖ Rf values
- ❖ Advantages
- ❖ Disadvantages
- ❖ Application

## THIN LAYER CHROMATOGRAPHY

## ❖ INTRODUCTION

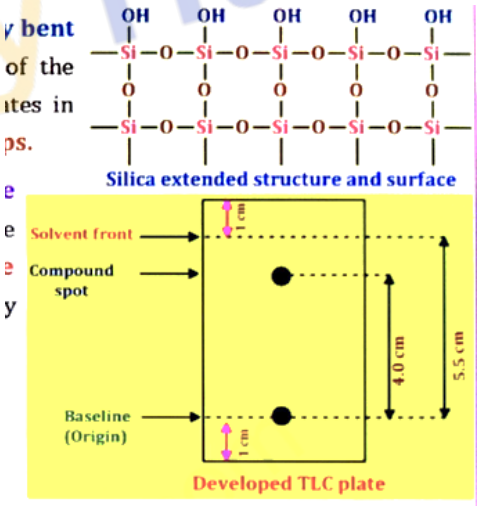
- TLC history dates to **1938** (Izmailov & Schreiber).
- **1944:** Consden, Gordon & Martin used filter papers for amino acids.
- **1950:** Kirchner used filter paper coated with alumina.
- **1958:** Stahl developed standard TLC equipment.

## ❖ PRINCIPLE




- TLC is a method of **separation or identification of mixture components** by using finely divided **adsorbent coated on a chromatographic plate**.
- The **mobile phase solvent** flows due to **capillary action (against gravity)**.
- Components move according to their **affinity** toward the adsorbent.
  - **Higher affinity ⟶ moves slower**
  - **Lower affinity ⟶ moves faster**

Thus separation occurs based on affinity.




- **Silica ($SiO_2$)** is a solid with extended tetrahedral silica atoms bridged by **oxygen atoms**.

**• Silica atoms bridged together by bent oxygen atoms.**

On the surface of the silica particles, the solid terminates in **very polar silanol (Si–O–H) groups**.

- The **silica is the stationary phase** because it remains adhered to the glass plate and **does not move** during the chromatography process.

## ❖ METHODOLOGY

**• The various steps involved in TLC are:**

- Stationary Phase
- Preparation of the Glass Plates
- Application of Sample
- Development Tank
- Mobile Phase
- Development technique
- Detecting or Visualizing Agents
- Qualitative analysis
- Quantitative Analysis
- Application of TLC

### ❑ Stationary Phase

- There are several adsorbents which can be **used as stationary phases**.
- Some of the stationary phases, their composition and the ratio in which they have to be mixed with **water or other solvents to form a slurry for preparing thin layer chromatographic plates**.

| Name | Composition | Adsorbent : Water Ratio |
|---|---|---|
| Silica gel H | Silica gel without binder | 1 : 1.5 |

| Name | Composition | Adsorbent : Water Ratio |
|---|---|---|
| Silica gel GF | Silica gel + Binder + Fluorescent indicator | 1 : 2 |
| Silica gel G | Silica gel + $CaSO_4$ (gypsum) | 1 : 2 |
| Alumina Neutral | $Al_2O_3$ without binder | 1 : 1.1 |
| Alumina Basic/Acidic | $Al_2O_3$ + binder | 1 : 2 |
| Cellulose powder | Cellulose without binder | 1 : 5 |
| Cellulose powder | Cellulose with binder | 1 : 6 |
| Kieselguhr G | Diatomaceous earth + binder | 1 : 2 |
| Polyamide powder | Polyamide | 1 : 9 ($CHCl_3$ : $CH_3OH$ = 2:3) |

- Silica gel and alumina are available with different specific surface areas and these grades are identified by a number, e.g., silica gel 60 (or 40 or 150), which indicates the mean pore size in Ångstroms **(10–10 m)**.
- The particle size of silica gel for TLC is **10–40 µm** (average 15 µm).

## ❑ Preparation of the Glass Plates

- The sizes of the glass plates for use with commercially available spreaders **are usually 20 × 20, 20 × 10, or 20 × 5 cm.**
- Microscopic slides can also be used for some applications like monitoring the progress of chemical reaction.
- In general, **the glass plates should be of good quality and should withstand temperatures used for drying the plates.**

## ✓ General method:

- Mix **30 g of the adsorbent** in a mortar to a smooth consistence with the required amount of water or solvent specified in the manufacturer's instruction and **transfer the slurry quickly** to the spreader.
- Spread the mixture over **4 to 5 plates (20 × 20 cm)** or a proportionately larger number of smaller plates and allow the thin layers to set (about 4 minutes when $CaSO_4$ is present).
- Transfer the plates carefully to a suitable holder and after a further **30 minutes, dry at 100–120°C for 1 hour** to activate the adsorbent.
- Cool and store the plates in a **desiccator over silica gel**.
- The thickness of the moist thin layer should be **about 0.25 mm.**

## ✓ Special methods:

### a. Preparative thin layer:

- Layers are **0.5–2 mm thick**, prepared as described under the general method, but using smaller quantity of water and allowing longer time for initial drying.

### b. Microscopic slides coating (dipping technique):

- Prepare slurry of the adsorbent by shaking with **chloroform or chloroform–methanol (2:1)**.
- Insert two microscope slides back-to-back into the slurry.
- Withdraw, allow draining, separate and dry the slides.

### c. Spray method:

- The slurry (normal preparation) is sprayed onto **glass plates using a laboratory spray gun**.

### d. Organic solvent slurry coating:

- Adsorbent mixed with organic solvent (e.g., chloroform or ethyl acetate) is evenly distributed over the glass plate by careful tilting.
- After evaporation, dry as usual.
- Plates should be cleaned at edges and backs before use.

### ❑ Application of Sample:

- To obtain **good spots**, the concentration of the sample or standard solution can be **2–5 µL of a 1% solution** using a capillary tube or micropipette.
- The spots can be placed randomly or equidistant from each other using a template.
- **The spot should be kept at least 2 cm above the base** of the plate.
- The spotting area should **not be immersed** in the mobile phase in the development tank.

### ❑ Development Tank:

- For the purpose of development, a **developing tank or chamber of different sizes** to hold TLC plates of standard dimensions are used.
- These require **more solvents for developing the chromatogram**. When a new method is developed, it is **better to develop in glass beakers or specimen jars, etc., to avoid more wastage of solvents**.
- When developed method or standard method is used, it is better to use development tank. In the new type of development **tanks have hump in the middle**, which require less solvent.
- The development chamber or tank should be lined inside with **filter paper moistened with the mobile phase** so as to saturate the atmosphere.
- If this kind of saturation of the atmosphere is not done, **"edge effect" occurs**, where the solvent front in the middle of the TLC plate moves faster than that at the edge.

## ❑ 5. Mobile Phase:

- **Selection of the mobile phase depends upon the below factors**
  i. Nature of the substances to be separated
  ii. Nature of the stationary phase used
  iii. Mode of chromatography (**Normal phase or Reverse phase**)
  iv. Separation to be achieved – **Analytical or Preparative**

- **Pure solvents or mixture of solvents are used.**
  The following gives a list of solvents (**of increasing polarity**):
  *Petroleum ether, Carbon tetrachloride, Cyclohexane, Carbon di-sulphide, Ether, Acetone, Benzene, Toluene, Ethyl acetate, Chloroform, Alcohols like methanol or ethanol, Water, Pyridine.*
  The solvent composition is done by trial and error method only but with a systematic approach.

## Development technique:

- Different development techniques are used for efficient separations. They are
  **i. Vertical development (One dimensional)**
  **ii. Two dimensional development**
  **iii. Horizontal development**
  **iv. Multiple development**

### i. Vertical development (One dimensional):

In this technique, the plates are kept **vertical** and the solvent flows **against gravity, because of capillary action.**

**ii. Two dimensional techniques:**

For complex mixtures this technique is used. First, the **plates are developed in one axis and the plates after drying are developed in the other axis.**

When large number of compounds cannot be separated by using one dimensional technique.

## Detecting or Visualizing Agents

- After the development of TLC plates, the spots should be visualized.
- Detecting colored spots can be done visually. But for detecting colorless spots, **any one of the following techniques can be used.**

**a. Specific methods:**

In this method particular detecting agents are used to find out the nature of compounds or for identification purposes. Examples are

**i. Ferric chloride – for phenolic compounds and tannins.**
**ii. Ninhydrin in acetone – for amino acids**
**iii. Dragendorff's reagent – for alkaloids**
**iv. 2,4 – Dinitrophenyl hydrazine – for aldehydes and ketones**

**b. Nonspecific methods:**

Where the number of spots can be detected, but not the exact nature or type of compound.

**Examples**

**i. Iodine chamber method:**

Where brown or amber spots are observed when the TLC plates are kept in a tank with few iodine crystals at the bottom.

**ii. Sulphuric acid spray reagent:**

**70–80% v/v of sulphuric acid with few mg of either potassium dichromate or potassium permanganate or few ml of nitric acid as oxidizing agent** is used.

This reagent after spraying on TLC plates is heated in an oven. **Black spots are seen due to charring of compounds.**

**iii. Using fluorescent stationary phase:**

When the compounds are not fluorescent, a **fluorescent stationary phase is used.**
When the plates are viewed under UV chamber, dark spots are seen on a fluorescent background.
Examples of such stationary phase is **Silica gel GF.**

### The detecting techniques can be categorized as:

**i. Destructive technique:**
Specific spray reagents, Sulphuric acid spray reagent, etc where the **samples are destroyed for detection.**

**ii. Non-Destructive technique:**
Like UV chamber method, Iodine chamber method, densitometry method, etc where the sample is **not destroyed even after detection.**
These detecting techniques are used in **TLC method development and in preparative TLC.**

### In densitometry method,

Densitometer is used which measures **quantitatively the density of the spots.**
When the optical density of the spots for the standards and test solution are measured, the quantity of the substance can be calculated.

- The plates are neither destroyed nor eluted with the solvents to get the compounds.
- **This method is also called as in-situ method.**

### Qualitative analysis

- The **Rf value** is calculated for **identifying the spots in qualitative analysis.**
- Rf value is the ratio of distance travelled by the **solute** to the **distance travelled by the solvent front.**

**Rf = (Distance travelled by solute) / (Distance travelled by the solvent front)**

- The **Rf value ranges from 0 to 1.**

- The **ideal value is 0.3 to 0.8.**
- The **Rf value is** constant for every compound in a particular combination of stationary and mobile phase.
  When the Rf value of a sample and reference compound is same, **the compound is identified by its standard.**
  When the Rf value differs, the compound may be different from its reference standard.

## Rx value

Rx value is the **ratio of distance travelled by the sample and the** distance travelled **by the standard.**
Rx value is always closer to **1.**

## Rm value

Is used to find out whether the compounds belong to a homologous series.

If they belong to a homologous series, the **ΔRm** values are constant.

The ΔRm values for a pair of adjacent member of a homologous series are determined by using the formula:

**Rm = log ( (1 / Rf) – 1 )**

## Quantitative Analysis

- **Indirect method:**

Quantitative analysis can be done after eluting the individual spots with solvent and filtering off the stationary phase.
The solution can be concentrated and the exact quantities of the compound determined by methods like:

- UV-Visible spectrophotometry
- Fluorescence method

- Flame photometric method
- Electrochemical methods of analysis

● **Direct method:**

After eluting the **individual spots with solvent** **and filtering off the stationary phase.**
The solution can be concentrated and examined by:

- UV-visible spectrophotometry
- Fluorescence method
- Flame photometric method
- Electrochemical methods etc.

## Application of TLC

**i. Separation of mixtures of drugs of chemicals or biological origin, plant extracts etc.**
**ii. Separation of carbohydrate, vitamins, antibiotics, proteins, alkaloids, glycosides etc.**
**iii. Identification of drugs.**

**Drug Examples Table**

| Drug | Stationary Phase | Mobile Phase | Detecting agent |
|---|---|---|---|
| **Amoxicillin trihydrate** | Silica Gel G.F.-254 | Buffer pH 6: acetone (4:1) | NaOH + Starch + glacial acetic acid + Iodine in potassium iodide |
| **Ampicillin for oral suspension** | Cellulose M.N.-300 | Citric acid : Butyl alcohol (5:1) | Starch iodide reagent |

**Test for impurities, decomposition & related substances**

(as per British Pharmacopoeia substances and preparations)

| Substance | Tested for | Mobile Phase | Detection |
|---|---|---|---|
| **Chlorpropamide** | p-Chlorobenzene sulphonamide and NN'-diisopropylurea (0.33%) | Chloroform : methanol : cyclohexane :13.5M ammonia (100:50:30:11.5) | Sodium hypochlorite followed by potassium iodide in starch mucilage |
| **Nitrazepam Tablets** | Decomposition and related substances 0.5% | Nitromethane : ethyl acetate (85:15) | 254nm radiation |
| **Desipramine Hydrochloride** | Iminodibenzyl (0.2%) | Toluene : ethyl acetate : ethanol : diethylamine (20:20:4:1) | Potassium dichromate (0.5%) in sulphuric acid : water (4:4) |

## ❖ ADVANTAGES

- TLC combines the advantages of paper and column chromatography, and in some cases is also superior to them. The main advantages of TLC are:

**1) Simple Equipment:** It requires simple equipment.

**2) Short Development Time:** A good separation on inorganic adsorbent layers can be achieved rapidly as the development time is only an hour; while **paper and column chromatographic techniques require several hours or days for development.**

**3) Wide Choice of Stationary Phase:** It can be employed for adsorption, partition (including reversed phase) or ion exchange chromatography.

**4) Early Recovery of Separated Components:** The powdery coating of the plates can be removed by scraping with a knife. This means that a spot can be removed quantitatively, **and the required component can be determined by calorimetric or spectrophotometric analysis** by dissolving in a suitable solvent.

**5) Separation Effects:** The separation effects in TLC are superior to those obtained by paper chromatography.

**6) Easy Visualisation of Separated Compounds:** The fluorescence compounds can be **easily detected under UV light because the inorganic background does not fluoresce.**

**7) Sensitivity:** Extremely sharp delineated spots are obtained, thus spray reagents can easily detect even small quantities in comparison to paper chromatography in which more diffused spots can only be detected. This **increased sensitivity is of the order of 10 to 100 folds** than that of paper chromatography.

**8) Variable Thickness of Thin Layers:** The method used in TLC analysis can be applied for the **preparative separation using thicker layers** as well as to most separations by column chromatography.

**9) Chemically Inert Stationary Phase:** The biggest advantage of using an inert stationary phase in TLC is that it affords a vigorous means of detection including the **application of strong heat or corrosive reagent, like concentrated $H_2SO_4$.**

## ❖ DISADVANTAGES

- The main disadvantages of TLC are:

1. There is a no longer stationary available in TLC plates, therefore, **its separation length is insufficient in comparison to other chromatographic techniques.**
2. The results obtained are difficult to reproduce.
3. Only soluble components of the mixtures can be separated.
4. It is not automatic.
5. It works in the open system, thus can get affected by temperature and humidity.

## ❖ APPLICATIONS

- Thin layer chromatography has the following applications:
  **1) Qualitative estimation**, and
  **2) Quantitative estimation.**

## ❑ Qualitative Estimation

- The qualitative applications of TLC are as follows:

**1) As a Check on Processes:**

TLC can control some other **separation and purification processes** (e.g., distillation fractions and purification by molecular distillation).

**2) In Organic Chemistry:**

It can be used for the isolation and separation of individual components of a mixture.

i) **For Checking the Purity of Sample:**
It is used for measuring the sample purity obtained by other methods.

ii) **As a Purification Process:**
A pure form of an organic compound is used in small quantity for characterization of the same compound by elemental analysis and other physical methods.

iii) **Examination of Reaction:**
It is used for measuring the reaction progress and its completion.

iv) **For Identifying Organic Compounds:**
It can be used for rapid isolation, separation and purification of organic substances.

**3) Applications of TLC for Separation of Inorganic Ions:**

It can be used for the separation of cations, anions, pure covalent species, and some organic derivatives of the metals.

**4) Separation of Amino Acids:**

It can be used for the separation of amino acids in a mixture.

**5) Separation of Vitamins by Thin-layer Chromatography:**

**$F_{254}$ pre-coated silica gel TLC plates (20 × 2 cm dimension)** are used for the separation of vitamins E, $D_3$ and A.

## ❑ Qualitative estimation

- The spot area is measured based on pre-calibration, and this helps in quantitative analysis of the components in the spot. The samples separated by TLC can be quantitatively analyzed by the following techniques:

**1) Photodensitometry or Visual Comparison:**

Photodensitometry is used in case of **transmission, reflectance or fluorescence modes.** Visual comparison of the **spot intensity** of the test sample with that of the standard (with known intensity) is done.

**2) The sample spots (eluted with a proper solvent)**

are determined with a suitable technique (**visual spectrophotometry or spectrofluorimetry**). Incorrect results are obtained if the eluate is directly measured in the UV region.
This is because the substances extracted from the adsorbent undergo inappropriate absorption.
**This incorrectness can be overcome by measuring the extracts at longer wavelength.**

**3) Measurement of Spot Areas:**

The spot area on the plate and the component quantity in that spot can be correlated by many available techniques.
Purdy and Truter in 1962 stated that **a linear relationship exists between the logarithm of the weight of the substance and the square root of the spot area.**

# UNIT–III PAPER CHROMATOGRAPHY (PART - 4)

**Points to be covered in this topic**

- **Introduction**
- **Methodology**
- **Development techniques**

➤ **Application**

➤ **Advantages**

➤ **Disadvantages**

**PAPER CHROMATOGRAPHY**

## ❖ INTRODUCTION

• Paper chromatography (PC) is a type of a **planar chromatography** whereby chromatographic procedures are run on a specialized paper.

• It 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 (1865).**

### ✓ Types of Paper chromatography:

**(i) Paper Adsorption Chromatography:**
Paper impregnated with **silica or alumina acts as adsorbent (stationary phase)** and solvent as mobile phase.

**(ii) Paper Partition Chromatography:**
Moisture / Water present in the pores of **cellulose fibers present in filter paper acts as stationary phase** & another mobile phase is used as solvent.
In general, paper chromatography mostly refers to paper partition chromatography.

## ❖ PRINCIPLE

• This technique is generally used in the **analysis, identification, purification and quantification** of the components of mixtures into individual compounds.

• Paper chromatography is based on the principle of **partition** and the substances are distributed according to their **migration rates between two liquids**, of which one is the

stationary phase **(stationary liquid held in the paper)** and the other is the **mobile phase (moving liquid).**

• The mobility of mixture components depends on the nature of stationary phase and partition coefficient.

## ❖ INSTRUMENTATION OF PAPER CHROMATOGRAPHY

1. **Stationary phase & papers used**
2. **Mobile phase**
3. **Application of sample**
4. **Developing Chamber**
5. **Detecting or Visualizing agents**

### ❑ Stationary phase and papers:

• Whatman filter papers of different **grades** like **No.1, No.2, No.3, No.4, No.17, No.20** etc. are used.

In general, the paper contains **98–99% of α-cellulose, 0.3–1% β-cellulose.**

• These papers differ in sizes, shapes, porosities and thickness.

• Other modified papers like Acid **or** base washed **filter paper, glass fiber type paper.**

• **Hydrophilic Papers** – Papers modified with **methanol, formamide, glycol, glycerol** etc.

• **Hydrophobic papers** – Acetylation of OH groups leads to hydrophobic nature, hence can be used for reverse phase chromatography.
Silicon pretreatment and organic non-polar polymers can also be impregnated to give reverse phase chromatographic mode.

• Impregnation of silica, alumina, or ion exchange resins can also be made.

• **Size of the paper used:** Paper of any size can be used. Paper should be kept in a chamber of suitable size.

## ❑ Application of sample:

• The sample to be applied is **dissolved in the mobile phase and applied using capillary tube or using micropipette**.

• Very **low concentration** is used to avoid larger zone.

## ❑ Paper chromatography mobile phase

• Pure solvents, buffer solutions or mixture of solvents can be used. Some of the examples:

### ✓ Hydrophilic mobile phases

• **Isopropanol : ammonia : water = 9 : 1 : 2**
• **Methanol : water = 4 : 1 or 3 : 1**
• **n-Butanol : glacial acetic acid : water = 4 : 1 : 5**

### ✓ Hydrophobic mobile phases

• **Kerosene : 70% isopropanol**
• **Dimethyl ether : cyclohexane**

• 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.

## ❑ 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 sizes depending upon paper length and development type.**

• The chamber atmosphere should be **saturated with solvent vapor.**

• **Development technique:**

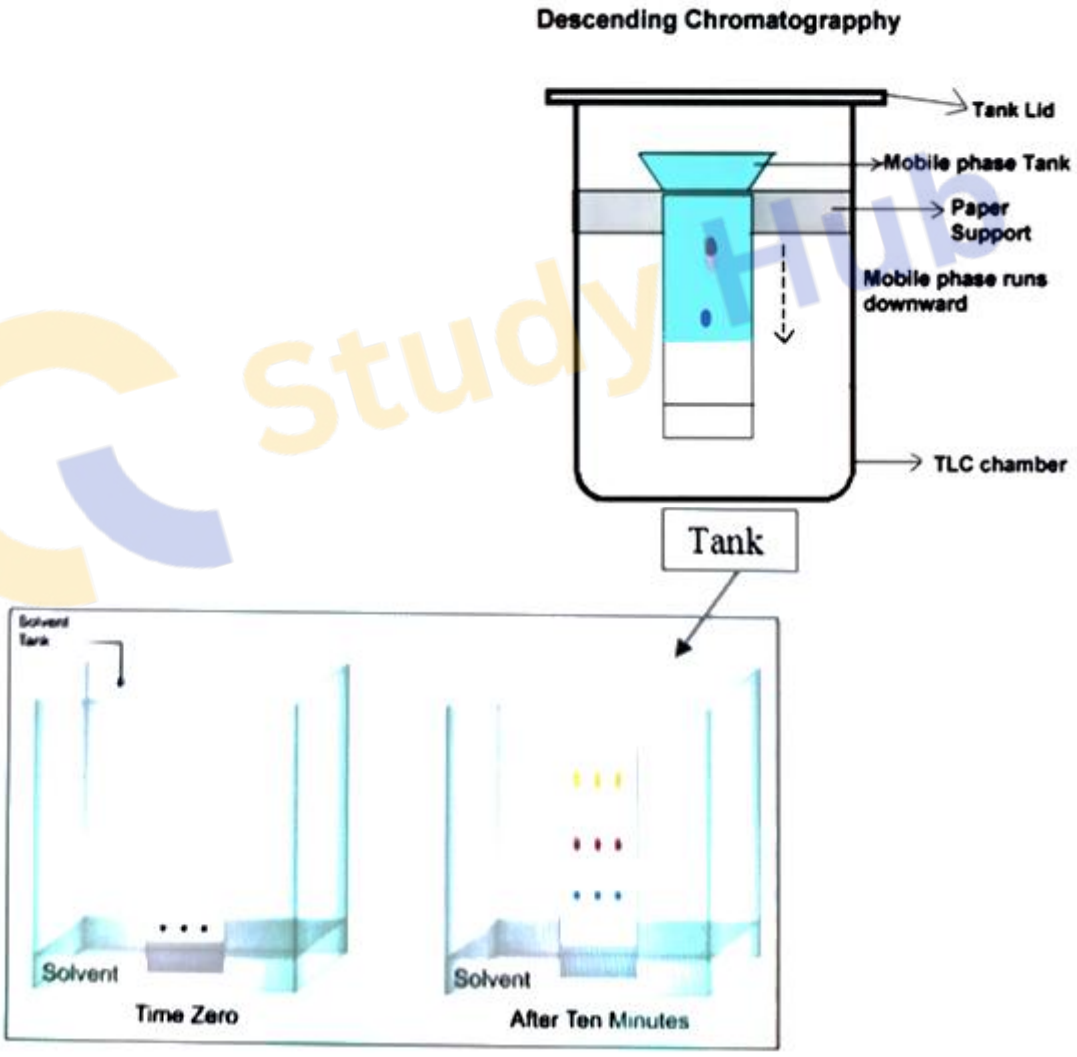
Sample-loaded filter paper is dipped carefully into the solvent not more than a **height of 1 cm** and **waited until the solvent** front reaches near the edge of the paper**.**

• Different types of development techniques can be used:

**a.** Ascending development

• Like conventional type, the solvent flows against gravity.

The spots are kept at the **bottom portion of paper** and kept in a chamber with **mobile phase solvent at the bottom**. (Same as in TLC).




**b.** Descending type**:**

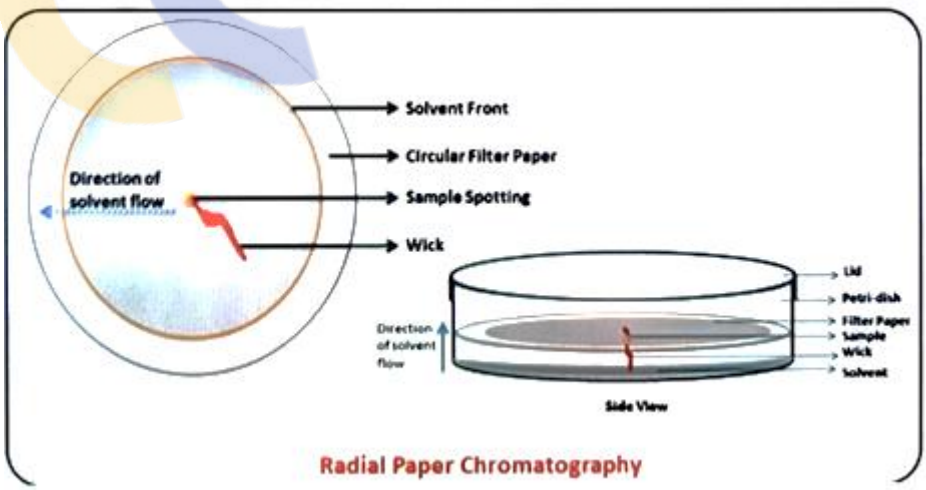
- This is carried out in a special chamber **where the solvent holder is at the top**.
- The spot is kept at the **top and the solvent flows down the paper**.
- In this method **solvent moves from top to bottom**, so it is called **descending chromatography**.

**c. Ascending – descending development:**

- A hybrid of the above **two techniques** is called **ascending–descending chromatography**.
- Only the **length of separation increases**; first *ascending* takes place followed by *descending*.

**d. Circular / radial development**

- Spot is kept at the **centre of a circular paper**.
- The solvent flows through a wick at the **centre and spreads in all directions uniformly**.
- Hence the **individual spots after development look like concentric circles**.
- By making perforations radially, **more quadrants** can be created allowing *more number of samples* to be spotted.

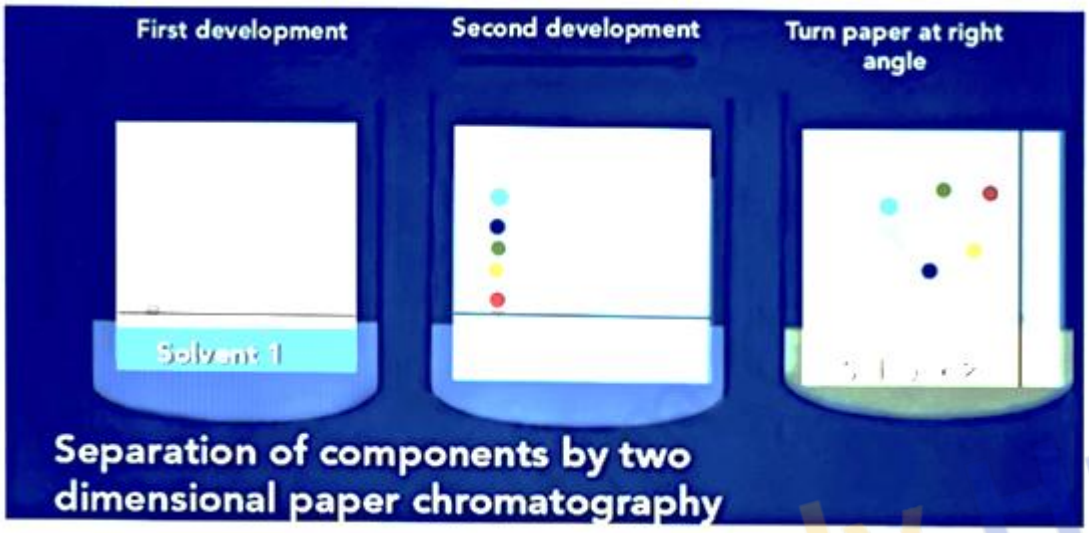


Radial Paper Chromatography

**e. Two-dimensional developments**

- This technique is **very** similar to 2-Dimensional TLC.

- Here, the **chromatogram develops in two directions at right angles**.
- Samples are spotted at **one corner** of rectangular paper and allowed for first development.
- Then the paper is **again immersed in the mobile phase at a right angle** to the previous development for the **second chromatogram**.
- In the second direction, **same or different solvent systems** may be used.




## Drying of Chromatogram

- After the development, the **solvent front is marked**, and the chromatogram is allowed to **dry in a dry cabinet or oven**.

## Detection

- After development, the **spots should be visualized**.
- **Colored spots** can be detected visually.
- For **colorless spots**, any one of the following techniques can be used:

### a. Nonspecific methods

- Brown or amber spots detected but do not reveal exact nature of compound.

**Examples:**

1. **Iodine chamber method**

   Brown or amber spots appear when developed papers are kept in a tank with **iodine crystals** at the bottom.

2. **UV chamber for fluorescent compounds**

   When compounds are viewed under **UV chamber**, at **254 nm (short λ)** or **365 nm (long λ)**, fluorescent compounds can be detected.

**b. Specific methods**

Specific spray reagents or visualizing agents are used for identification.

1. **Ferric chloride** – for phenolic compounds and tannins
2. **Ninhydrin in acetone** – for amino acids
3. **Dragendorff's reagent** – for alkaloids
4. **3,5-Dinitro benzoic acid** – for cardiac glycosides
5. **2,4-Dinitrophenyl hydrazine** – for aldehydes and ketones

**The detecting techniques can also be categorized as:**

**a. Destructive techniques**

- Samples destroyed before detection.
  Example: **Ninhydrin reagent**

**b. Non-destructive techniques**

- Sample **not destroyed even after detection**
  Examples: UV chamber, iodine chamber, densitometric method, etc.
- For **radioactive materials**, detection is by **autoradiography** or **Geiger–Muller counter**.
- For **antibiotics**, chromatogram is laid on nutrient agar inoculated with appropriate strain and **zone of inhibition is compared**.

**Quantitative Analysis**

• **Direct technique (Densitometry):**

- A **densitometer** quantitatively measures **density of spots**.
- Optical densities of spots for standard and test solutions are compared to calculate substance quantity.
- Papers are **neither destroyed nor eluted with solvents** ⟶ *in-situ method.*

• **Indirect techniques:**

- Spots are cut and **eluted with solvents**.
- Eluate is analyzed through conventional techniques: **spectrophotometry, electrochemical methods**, etc.

**Qualitative Analysis: a. Rf value**

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

- **Rf ranges from 0 to 1**, ideal values: **0.3–0.8**.

**b. Rx value**

- Always closer to 1.

$$Rx = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by the standard}}$$

**c. Rm value**

- Used to check whether compounds belong to a **homologous series**.
- ΔRm values are constant for adjacent members.

$$Rm = \log\left(\frac{1}{Rf} - 1\right)$$

APPLICATION

**(i) To check the control of purity of pharmaceuticals**

**(ii) For detection of impurities**

| Drug | Mobile phase | Detecting agent |
|---|---|---|
| **Hydroxocobalamin** | Butyl alcohol : acetic acid : potassium cyanide | Elution + measurement of absorbance at **361 nm** |

**(iii) Detect contaminants in foods and drinks**

**(iv) For detection of drugs**

| Drug | Mobile phase | Detecting agent |
|---|---|---|
| **Gentamycin** | Chloroform : Methanol : Ammonia : Water (10:5:3:2) | **Ninhydrin** in pyridine–acetone mixture |
| **Vancomycin** | t-Amyl alcohol : Acetone : Water (2:1:2) | Nutrient agar containing **Bacillus subtilis** |

**(v) In analysis of cosmetics**

**(vi) Analysis of the reaction mixtures in biochemical labs.**

**(vii) Identification of decomposition products**

**(viii) Analysis of metabolites of drugs in blood, urine etc.**

**(ix) In the study of ripening and fermentation**

## ❖ ADVANTAGES OF PAPER CHROMATOGRAPHY:

1. **Simple and Rapid**
2. **Paper Chromatography requires very less quantitative material.**

3. **Paper Chromatography is cheaper compared to** other chromatography methods.
4. **Both unknown inorganic as well as organic compounds can be identified by paper chromatography method.**
5. **Paper chromatography does not occupy much space compared to** other analytical methods or equipment's.

## ❖ LIMITATIONS OF PAPER CHROMATOGRAPHY

1. Large quantity of sample cannot be applied on paper chromatography.
2. In quantitative analysis paper chromatography is not effective.
3. Complex mixture cannot be separated by paper chromatography.
4. **Less Accurate compared to HPLC or HPTLC**

# UNIT–III ELECTROPHORESIS (PART - 5)

**Points to be covered in this topic**

- ➤ ❖ **Introduction**
- ➤ ❖ **Factors Affecting Electrophoretic Mobility**
- ➤ ❖ **Techniques of paper**
- ➤ ❖ **Gel**
- ➤ ❖ **Capillary electrophoresis**
- ➤ ❖ **Applications**

**ELECTROPHORESIS**

## ❖ INTRODUCTION

• Electrophoresis is a phenomenon describing the **motion of particles in a gel or fluid within a relatively uniform electric field.**

• Electrophoresis is used for separating the molecules **based on their charge, size and binding affinity.**

• Electrophoresis of anions (**negatively charged particles**) is termed anaphoresis, while the electrophoresis of cations (**positively charged particles**) is termed **cataphoresis.**

• **Ferdinand Frederick Reuss** (of Moscow State University) first discovered the technique of electrophoresis in **1807** when he noticed that clay particles migrated in water subjected to a continuous electric field.

## ❖ PRINCIPLE

• Electrophoresis is a **physical method of analysis based on the migration of electrically charged proteins, colloids, molecules** or other particles dissolved or dispersed in an electrolyte solution in the direction of the electrode bearing the opposite polarity when an electric current is passed through it.

• The **electrophoretic mobility** is the rate of movement in meter per second of the charged particles under the action of an electric field of 1 volt per meter and is **expressed in square meters per volt second.**

• For practical reasons, it is given in square centimeters per volt second, $cm^2V^{-1}S^{-1}$. The mobility is specific for a given electrolyte under precisely determined operational conditions.

**Where μ = Electrophoretic mobility**

Q – Net charge on the ion
r – Ionic radius of the solute

η – viscosity of the medium

$$\mu = Q / 6\pi\eta r$$

• The electrophoretic mobility is **directly proportional to net charge** and **inversely proportional to molecular size and viscosity** of the electrophoresis medium.

• The **pH of the solution affects the mobility of the ion**.

Depending on the method used, the electrophoretic mobility is either measured directly or compared with that of a reference substance.

• Based upon the type of apparatus used, electrophoretic methods may be divided into **two categories**,

➤ one called **free or moving boundary**, and

➤ the other called **zone electrophoresis** (using a supporting medium).

Zone electrophoresis include paper, gel such as agar, starch or polyacrylamide.

## ❖ ADVANTAGES OF PAPER CHROMATOGRAPHY

1. Simple and Rapid
2. Paper Chromatography requires **very less quantitative material.**
3. **Paper Chromatography is cheaper compared to** other chromatography methods.
4. Both unknown inorganic as well as organic compounds can be **identified by paper chromatography method.**
5. Paper chromatography **does not occupy much space** compared to other analytical methods or equipment's.

## ❖ LIMITATIONS OF PAPER CHROMATOGRAPHY

1. Large quantity of sample cannot be applied on paper chromatography.
2. In quantitative analysis paper chromatography is not effective.
3. Complex mixture cannot be separated by paper chromatography.
4. **Less Accurate compared to HPLC or HPTLC**

## ★ FACTORS AFFECTING ELECTROPHORETIC MOBILITY

• Electrophoretic mobility is the solute's response to the applied electrical field. The following factors affect electrophoretic mobility:

**1) Sample:**

The charge/mass ratio of the sample indicates its electrophoretic mobility. The mass depends on molecular size and shape.

**i) Charge:**

Higher the charge, greater is the electrophoretic mobility. However, the charge is dependent on medium pH.

**ii) Size:**

Bigger the molecule, greater are the frictional and electrostatic forces exerted on it by the medium.

Thus, larger particles have smaller electrophoretic mobility.

**iii) Shape:**

Rounded contours produce lesser friction and electrostatic retardation than the sharper contours.

For example, among globular and fibrous proteins, the former will migrate faster.

**2) Electric Field:**

The migration rate increases with an increase in potential gradient. Buffer ions carry the current in a solution placed between two electrodes.

An increase in potential difference increases the current.

Resistance to current flow and the migration rate are **inversely proportional**; therefore, a power pack has to be made available to control the current to a constant level without producing heat.

**3) Medium:**

An inert supporting medium is generally selected for electrophoresis.

This medium can exert adsorption or molecular sieving effect on the particles, thus influencing the migration rate.

**4) Buffer:**

The buffer maintains the pH of supporting medium and also affects the electrophoretic mobility of the sample.

**i) Composition:**

The commonly used buffers are formate, phosphate, citrate, acetate, barbitone, etc.

The choice of buffer depends on the type of sample to be separated by electrophoresis.

**Buffer affects the electrophoretic mobility if it binds to a sample component being separated.**

**ii) Ionic Strength:**

Buffer having ionic strength between 0.05 and 0.1 M is usually selected.

If the ionic strength of buffer increases, the buffer ions will carry a larger share of the current, while the sample ions will carry a smaller part.

This slows down the migration of sample components.

If ionic strength decreases, sample ions will carry a larger share of the current, resulting in faster separation.

However, this causes diffusion of the smaller components to be high with associated loss of resolution.

**iii) pH:**

The direction and extent of migration of ampholytes depend on the buffer pH.

Buffers having pH between 1 to 11 are used for achieving the desired separation.

**5) Medium:**

The inert medium can exert adsorption and molecular sieving effects on the particle, thus influencing its migration rate.

**i) Adsorption:**

It indicates a component's retention on the supporting medium surface.

Adsorption can reduce the rate and resolution of electrophoretic separation.

**ii) Molecular Sieving:**

Polyacrylamide, agar, starch, and sephadex media have cross-linked structures that give rise to pores within the gel beads.

**a) Sephadex:**

Molecules larger than the pores do not enter the gel beads and migrate faster.

**b) Polyacrylamide, Starch, and Agarose:**

Larger molecules squeeze through the pores.

The smaller molecules easily pass through the pores, but the larger ones are retarded.

## ★ APPLICATIONS OF ELECTROPHORESIS

✓ Electrophoresis has the following applications:

1. It is used for determining the high molecular weight compounds (e.g., amino acids, proteins, and nucleic acids).
2. It is used for DNA sequencing.
3. It is used for DNA hybridisation.
4. It is used for determining **biological fluid constituents**.
5. It is used in the **analysis of antibiotics**.
6. It is used in the **purification of vaccines**.
7. It is used in **chiral analysis**.
8. It is used for **determining impurities**.
9. It is used in the **assay of atropine phosphate**.
10. It is used in the **assay of codeine**.
11. It is used in the **diagnosis of genetic toxicology**.
12. It is used in the **diagnosis of hereditary diseases**.
13. It is used in the **analysis of carbohydrates**.
14. It is used in the field of **forensic science**.
15. It is used in the field of **molecular biology**.

## ★ Types of Electrophoresis

Electrophoresis relies on the principle of migration of charged particles under the influence of electric field.

The technique of electrophoresis is of the following two types:

**1) Zone Electrophoresis**

i) Paper electrophoresis
ii) Gel electrophoresis
iii) Thin layer electrophoresis
iv) Cellulose acetate electrophoresis

**2) Moving Boundary Electrophoresis**

i) Capillary electrophoresis
ii) Isotachophoresis
iii) Isoelectric focusing
iv) Immunoelectrophoresis

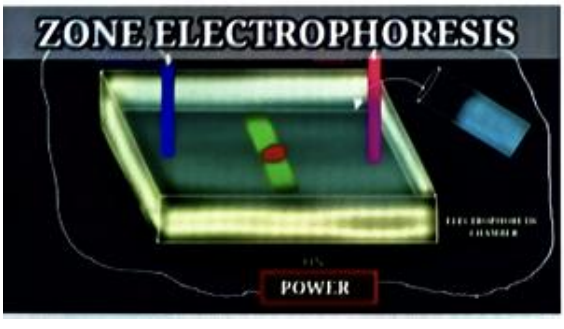
## ★ TECHNIQUE OF PAPER ELECTROPHORESIS

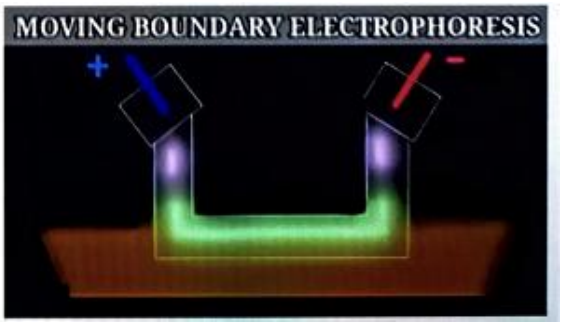
### ❑ Introduction

• Paper electrophoresis is a type of zone electrophoresis, and is a very important laboratory method.

### ❑ Principle

• The charge a molecule carries depends on the medium pH.
• Electrophoresis at low voltage cannot separate low molecular weight compounds because of diffusion; however, it is easy to illustrate the relationship between charge and pH using amino acids, instead of proteins or other macromolecules.



## ❑ Filter Paper

• Paper of good quality and containing approximately 95% of α-cellulose should be used.
• The adsorption capacity of the selected paper should be very low.

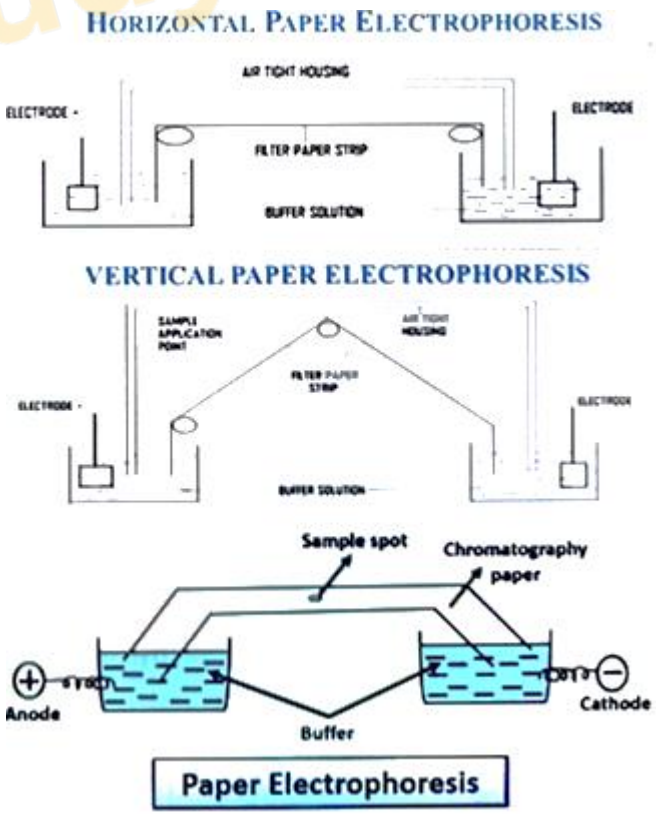
## ❑ Apparatus

• The equipment of electrophoresis consists of a power pack and an electrophoretic cell.
• The power pack provides a stabilized direct current and can be programmed to produce a **constant voltage** or a **constant current**.
• The electrophoretic cell comprises:
– platinum electrodes,
– two buffer reservoirs,
– a supporting medium for paper,
– a transparent insulating cover.
• Each buffer reservoir is divided into two sections that are interconnected.

• One section contains the electrode and the other is in contact with the supporting medium.

• If the electrodes undergo any *change in pH*, the *buffer in contact with the supporting medium* ***should remain unaffected***; thus, separate compartments are necessary. Wicks made up of several layers of filter paper or gauze are used to keep the **buffer reservoir** and the **supporting medium** in contact.

• Paper is placed on an **insulating material** (usually a Perspex sheet). Then *the entire electrophoretic unit is covered with an insulating material* for reducing evaporation during run and for **assuring electrical insulation**.



• **The filter paper strips are *commonly used in horizontal and vertical arrangements.***

## Sample Application

● **The sample is applied as a spot (of 0.5 cm diameter) or as a narrow uniform streak.**

Special devices are commercially available for sample application.
The sample can be **applied before or after equilibrating the paper with buffer.**

## Electrophoretic Run

● **After applying the sample to the paper and equilibrating the paper with buffer, the current is switched on.**

The **electrophoretic equipment is equipped with devices that provide stable voltage or current**, still the entire unit is placed in a cold room to avoid overheating.
The separation process completes within two hours, after which the power is switched off, the paper is removed, and vacuum **dried in oven at 110°C temperature**.

## Detection and Quantitative Assay

● **The unknown electropherogram can be identified by comparing the unknown electogram with the standard one under standard conditions.**

Individual compounds are identified as per their **physical properties by the following methods:**

**1) Fluorescence**

i) The electrophoretogram is **stained with ethidium bromide** and visualized under UV light.
The **DNA and RNA fluoresce**, thus facilitating their detection.

ii) The electrophoretogram is **stained with fluorescamine** to detect amino acids, amino acid derivatives, peptides, and proteins.

iii) Dansyl chloride can be used **instead of fluorescamine**.

### 2) UV Absorption

Proteins, peptides, and nucleic acids **absorb in the range of 260–280 nm**, thus enabling their detection.

### 3) Staining

The compounds given in table are stained with the following dyes:

| Compounds | Dyes | Comments |
|---|---|---|
| **Proteins** | Bromophenol blue in acetic acid<br>Fluorescamine | Visual, quantitative<br>Fluorescent, very sensitive |
| **Nucleic acids** | Methyl green-pyronin<br>Ethidium bromide | DNA-Blue, RNA-Red, sensitive<br>Fluorescent, very sensitive |
| **Polysaccharides** | Iodine | Visual, sensitive |
| **Lipoproteins** | Sudan black in 60% ethanol | Visual, sensitive |
| **Glycoproteins** | Alcian blue | Visual, sensitive |

### 4) Detection of Enzymes *in situ*

When the component to be separated is an enzyme, special techniques are used for its detection.

The paper strip having the enzyme **to be separated is saturated with the substrate** to facilitate enzyme separation.

Thereafter, the paper strip is placed in a suitable buffer along with the electrophoretogram. Colour bands appear indicating the enzyme position.

### 5) Quantitative Estimation

On multiplying the colour density of the zone with the area of zone, the resulting value gives an estimate of the component concentration.

## ▼ Applications

Paper electrophoresis has the following uses:

1. It is used for serum analysis for diagnostic purpose.
2. It is used for the analysis of **muscle proteins, egg white proteins, milk proteins, and snake and insect venom.**

## ❖ TECHNIQUES OF GEL ELECTROPHORESIS

### ▼ Introduction

● **Gel electrophoresis is a method utilised for the separation and analysis of macromolecules (DNA, RNA, and proteins) and their fragments**, based on their size and charge.

● Used in clinical chemistry for separating proteins.
Also used in biochemistry and molecular biology fields for separating a mixed population of DNA and RNA fragments based on their length.

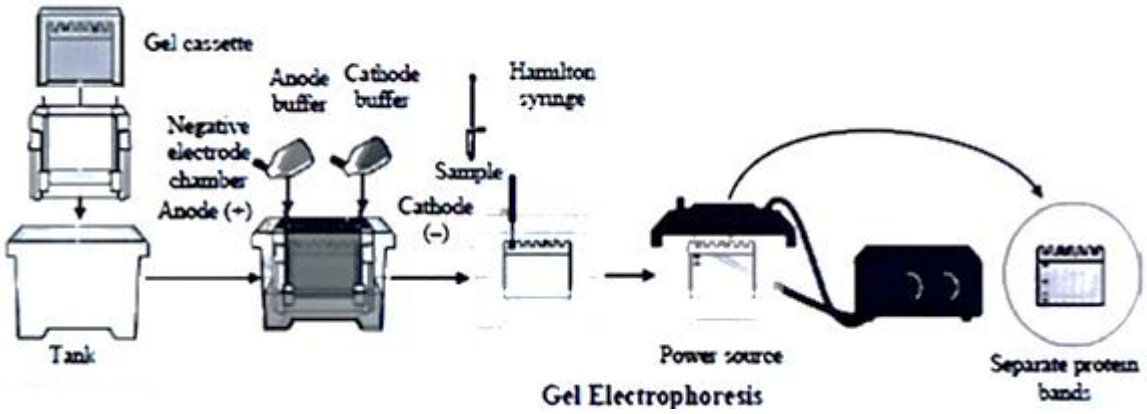
● In gel electrophoresis, **a gel is used as an anti-convective medium**, suppressing thermal convection due to electric field.
The gel also serves as a **sieving medium** and retards passage of molecules.
Stain can be applied post-electrophoresis.

● **DNA gel electrophoresis** is used for analytical purposes, often after amplification of DNA via PCR.
It may also be used as a preparative technique before:

- mass spectrometry
- RFLP
- PCR
- cloning
- DNA sequencing

- Southern blotting

for further characterization.



Gel Electrophoresis

## Theory

● A molecule with a net charge migrates in an electric field.
This phenomenon is termed **electrophoresis**, effectively separating proteins, DNA, and RNA.

● A molecule's **migration velocity (v)** in an electric field depends on **electric field strength (E)** and **electrophoretic mobility (μ)**.

$$v = \mu E \text{———} \text{ Equation 1}$$

**In gel electrophoresis, the following relation is found:**

$$\log \mu = \log \mu_0 - K_r \tau \text{———} \text{ Equation 2}$$

Where:

**$\mu_0$** = Free electrophoretic mobility of molecule.

**$K_r$** = Retardation coefficient.

**$\tau$** = Concentration of gel.

## Principle

● On placing a **mixture of electrically charged biomolecules** in an electric field (E), the particles freely move toward the oppositely charged electrode.
Depending on physical conditions, different molecules move at different rates.

A charged molecule's velocity (v) depends on:

$$v = \frac{E \cdot q}{f}$$

Where:
**f** = Frictional coefficient
**q** = Net charge on the molecule

## 🔻 Types of Gel

Different types of gels used in gel electrophoresis:

1. **Starch Gel:** Potato starch is hydrolysed with acidified acetone at 37°C.
2. **Agar:** Made of agarose and agaropectin (galactose-based polymers).
3. **Polyacrylamide:** Components include acrylamide monomer, N,N'-methylene-bis-acrylamide, ammonium persulfate, TEMED.

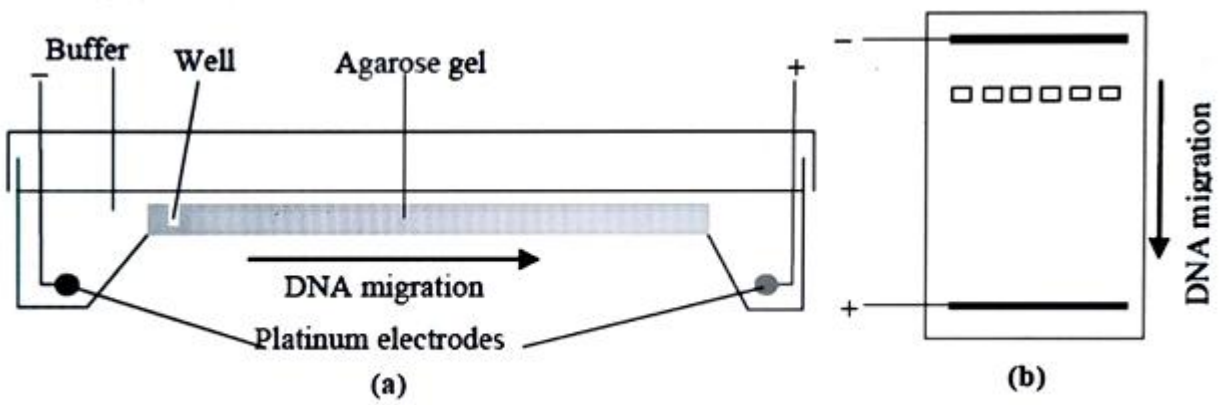
## 🔻 Agarose Gel Electrophoresis

● A **horizontal slab gel** is commonly used.

● The gel is poured on a glass/plastic tray, then **installed on a platform in the electrophoresis tank**.

● The gel is **immersed beneath the buffer surface** and electrophoresis is performed.

• **The resistance of gel to electric current is similar to that of the buffer, and thus a**




Agarose Gel Electrophoresis Apparatus a) Side view b) Top Down View

• **The resistance of gel to electric current is similar to that of the buffer, and thus a significant portion of the applied current passes along the length of the gel.**

## ❑ Preparation and Staining of Gel

• Agarose powder is **boiled and melted with a buffered solution**.

**The resultant solution is poured in a gel tray** and allowed to cool at room temperature, as a result of which polymerization occurs.

• By staining with **ethidium bromide** (a fluorescent dye), DNA in **agarose gels can be conveniently visualized.**

## ❑ Factors Affecting the Rate of DNA Migration

• There are many factors that affect the velocity of DNA molecules in the gel (v). Some of these factors are:

### 1) Voltage Applied:

Voltage **directly influences the electric field strength (E)** of the gel.

### 2) Agarose Concentration:

Equation (2) suggests a **linear relationship between the logarithm of electrophoretic mobility of DNA and agarose concentration** in the gel.

### 3) Molecular Size of the DNA:

Electrophoretic mobility of linear double-stranded DNA and the **$\log_{10}$ of the number of base pairs** (size component of Kr) are inversely proportional.

## ❑ Preparation and Staining of Gel

• Acrylamide **and** bisacrylamide mixture is prepared in a buffer, and **added with ammonium persulfate** to provide free radicals that initiate polymerization.
The obtained solution is poured between two glass plates to attain the shape of a gel and to hold it in place.

• Different staining techniques are used for revealing the protein in the polyacrylamide gels.
**Two of such techniques are:**

**1) Staining Resulting from a Chemical Reaction:**

In silver staining, **$Ag^+$ ions react with glutamate, aspartate and** cysteine residues of the proteins and form complexes.

**2) Staining with Protein Binding Dyes:**

Different dyes that bind to the proteins are used, but the most common one is **Coomassie brilliant blue.**

## ❑ Factors Influencing the Migration

• The migration of **SDS-protein complexes** in the resolving gel is **affected by the molecular weight**, the acrylamide-bisacrylamide ratio (affecting the gel properties component of Kr), and the total polymer concentration **(τ).**

• **Cross-links** formed from bisacrylamide provide rigidity and tensile strength to the gel and form pores to allow the **passage of SDS-polypeptide complexes.**

• These **pore sizes decrease with the increase in** bisacrylamide-acrylamide ratio, and become minimum when the ratio is approximately **1:20**.
Most SDS-polyacrylamide gels are cast with a molar ratio of **1:29**, which can resolve the polypeptides of different sizes by as little as **3%.**

## ❑ Applications

Given below are the applications of gel electrophoresis:

**1) It is used for estimating the size of DNA molecules after restriction enzyme digestion, e.g., in restriction mapping of cloned DNA.**

**2) It is used for analysing PCR products, e.g., in molecular genetic diagnosis or genetic fingerprinting.**

**(3) It is used for separating restricted genomic DNA before Southern transfer, or for separating RNA before Northern transfer.**

**(4) It is used in the field of forensic science, molecular biology, genetics, microbiology, and biochemistry.**

**(5) Western blot technique that is slightly different but related to gel electrophoresis is used for separating proteins and probing with labelled antibodies for specific proteins.**

**(6) It also helps in comparing similarities and differences between species.**

**7) It is used for determining genetic relationship among species.**

# ❖ TECHNIQUES OF CAPILLARY ELECTROPHORESIS

## ❑ Introduction

• The technique of capillary electrophoresis is used for **separating ions on the basis of their electrophoretic mobility by applying voltage.**
Electrophoretic mobility depends on the **molecular charge, viscosity, and atomic radius.**

## ❑ Instrumentation

• A quite simple in design instrumentation is required for capillary electrophoresis.
The basic instrument includes:

**an auto-sampler, a detection module, a high-voltage power supply, a capillary, and a computer.**

• The capillary ends are placed in different buffer reservoirs, each carrying an electrode connected to a high-voltage power supply (can supply voltage of 30 kV).
Sample is injected into the capillary by temporarily replacing one of the buffer reservoirs (at the anode) with a sample reservoir.
Then either an electric potential or external pressure is applied across the capillary for a few seconds, and the separation is performed.

• The separated analytes are **optically detected (UV-visible or fluorometric)** through the capillary wall near the opposite end (near the cathode).

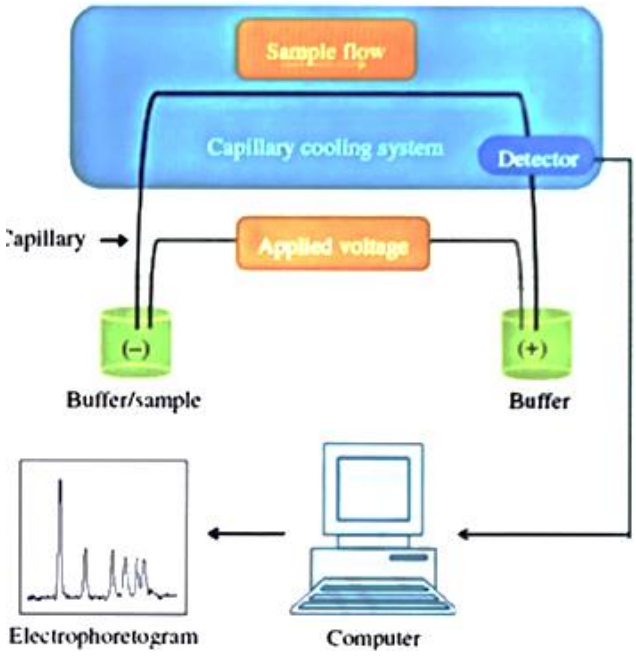
• Capillary electrophoresis is suited to automation, and the arrangement of its commercial instruments is much familiar to that of modern HPLC.

## ❑ Introduction

- The technique of capillary electrophoresis is used for **separating ions on the basis of their electrophoretic** mobility by applying voltage. Electrophoretic mobility depends on the molecular charge, viscosity, and atomic radius.

## ❑ Electrophoretic Mobility

- In the process of electrophoresis, the sample ions move under the influence of an applied voltage. The ions undergo a force equal to the product of the **net charge and** the electric field strength.
- The sample ions are also **affected by a drag force equal to the product of the translational friction coefficient and the velocity.** This results in the expression for electrophoretic mobility:



$$\mu_{ep} = \frac{q}{f} = \frac{q}{6\pi\eta r}$$

**------Equation 3**

**Where:**

- **f** = Translational friction coefficient for spherical particle (given by Stokes' law)
- **η** = Viscosity of the solvent
- **r** = Radius of the atom
- Migration rate of sample ions is **represented by the charge-to-mass ratio.**
- The actual **velocity of the ions is proportional to the magnitude of the electrical field (E)** and can be determined using:

$$v = \mu_{ep} E$$

**----------Equation 4**

- This **relationship indicates that in the presence of greater voltage, the migration of ionic species will hasten.**

## ❑ Electro-Osmotic Flow (EOF)

- When a **high voltage is applied to an** electrolyte-filled capillary, electro-osmotic flow occurs.
- This flow results when the pH of the buffer running through the silica capillary is more than 3, and the SiOH groups lose a proton to become $SiO^-$ ions.
- Consequently, the capillary wall has a negative charge that develops a double layer of cations attracted to it.
- The **inner cation layer is** stationary and the outer one freely **moves** along the capillary.

- Under the influence of the applied electric field, the free cations move towards the cathode and create a powerful bulk flow.
- The electro-osmotic flow rate is governed by:

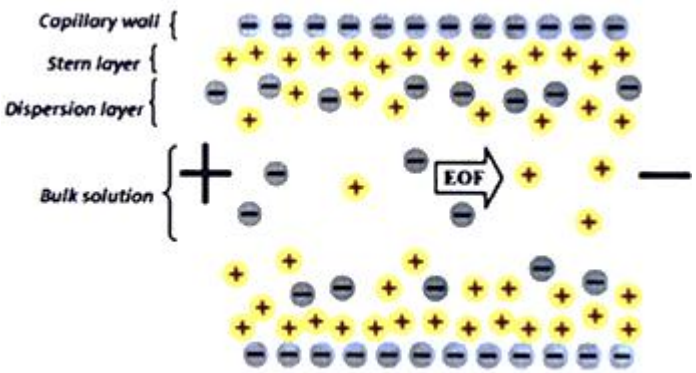
$$\mu_{EOF} = \frac{\varepsilon}{4\pi\eta}\zeta E$$

**------Equation 5**

**Where:**

- **ε** = Dielectric constant of the solution
- **η** = Viscosity of the solution
- **E** = Field strength
- **ζ** = Zeta potential
- The electrophoretic mobility is greater than the electro-osmotic flow; thus the negatively charged particles (naturally attracted to the positively charged anode) separate out.

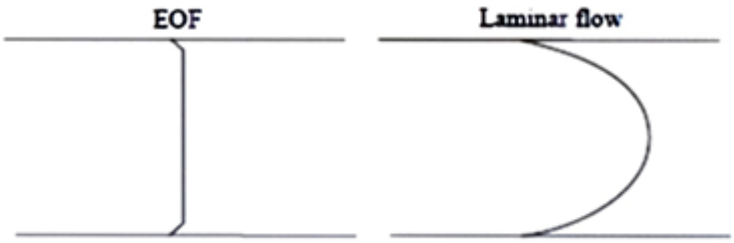
## ❑ Zeta Potential




- Zeta potential and the pH-dependent charge density on the capillary wall are proportional to each other.
- Therefore, mobility of EOF varies with the buffer pH; i.e., the EOF mobility will be comparatively greater at high pH.

## ❑ Flow Profile in CE

- Another key feature of EOF is that along with the parabolic flow profile (generated by an external pump), it has a **flat flow profile since its driving force (charge on the capillary wall) is uniformly distributed along the capillary.**
- This yields a parabolic or laminar flow profile.



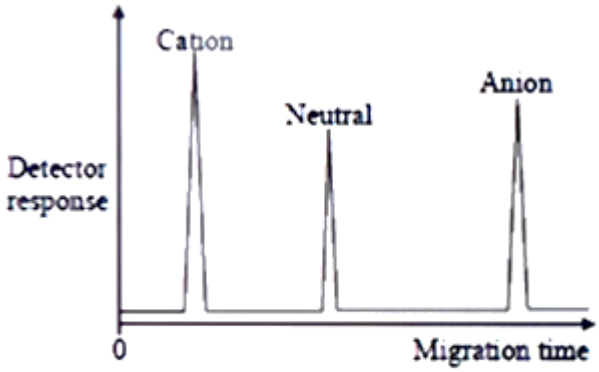

**Flow Profiles of EOF**

- The **flat flow profile of EOF minimizes zone broadening** and enhances high separation efficiencies, allowing separations based on mobility differences as small as 0.05%.

## ❑ Electropherogram

- The data output from **capillary electrophoresis is presented as an electropherogram** (similar to a chromatogram), which is a plot of migration time versus detector response.

## ❑ Applications of CE




**Typical Electropherogram Showing the Separation of a Cation, a Neutral Ion and an Anion**

- Capillary electrophoresis is used in **pharmaceutical industries for quality testing** to ensure the absence of side products or interferents.
- Since the capillary walls can be **neutralized with an acidic pH**, drugs having a basic amino group will not stick to the capillary, and thus capillary electrophoresis can be used for their separation.
- **Capillary gel electrophoresis is used for sequencing the human genome and separating DNA.**

For such separations, the capillary is injected with a polymer, which provides an additional mode of separation **based on the size**, as the smaller fragments travel faster through the gel. This method is termed **sieving**.