BP701T Instrumental Method of Analysis

Unit III:

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A. Chromatography

1. Introduction to Chromatography
2. Column Chromatography
3. Thin layer Chromatography
4. Paper Chromatography

B. Electrophoresis

1. Paper Electrophoresis
2. Gel electrophoresis
3. Capillary electrophoresis

A. Chromatography

Introduction:

Chromatography was invented by the Russian botanist Mikhail Tswet in the year 1903. He employed the technique to separate various plant pigments (i.e. Chlorophylls and Xanthophylls) by passing solutions of these substances (in petroleum-ether extract) through a glass column packed with finely powdered $CaCO_3$. The separated species appeared as separate bands having colored bands; the various pigments migrating through the column at different rates (because of differences in their distribution ratios). The separated species appearedasseparate bands having colored bands: the various pigments migrating through the column at different rates (because of differences in their distribution of ratios). The various solutes were isolated by cutting and sectioning of the chalk packing. Twswett choose to designate the name of such a process of separation as chromatography (Chroma-color, graphein-writing). Tswett's original experiments remained unnoticed in the literature for several decades. It was not until 1931 when kuhan and Lederer investigated polyene pigments that interest in such a technique was renewed.

But later on a diversify group of techniques which allow the separation of closely related components of the complex mixtures. In this technique, the sample is moved in a mobile phase, may be a gas, a liquid or a supercritical fluid. Such a mobile phase is then allowed to flow through an immiscible stationary phase.

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.

Classification of Chromatographic methods:

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)	HighPerformance liquid Chromatography (HPLC)

General Classification	Types of method	Stationary Phase	Mobile Phase	Type of equilibration Process	Name of the Technique
	Ion-Exchange	Solid (ion-exchange resign)	Liquid	Partition/Sieving	Ion-exchange chromatography (IEC)
	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.
Gas Chromatography	Gas – Solid (or adsorption) Gas – liquid (or Partition)	Solid Liquid adsorbed on a solid	Gas Gas	Adsorption Partition between Gas and liquid	Gas-solid chromatography (GSC) Gas-Liquid Chromatography (GLC)
Supercritical fluid chromatography (SFC)	Bio specific adsorption or bio affinity	Organic Species bonded to a solid surface	Supercritical fluid	Partition between Super-critical fluid and bonded species	Super-critical fluid chromatography (SFC) or Bioaffinity chromatography (BC)

Column Chromatography

When a column of stationary phase is used, the technique is called as 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 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 with the adsorbent present in the stationary phase.
2. The molecules having higher affinity remain adsorbed for a longer time decreasing their speed of movement through the column.
3. However, the molecules with lower affinity move with a faster movement, thus allowing the molecules to be separated in different fractions.
4. Here, the stationary phase in the column chromatography also termed the adsorbent is a solid (mostly silica) and the mobile phase is a liquid that allows the molecules to move through the column smoothly. The type of interaction between the stationary phase (adsorbent) & the solute is reversible in nature.

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

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

The equation can be simplified as follows:

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

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

$$\mathbf{R = \frac{Am}{Am + \alpha\ As}}$$

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

Am is the average cross section of mobile phase

As is the average cross section of stationary phase

Practical Requirement

1. Stationary Phase
2. Mobile Phase
3. Column characteristics
4. Preparation of the column
5. Introduction of sample
6. Development technique (elution)
7. Detection of components
8. Recovery of components

Stationary Phase

Adsorbents are used in this technique may be organic and inorganic classes of compounds. The ideal requirements of adsorbent are:

i. It should produce only adsorption of the analyte over it
ii. The particles should have uniform size distribution and have spherical shape. Particle size: 60-200µ.
iii. It should have high mechanical stability
iv. It should be inert & should not react with the solute or other components.
v. Insoluble in the solvents or mobile phases used.
vi. It should be colorless to facilitate observations of zones and recovery of components.

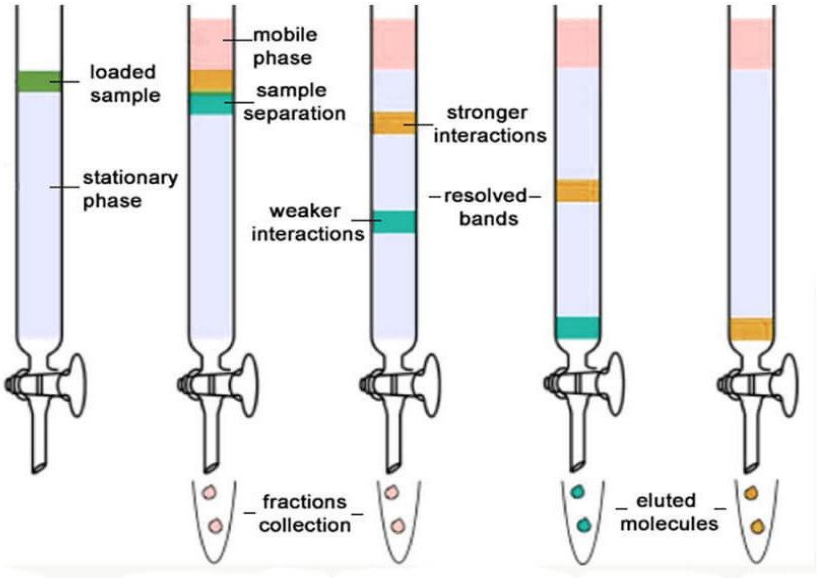


Figure: 1 Column chromatography.

Table : 1 Adsorbents and Solvents		
	Adsorbents	**Solvent**
Weak	Sucrose	Petroleum ether
	Starch	Carbon tetrachloride
	Inulin	Cyclohexane
	talc	Carbon di-sulphide
	Sodium carbonate	Ether (ethanol free)

Medium	Calcium carbonate	Acetone
	Calcium phosphate	Benzene
	Magnesium carbonate	Toluene
	Magnesium oxide	Esters
	Calcium hydroxide	Chloroform
	Activated magnesium silicate	Acetonitrile
Strong	Activated alumina	Alcohols
	Activated charcoal	Water
	Activated magnesia	Pyridine
	Activated silica	Organic acids
	Fuller's earth	Mixtures of acids or bases with ethanol or pyridine

The most commonly used adsorbent is Silica gel of 80-100 mesh or 100 – 200 mesh size which has a particle size of 60-200µ.

Selection of Stationary Phase

The selection of stationary phase in column chromatography depends on the following:

1. Removal of impurities: When a small quantity of impurity is present and there is difference in affinity when compared to the major component, a weak adsorbent is sufficient.
2. No. of components to be separated: When few components are to be separated, weak adsorbent is used. When more components are to be separated, a strong adsorbent is used.
3. Affinity differences between different components: When components have similar affinities, a strong adsorbent will be effective. When there is more differences in affinities, a weak adsorbent is selected.
4. Length of the column used: When a shorter column is used, strong adsorbent has to be used. When a longer column is used, a weak adsorbent can be used.
5. Quantity of adsorbent used: 20 or 30 times the weight of the adsorbent is used for effective separation.
   Adsorbate: Adsorbent = 1: 20 or 1: 30.

Mobile Phase: Mobile Phase is the very important and they are several functions. Mobile is acting as solvent, developer, and as eluent. The functions of a mobile phase are:

To introduce the mixture into the column – As solvent

To develop the zones for separation – As developing agent

To remove pure component out of the column – As eluent

Different mobile phases used: It is used in increasing order of polarity or elution strength. The solvents are given in the above Table 1. These solvents can be used in either pure form or as a mixture of solvents of varying compositions

ColumnCharacteristics

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

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 botton of the column before packing the stationary phase.
- After packing, a paper disc kept on the top, so that the stationary layer is not disturbed during the introduction of sample or mobile phase.

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

1. Dry packing / dry filling

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

2. Wet packing / wet filling

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

- Before using column, it should be washed properly and dried.

Introduction of the Sample

- The sample which is usually a mixture of components is dissolved in minimum quantity of the mobile phase or a solvent of minimum polarity
- The entire sample is introduced into the column at once and gets adsorbed on the top portion of the column.
- From this zone, individual sample can be separated by a process of elution.

C. Elution (Development technique)

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

Eg. Use of chloroform alone or Pet.ether: Benzene = 1:1 only, etc.

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

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

Other techniques like Frontal analysis and Displacement analysis where a graph of concentration of eluate Vs. volume of eluate will give an idea of how compounds are eluted out from the column.

D. Detection of Components

1. If the compounds separated in a column chromatography procedure are colored, the progress of the separation can simply be monitored visually.
2. If the compounds to be isolated from column chromatography are colorless. Then the technique depends upon the properties of the components. Different properties which can be used are
3. Absorption of light (UV/Vis) – Using UV-Visible Spectropotometer
4. Flourescence or light emission characteristics – Using fluorescence detector
5. By using flame ionization flame detector
6. Refractive index detector- based on the refractive index difference between the mobile phase and mobile phase + component
7. Evaporation of the solvent and weighing the residue
8. Small fractions of the eluent are collected sequentially in labeled tubes and the composition of each fraction is analyzed by TLC (thin layer chromatography).

Recovery of components: Earlier, recoveries of the components were done by cutting the column into several distinct zones. Later, extrusions of the column into zones were done by using plunger. The best technique is to recover the components by a process called as***elution*.** The components are called as **eluate,** the solvent called as **eluent** and the process of removing the components from the column is called as **elution.** The different elution techniques like isocratic elution technique and gradient elution technique.Recovery is done by collecting different fractions of mobile phase of equal volume like 10ml, 20ml etc or unequal volume. They can also be collected time wise i.e. a fraction every 10 or 20 minutes etc. The recovered fractions are detected by using the techniques discussed earlier. Similar fractions are mixed so that the bulk of the compound of each type is obtained in a pure form. If a fraction still contains several components, it can be resolved by using another column.

Applications:

1. Separation of mixture of compounds: Separation of glycosides, amino acids, plant extracts
2. Removal of impurities Isolation of the active constituents from the plant extract or from formulations
3. Isolation of metabolites from the biological fluids: 17-ketosteroids from urine, cortisol
4. Estimation of drugs in formulations or crude extracts
    i. Determination of % w/w of strychnine in syrup of ferrous phosphate with quinine and strychnine

ii. Separation of diastereomers.
iii. Separation of tautomers and racemates

Factor affecting Column efficiency

1. Dimensions of the column
2. Particle size of the adsorbent
3. Nature of the solvent
4. Temperature of the column
5. Pressure

Advantages:

1. Any type of mixture can be separated by column chromatography.
2. Any quantity of the mixture can also be separated (µg to mg of substance).
3. Wider choice of mobile phase.
4. In preparative type, the sample can be separated and reused.
5. Automation is possible.

Limitation or Disadvantages of Column chromatography

1. Time consuming method.
2. More amounts of solvents are required which may be expensive.
3. Automation makes the technique more complicated and costly.

Thin layer Chromatography (TLC)

Introduction: The history of thin layer chromatography dates back to 1938 when Izmailov and Shraiber separated plant extracts using 2mm thick and firm layer of alumina set on glass plate. In 1944, Consden, Goden and Martin used filter papers for separating amino acids. In 1950, Kirchner identified terpens on filter paper and later glass fibre paper coated with alumina. Only in 1958, Stahl developed standard equipment for analyzing by Thin layer chromatography.

Principle:

Thin Layer Chromatography can be defined as a method of separation or identification of a mixture of components into individual components by using finely divided adsorbent coated or spread over a chromatographic plate. The mobile phase solvent flows through because of capillary action (against gravitational force). The components move according to their affinities towards the adsorbent. The component with more affinity towards the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster. Thus the components are separated on a thin layer chromatographic plate based on the affinity of the components towards the stationary phase.

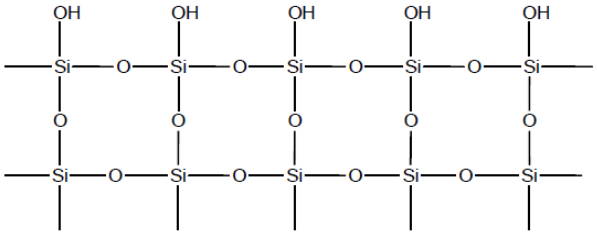


Fig 2: Silica extended structure and surface.

Silica (SiO2) is a solid with an extended structure of tetrahedral 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.

The Silica extended structure and surface is shown in the Fig 2. The developed TLC plate is shown in the Fig 3.

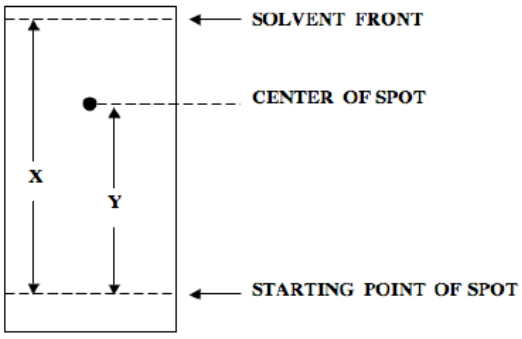


Fig 3: Developed TLC Plate

Advantages of TLC

1. It is a simple process with a short development time.
2. It helps with the visualization of separated compound spots easily.
3. It helps in isolating of most of the compounds.
4. The separation process is faster and the selectivity for compounds is higher (even small differences in chemistry is enough for clear separation).
5. The purity standards of the given sample can be assessed easily.
6. It is a cheaper chromatographic technique.
7. TLC offers a faster and more efficient separation than paper chromatography and the majority of paper chromatographic separations have now been superseded by the TLC Procedures.

Practical Requirement:

1. **Stationary Phase:** There a 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 are given in the below Table 2:

Name	Composition	Adsorbent: Water ratio
Silicagel H	Silicagel without binder	1:1.5
Silicagel GF	Silicagel + Binder + Fluorescent indicator	1:2
Silicagel G	Silicagel + $CaSO_4$(gypsum)	1:2
Alumina Neutral Basic Acidic	Al_2O_3 without binder	1:1.1
Al_2O_3 G	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 (CHCl3 : CH3OH= 2:3)

Fluoroscent indicator Zinc silicate

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 Angstroms (10^{-10}m).The particle size of silica gel for TLC is 10-40 µm (average 15 µm).

2. **Preparation of the Glass Plates:** The sizes of the glass plates for use with commercially available spreaders are usually 20 X 20, 20 X 10 or 20 X 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 be withstand temperatures used for drying the plates.

   General method:

   Mix 30gm of the adsorbent in a mortar to a smooth consistence with the requisite 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 X 20cm) or a proportionately larger number of smaller plates and allow the thin layers to set (about 4minutes when CaSO4 is present). Transfer the plates carefully to a suitable holder and after a further 30minutes, dry at 100-120 ^{0}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. The layers are 0.5 – 2mm thick, prepared as described under the general method, but using a smaller quantity of water and allowing a longer time for the initial drying of the plate.
   b. Microscopic slides are conveniently coated by a dipping technique in the following way: prepare slurry of the adsorbent by shaking withchloroform or chloroform-methanol (2:1) and insert two microscope slides (back to back) into the slurry. Withdraw the slides; allow draining, separating the slides and drying.
   c. The slurry, prepared in the normal way, is sprayed onto the surface of glass plates, using a laboratory spray gun.
   d. Theadsorbent, mixed with an organic solvent, e.g., chloroform or ethyl acetate, is distributed evenly over a glass plate by careful tilting, and, after evaporation of solvent, is dried in the normal way.

      In all the methods the plates should be tidied before use by cleaning the edges and backs (microscope slides).

3. **Application of Sample:** In order to get good spots, the concentration of the sample or standard solution can be 2-5µl of a %1 solution of either standard or test sample is spotting using a capillary tube or micropipette. The spots can be placed at random or equidistant from each other by using a template, with markings. The spot should be kept at least 2cm above the base of the plate and the spotting area should not be immersed in the mobile phase in the development tank.

4. **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 of the edge. The development tank examples are shown in the below Fig 3.

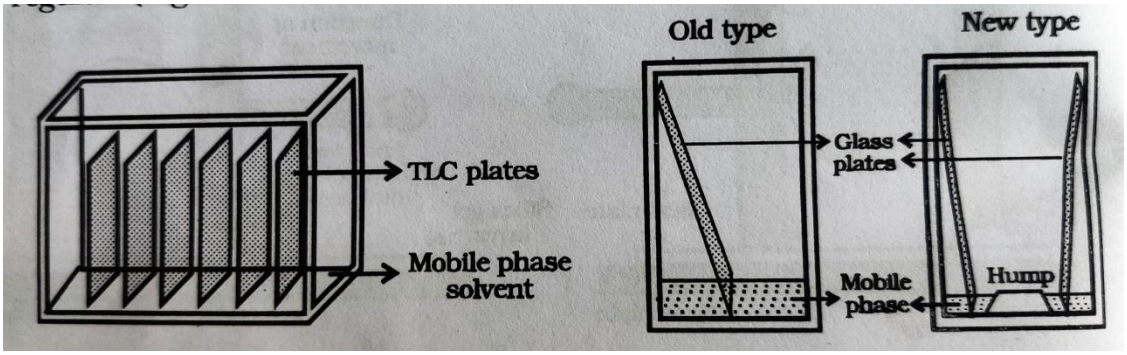


Fig 3: Development tank

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 review of literature and other logical considerations like solubility of the substance, polar or non-polar character of the samples, etc.

6. **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 compounds cannot be separated by using one dimensional technique.

7. **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. Dragendroff'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 compound s are not flourscent, 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, densitometric 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 densitometric 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-situmethod***.

8. **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.

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

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.

$\boldsymbol{Rx}$ value is the ratio of distance travelled by the sample and the distance travelled by the standard. Rx value is always closer to 1.

$\boldsymbol{Rm}$ **v**alue 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:

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

9. **Quantitative Analysis**

Indirect method: Quantitative analysis can be done after eluting the individual spotswith solvent and filtering off the stationary phase. The solution can be concentrated and the exact quantities of the compound determined by the methods like UV-Visible spectrophotometry, fluorescence method, flame photometric method, electrochemical methods of analysis.

Direct method: It can be done after eluting the individual spots with solvent and filtering off the stationary phase. The solution can be the concentrated and the exact amount of the compound deterimined by the various methods like UV-visible spectrophotometry, flourscence method, flame photometric method, electrochemical methods of analysis etc.

10. **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	**Stationary Phase**	**Mobile Phase**	**Detecting agent**
Amoxycillin trihydrate	Silica Gel G.F-254	Buffer pH 6: acetone (4:1)	NaOH+ Starch+glacial acetic acid+Iodinein 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 in pharmaceutical products (as per British Pharmacopoeia substances and preparations)

Substance	**Tested for**	**Mobile Phase**	**Detection**
Chlorpropamide	p-Chlorobenzene sulphonamide and NN'-diropylurea (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)

Paper Chromatography

Paper chromatography (PC) is a type of a planar chromatography whereby chromatography 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 of Separation

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

Instrumentation of Paper chromatography

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

1. STATIONARY PHASE AND PAPERS: Whatmann 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, alumna, 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.

2.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

3. PAPER CHROMATOGRAPHY MOBILE PHASE

Pure solvents, buffer solutions or mixture of solvents can be used. Some of the Examples of

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.

4.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) (Fig 4)

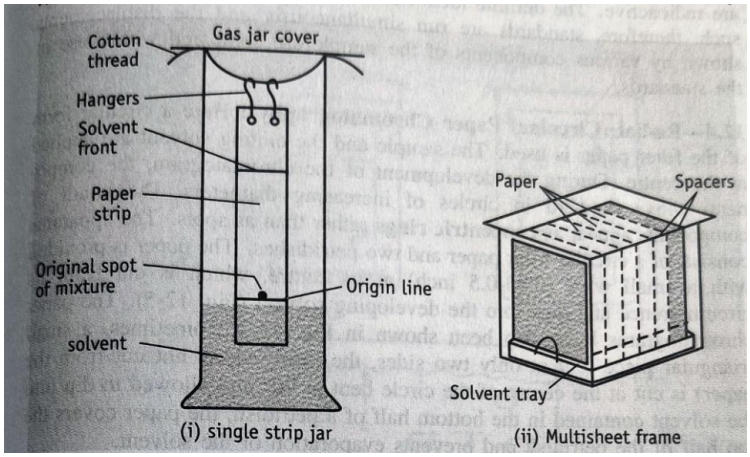


Fig 4

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 (Fig: 5).

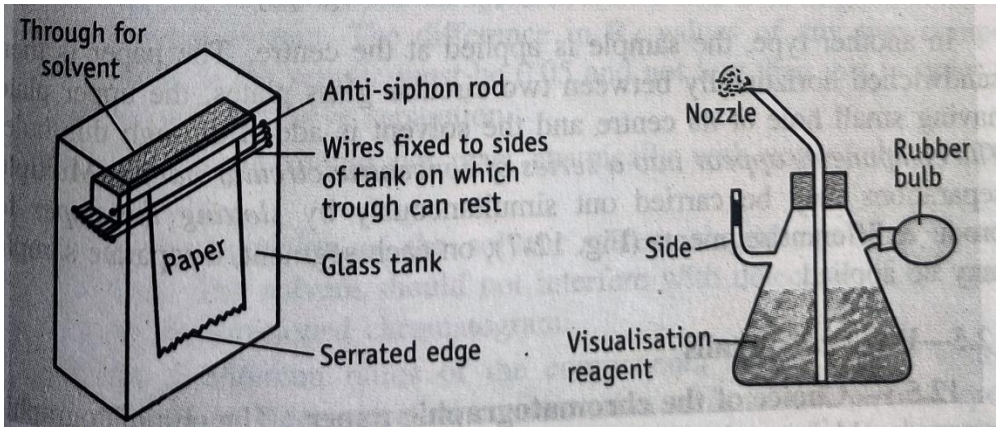


Fig 5: Descending technique and sprayer to spray the visualizing agent

c. **ASCENDING – DESCENDING DEVELOPMENT:** A hybrid of above two techniques is called ascending-descending chromatography.Only length of separation increased, first ascending takes place followed by descending.

d. CIRCULAR / RADIAL DEVELOPMENT

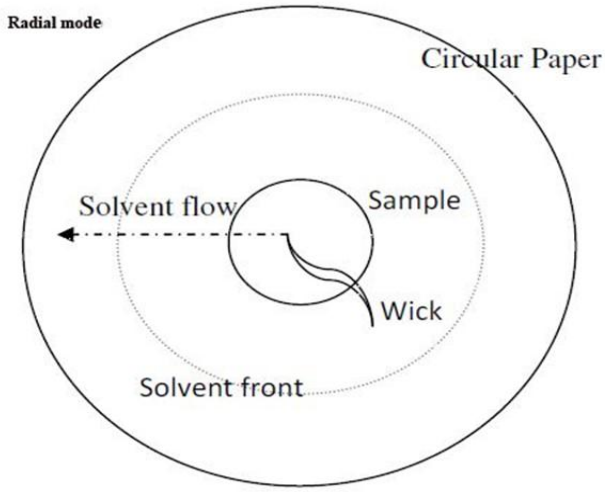


Fig 6: Circular/Development technique

Spot is kept at the centre of a circular paper.The solvent flows through a wick at the centre & spreads in all directions uniformly. Hence the individual spots after development look like concentric circles. By making perforations radially, number of quadrants can be created allowing more number of samples to be spotted (Fig 6).

e. **Two dimensional developments:** This technique is very similar to 2-Dimensional TLC. Here the chromatogram development occurs in two directions at right angles (Fig 7). In this mode, the samples are spotted to 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, either the same solvent system or different solvent system can be used for development.

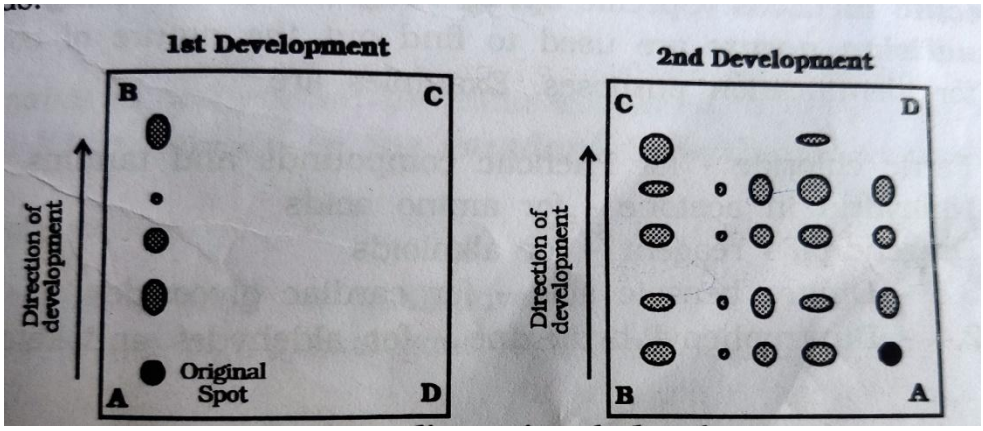


Fig 7: Two dimensional techniques

f. **Drying of Chromatogram:** After the development, the solvent front is marked and the left to dry in a dry cabinet or oven.

g. **Detection:** After the development of chromatogram, the spots should be visualized. Detecting colored spots can be done visually. But for detecting colorless spots, any one of the following technique can be used.

a. Nonspecific methods: where brown or amber of spots can be detected, but not the exact nature or type of the compound.

Examples

(i) Iodine chamber method where brown or amber spots are observed when the developed papers are kept in atank with few iodine crystals at the bottom.

(ii) UV chamber for fluorescent compounds: When compounds are viewed under UV chamber, at 254nm (short λ) or at 365nm (long λ), fluorescent compounds can be detected. Bright spots can are seen against a dark background.

b. Specific methods: Specific spray reagents or detecting or visualizing agents are used to find out the nature of compounds or identification purposes.

a. Ferric chloride- For phenolic compounds and tannins
b. Ninhydrin in acetone- for amino acids
c. Dragendroff's reagent- for alkaloids
d. 3,5-Dinitro benzoic acid- for cardiac glycosides
e. 2,4- Di-nitrophenyl hydrazine- for aldehydes and ketones

The detecting techniques can also be categorized as

a. **Destructive technique:** Specific spray reagents etc where the samples are destroyed before detection e.g. Ninhydrin reagent.
b. **Non-destructive technique:** UV chamber method, Iodine chamber method, densitometric method, e.t.c, where the sample is not destroyed even after detection.

   For radioactive materials, detection is by using autoradiographyor Geiger muller counter.

   For antibiotics, the chromatogram is layed on nutrient agar inoculated with appropriate strain and the zone of inhibition is compared.

Quantitative Analysis

Direct technique: Densitometer is an instrument whichmeasures quantitatively the density of the spots. When the optical densities of the spots for the standard and test solution are determined, the quantity of the substance can be calculated. The papers are neither destroyed nor eluted with solvents to get the compounds. The method is also known as in-situ method.

Indirect techniques: In this technique, the spots are cut into portions and eluted with solvents. The solution can be analyzedby any conventional techniques of analysis like spectrophotometry, electrochemical methods, etc.

Qualitative Analysis: a. Rf value

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

The Rf value ranges from 0 to 1. But the ideal values are from 0.3 to 0.8.

b. Rx value: It is always closure to 1.

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

c. Rm value: It is mainly used to find out whether the compounds belong to a homologous series. If they belong to a homologous series, the **Δ**Rm values are constant. The **Δ**Rm values for a pair of adjacent member of a homologous series are determined by using the below formula:

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

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 and measurement of absorbance at 361nm.

(iii) Detect the contaminants in foods and drinks,

(iv) For the 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 Bacilus 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

B. ELECTROPHORESIS

Electrophoresis is a physical method of analysis based on themigration of electrically charged proteins, colloids, moleculesor other particles dissolved or dispersed in an electrolytesolution in the direction of the electrode bearing the oppositepolarity when an electric current is passed through it.

The electrophoretic mobility is the rate of movement in metre per second of the charged particles under the action of an electric field of I volt per metre and is expressed in square metres per volt second. For practical reasons it is given in square centimetres per volt second, $cm^2V^{-1}S^{-1}$. The mobility is specific for a given electrolyte under precisely determined operational conditions.

$$\mu = \frac{Q}{6\pi r\eta}$$

Where μ = Electrophoretic mobility

Q – Net charge on the ion

r – Ionic radius of the solute

η – viscosity of the medium

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 by

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 poly acrylamide.

Paper electrophoresis:

It is separation technique, where ions of different charges are separated on a medium of paper (moistened with a buffer), by the application of a voltage between two electrodes, which are in contact with the paper.

Principle of Separation: A mixture of ions or ionisable substances is applied on the centre of a paper, previously immersed in a buffer of known ionic strength. This paper is placed across two trays, filled with buffer, into which two electrodes are immersed. When a voltage is applied across these electrodes, the ions or ionisable substances migrate towards anode or cathode, based on their charge and other factors. Neutral or non-ionisable substances do not migrate. Anionic substances move towards anode and cations move towards cathode. Ultimately, there is separation of anionic, cationic and non-ionic / zwitterionic substances. The spots/bands which migrate can be detected bu using appropriate spray reagents or visualizing agents as in paper chromatofraphy and can be quantified by using densitometer. Both qualitative and quantitative analysis can be performed in paper electrophoresis.

Components of paper electrophoresis

a. Normally Whatman® filter paper (Grade 3 MM or No. 1) of suitable dimension (2.5cm to 5cm) with a length so that ends of the strip of paper touch the buffer solution, kept in the electrode vessels. The paper to be used is washed with double distilled water followed by 0.1 M HCl or 0.01 M EDTA to remove impurities.

b. Electrodes and voltage to be applied

   The electrode in the form of a thin wire is made up of carbon or platinum. A DC voltage of about 8-15 V/cm length of paper is normally applied. In low voltage electrophoresis, the voltage across two electrodes is about 100-300V, with a current of 0.4mAmp per cm width or 1.5mAmp/strip.

   In high voltage electrophoresis, a potential of about 50-215V/cm(Total 10,000V/strip) is applied across the electrodes.

c. Buffers used: The pH of buffer to be used depends upon the types of compounds to be separated.

   The following are some of the buffers used:

   1. Barbitone buffer (Veronal buffer) (0.07mole/litre, pH 8.6). Ionic Strength – 0.05.
   2. Tris-acetate buffer (0.07 mole/litre, pH 7.6)
   3. Citrate buffer (0.07 mole/litr, pH 3.0 or pH 6.8)

   Other buffers of different pH and ionic strength can also be for separation, based on the type of compounds. Usually ionic strengths (IS) of 0.05-0.5 is used in most separations.

Types of paper electrophoresis (PE)

A. Horizontal/vertical/Continuous Electrophoresis

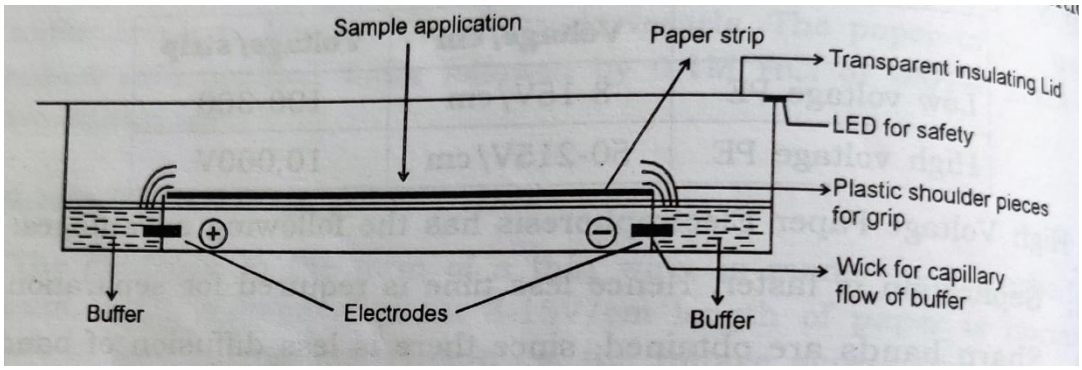
   There are different types of electrophoresis instruments based on the design of the instrument. The diagrams of these 3 types of instruments are shown in Fig 1 to 3.

Horizontal and vertical modes are used in analytical scale; whereas continuous electrophoresis is used on a preparative scale (i.e. large amount of sample mixture is used). The principles involved in all the modes are same, but the design of each instrument varies.

In Horizontal type / Vertical (Fig 1 and 2), buffer solution of known pH and ionic strength is filled into two troughs. Appropriate grade of Whatmann filter paper and suitable width and length of filter paper are immersed in buffer solution. **10-20µl** of sample solution is applied at the centre of the paper and fixed in position.

The transparent lid is closed for safety as well as to prevent evaporation of buffer/solvent. A suitable potential (100-300V) is applied across two electrodes dipped in buffer solution.

Fig 1: Horizontal Paper electrophoresis



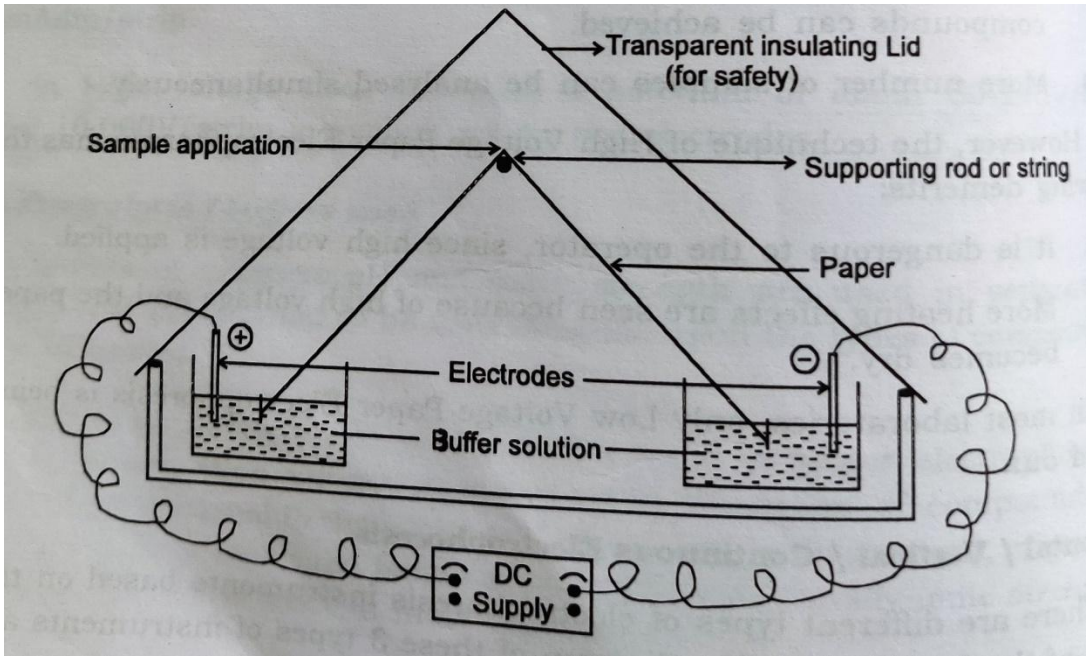


Fig: 2: Vertical Paper Electrophoresis

When such potential is applied across the electrodes, migration of cations and anions take place towards cathode and anode respectively. Non-ionisable / neutral substances do not move. Hence the separation of compound from the mixture takes place.

In this vertical mode, the migrations of ions are assisted by gravity and hence a typical separation takes place in about 6-8 hours. After sufficient migration, the paper is taken out and dried, to fix the spots / bands. Then the compounds / bands /spots can be visualized by using the visualizing agent. The quantitation of spots can be done by densitometer.

The horizontal mode is similar to the vertical mode, in principle. However, the paper is placed on a flat bed, as shown in Fig 2. The procedure to be followed is same as that of vertical type. In horizontal mode, it takes about 12-14 hours for separation.

Continuous electrophoresis (Fig. 3) is meant for preparative samples, where a predetermined sample volume through a valve device is applied continuously on the centre of paper. The application of voltage causes migration of samples and hence compounds are separated as bands. Thus each band is made to fall down and pure compounds are collected in separate containers. The solvent is evaporated and pure fractions are reused.

Various factors like charge of ions, size of the ions, viscosity of the medium, applied voltage, pH of buffer and ionic strength affect the migration of ions in paper electrophoresis

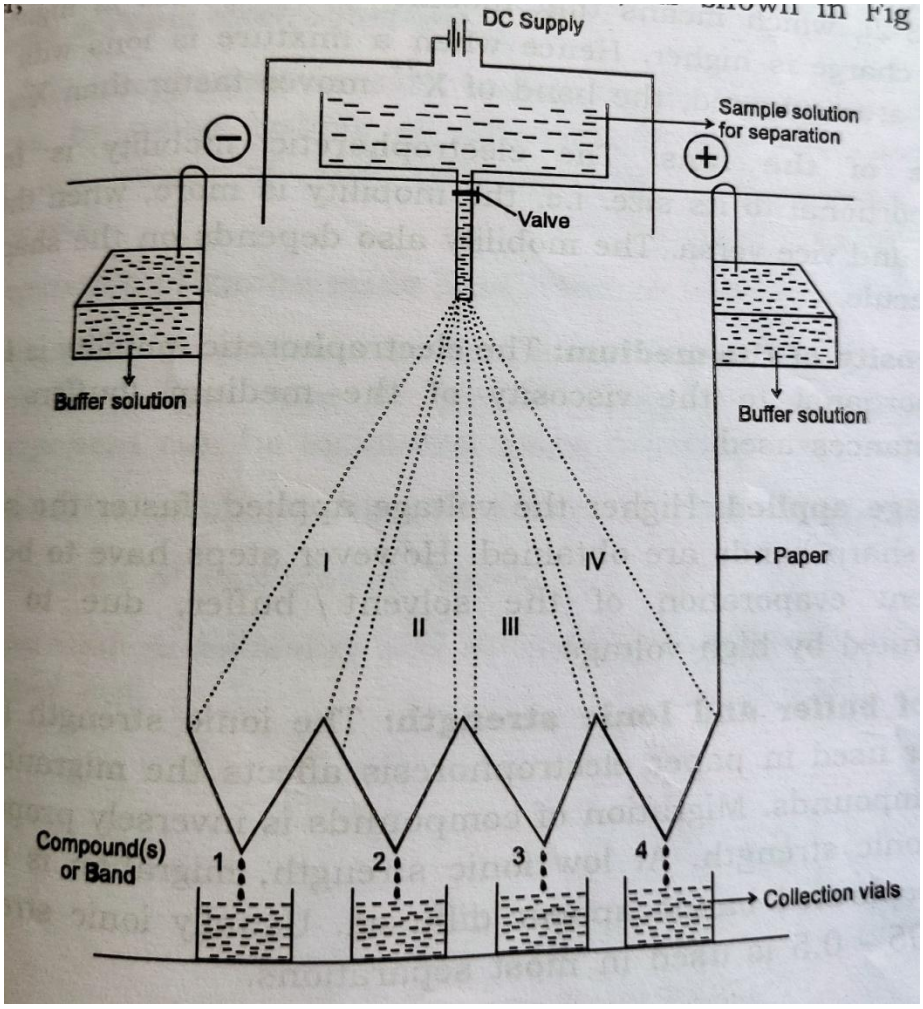


Fig 3: Continuous paper electrophoresis

B. There are two types of paper electrophoresis based on the voltage applied, i.e. Low voltage or High voltage Paper electrophoresis

	Voltage/cm	**Votage/strip**
Low voltage PE	8-15 V/ cm	100-300
High voltage PE	50-215V/ cm	10,000v

High voltage paper Electrophoresis has the following advantages:

1. Separation is faster, hence less time is required for separation
2. Sharp bands are obtained, since there is less diffusion of bands.
3. As sharp bands are obtained, separation of closely related compounds can be achieved.
4. More number of samples can be analysed simultaneously.

   Disadvantages of High Voltage Paper Electrophoresis
   1. It is dangerous to the operator, since high voltage is applied.
   2. More heating effects are seen because of high voltage and the paper becomes dry.

   So in most of the laboratories, low voltage paper electrophoresis is used.

Advantages of Paper Electrophoresis:

1. The technique is easy to follow
2. Less expenditure
3. Number of samples can be separated on a sample paper, at a time.
4. Wide variety of ionisable substances such as amino acids, proteins and peptides, antibiotics, alkaloids etc., can be separated.

Disadvantages

1. The time required for separation is more, i.e. 6-8 hours in vertical mode and 12-14hours in horizontal mode.
2. Use of high voltage may be dangerous, unless precautions are taken.

Application of Paper Electrophoresis

Paper electrophoresis is used mainly for the separation of ionizable substances, by using buffers of different pH and ionic strength. The following are some of the pharmaceutical applications of paper electrophoresis.

1. Separation of amino acids into acidic or basic or zwitterionic type
2. Separation of proteins in serum (into albumin, α1, α2, β and gamma globulins). The type of protein and the percentage of each component can be estimated using densitometer.

3. Separation of lipoproteins in serum (in case of hyperlipidemia)
4. Separations of enzymes in blood.
5. Separation of alkaloids and antibiotics in different samples can be carried out.

GEL ELECTROPHORESIS:

It is a separation technique. Gel is used as medium. The gel may be agar or agarose gel or polyacrylamide gel.

The device consists essentially of a glass plate over the whole surface ofwhich is deposited a firmly adhering layer of gel of uniform thickness. The connection between the gel and the conducting solution is effected in various ways according to the type of apparatus used. Precautions are to be takento avoid condensation of moisture or drying of the solid layer.

Vertical Gel Instrument- The schematic diagram of a vertical gel electrophoresis apparatus is given in Figure 4. It has two buffer chambers, upper chamber and a lower chamber. Both chambers are fitted with the platinum electrodes connected to the external power supply from a power pack which supplies a direct current or DC voltage. The upper and lower tank filled with the running buffer is connected by the electrophoresis gel casted in between two glass plates (rectangular and notched). There are additional accessories needed for casting the polyacrylamide gel such as comb (to prepare different well), spacer, gel caster etc.

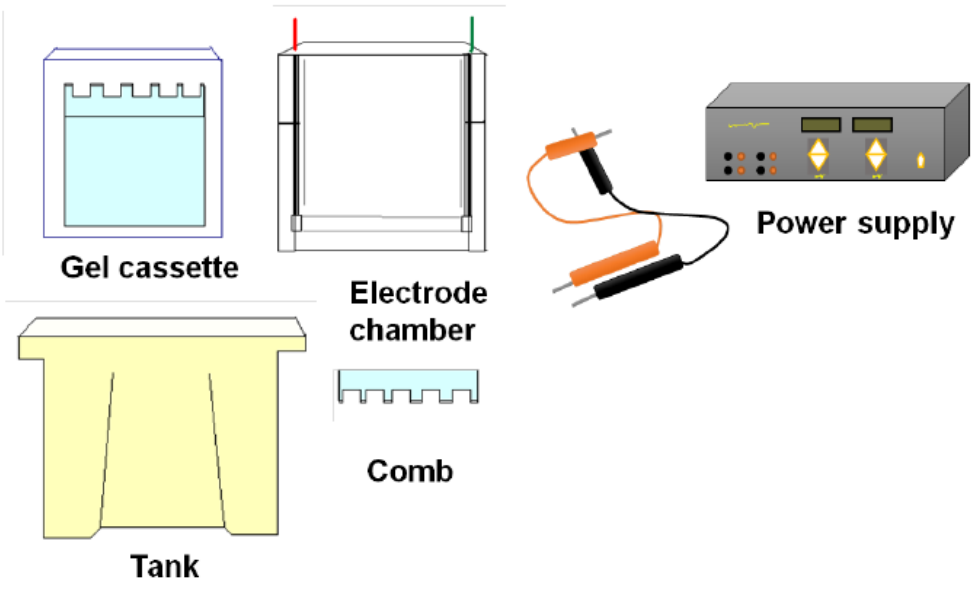


Fig 4: Different components of the vertical gel electrophoresis apparatus

Casting of the gel: The acrylamide solution (a mixture of monomeric acrylamide and a bi-functional cross linker bis-acrylamide) is mixed with the TEMED and APS and poured in between the glass plate fitted into the gel caster. What is the mechanism of acrylamide polymerization? Ammoinum persulfate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acrylamide monomer to form a linear polymer. These linear monomers are interconnected by the cross linking with bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is controlled by the concentration of acrylamide and amount of bis-acrylamide in the gel. In a vertical gel electrophoresis system, we cast two types of gels, stacking gel and resolving gel. First the resolving gel solution is prepared and poured into the gel cassette for polymerization. A thin layer of organic solvent (such as butanol or isopropanol) is layered to stop the entry of oxygen (oxygen neutralizes the free radical and slow down the polymerization) and make the top layer smooth. After polymerization of the resolving gel, a stacking gel is poured and comb is fitted intothe gel for construction of different lanes for the samples. Different steps involves the vertical gel electrophoresis is shown in the below Fig 5.

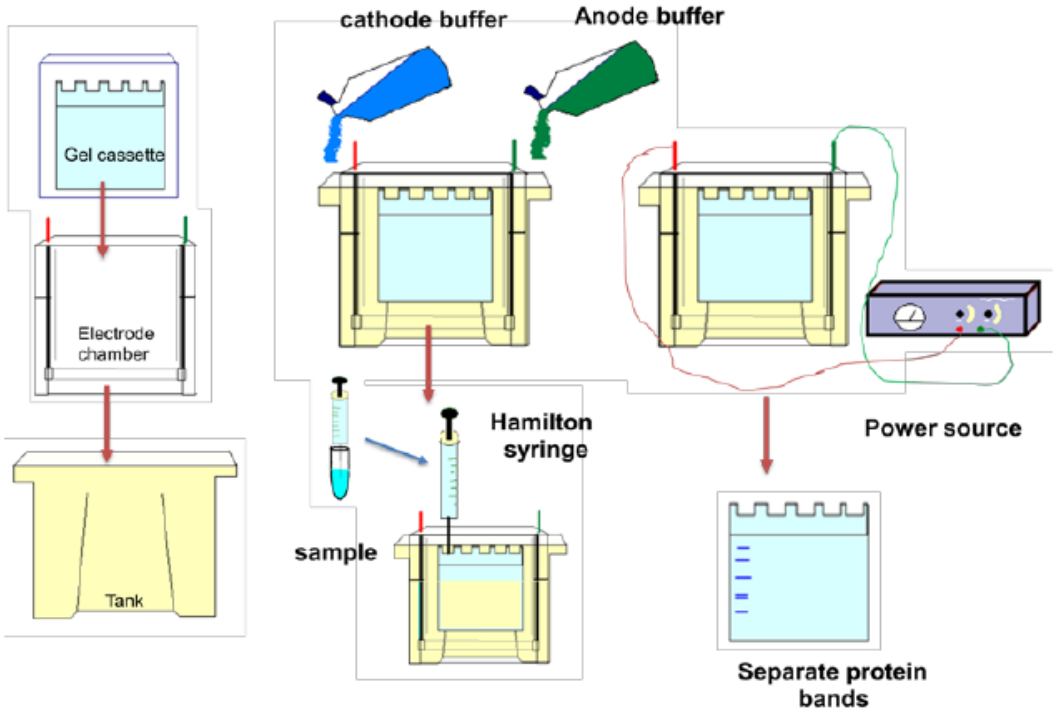


Fig 5: Different steps in performance of vertical gel electrophoresis to resolve sample

Running of the gel: The sample is prepared in the loading dye containing SDS, β-mercaptoethanol in glycerol to denature the sample and presence of glycerol facilitates the loading of sample in the well. As the samples are filled vertically there is a distance drift between the molecules at the top

Vs at the bottom in a lane. This problem is taken care once the samples run through the stacking gel. The pH of the stacking gel is 6.8 and at this pH, glycine is moving slowly in the front whereas Tris-HCl is moving fast. As a result, the sample gets sandwiched between glycine-Tris and get stacked in the form of thin band. As the sample enters into the resolving gel with a pH 8.8, the glycine is now charged, it moves fast and now sample runs as per their molecular weight (due to SDS they have equal negative charge). After tracking dye reaches to the bottom of the gel, gel is taken out from the glass plate with the help of a spatula. Gel is stained with coomassie brilliant blue R250 dye. The dye stains protein present on the gel. A typical SDS-PAGE pattern is given in the Fig. 6.

Potentials of discontinuous PAGE:

1. Number of disulfide bonds: Comparison of reducing and non-reducing denaturing gels can be used to provide information related to the number of disulfide bonds present in the protein.

2. Seperating Proteins based on size alone: In the presence of SDS and reducing environment, PAGE gel resolves two proteins of on the basis of molecular masses and the concentration of gel concentration.

In SDS-PAGE, the relative mobility and the log molecular weight as given by

$$v' = \mathbf{Vo}\frac{A - logM}{A}$$

Molecular weight of a protein can be determined by plotting relative migration Rf with the log molecular weight of standard protein.

$$Rf = \frac{\text{migration of protein from the lane}}{\text{migration of tracking dye}}$$

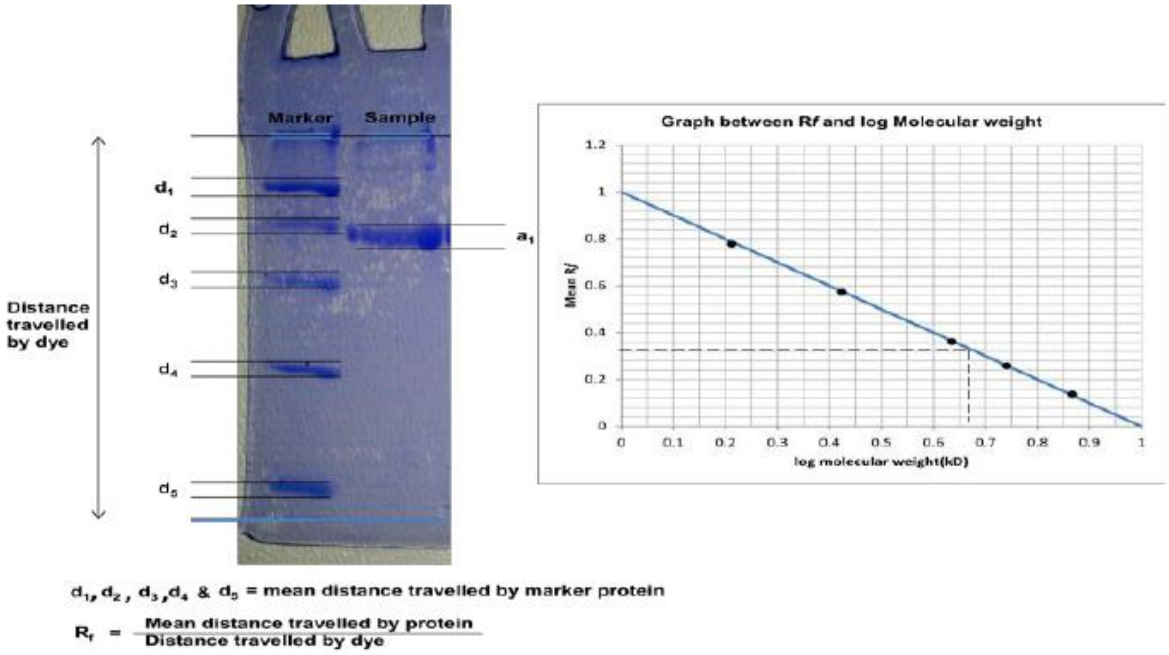


Fig 6: Determination of molecular weight using SDS-PAGE and Determination of Rf

Calculation of molecular weight of the unknown protein sample is a 5 step process:

1. Resolve the protein sample on the SDS-PAGE along with the molecular weight markers.
2. Calculate the relative mobility (Rf) using the following formula:

$$Rf = \mathbf{migrationofproteinfromthelane/migrationoftrackingdye}$$

3. Plotlog molecular mass (y-axis) versus relative mobility (x-axis) of the standards.
4. Perform a linear regression using a calculator or using regression software such as Microsoft Excel.
5. Use the linear regression equation **(Y = mx + c)** to estimate the mass of the unknown protein.

Log Molecular Weight = (slope) (mobility of the unknown) + Y intercept

Buffer and reagent for electrophoresis- The different buffer and reagents with their purpose for vertical gel electrophoresis is as follows-

1. N, N, N', N'-tetramethylethylenediamine (TEMED)-it catalyzes the acrylamide polymerization.

2. Ammonium Persulfate (APS)-it is an initiator for the acrylamide polymerization.

3. Tris-HCl- it is the component of running and gel casting buffer.

4. Glycine-it is the component of running buffer

5. Bromophenol blue- it is the tracking dye to monitor the progress of gel electrophoresis.

6. Coomassie brilliant blue R250-it is used to stain the polyacrylamide gel.

7. Sodium dodecyl sulphate-it is used to denature and providenegative charge to the protein.

8. Acrylamide- monomeric unit used to prepare the gel.

9. Bis-acrylamide- cross linker for polymerization of acrylamide monomer to form gel.

Capillary Electrophoresis

Capillary electrophoresis employing a narrow bore fused quartz silica capillary tube usually 50-75cm long with an i.d. of 25-100µm (and an o.d. of 400 µm) containing an appropriate electrolyte using a direct current (DC) high voltage source, capable of producing a current of 250 µA at voltage ranging from 1000 to 30,000volts and on-line detector that similar to those HPLC are involved (high voltage electrophoresis).

A cross – sectional view of such a capillary is shown in the Fig 7. The capillary is protected with an outer layer of a polyimide (polymer of imide monomer)

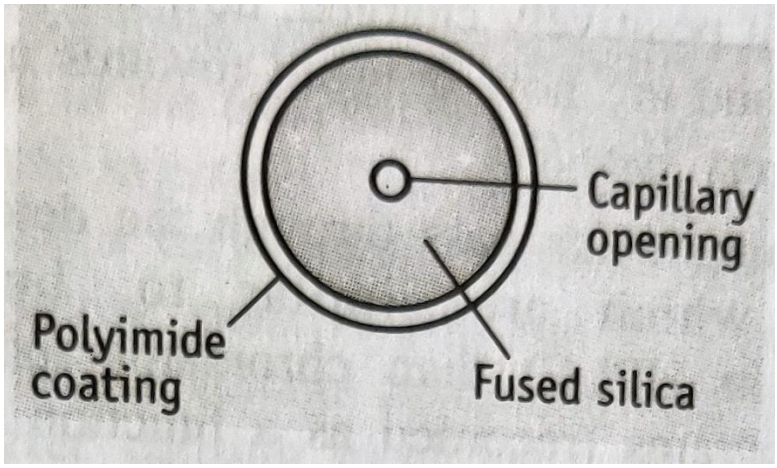


Fig 7: A cross – sectional view of such a capillary is shown in the

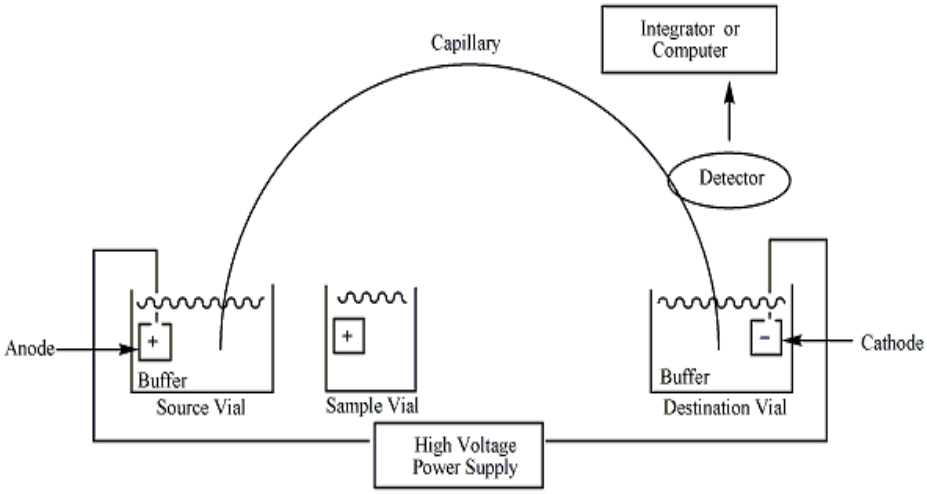


Fig 8: Capillary electrophoresis system

A basic schematic of a capillary electrophoresis system is shown in fig. 8. The system's main components are a sample vial, source and destination vials, a capillary, electrodes, a high voltage power supply, a detector, and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample. Sample is introduced into the capillary via capillary action, pressure, siphoning, or electrokinetically, and the capillary is then

returned to the source vial. The migration of the analytes is initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. In the most common mode of CE, all ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow (EOF) (Fig 9). The analytes separate as they migrate due to their electrophoretic mobility, and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different retention times in an electropherogram.

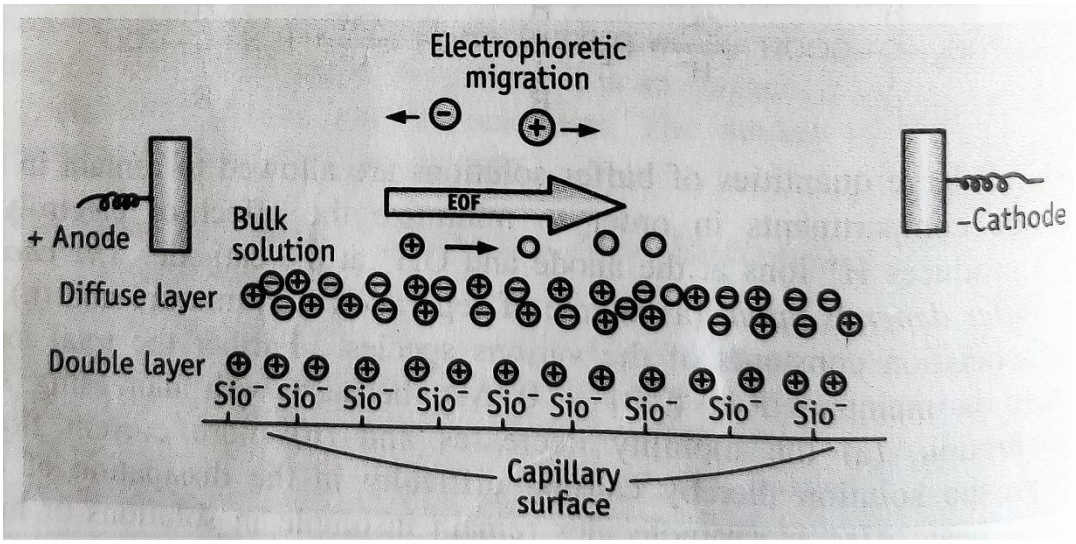


Fig 9: Various events of migration of species and EOF in capillary electrophoresis

The efficiency, N (Number of theoretical plates) may be expressed by the equation:

$$N = \frac{\mu E d}{2\,D}$$

Where, D = the diffusion coefficients of migration species,

d = the distance travelled

μ = electrophoretic mobility of the species, and

E = the applied electric field

CE mechanism is entirely different from a chromatographic distribution mechanism, in that it is readily applicable smaller as well as macromolecules. Thus it is used for the separation of large biomolecules.

Detection in CE: The most commonly used detectors are a UV absorbance or a fluorescence monitor or a diode array spectrometer producing absorbance data at multiple wavelengths, on account of the very small volume (< 10^{-9}L) of the separated analytes, the detection is carried out on column (or on-line detection). For this a small outer part of the protective polyimide coating from the capillary surface is removed either by burning, dissolving or scratching. This small opening of the outer capillary surface then serves the purpose of a detector cell (optical window). The path length for the focused beam to be passed through such an opening is very small (50-100μm) which

utilizes the small volumes. One example of representative Electropherogram which was obtained from the seized heroin sample is showing in the Fig 10.

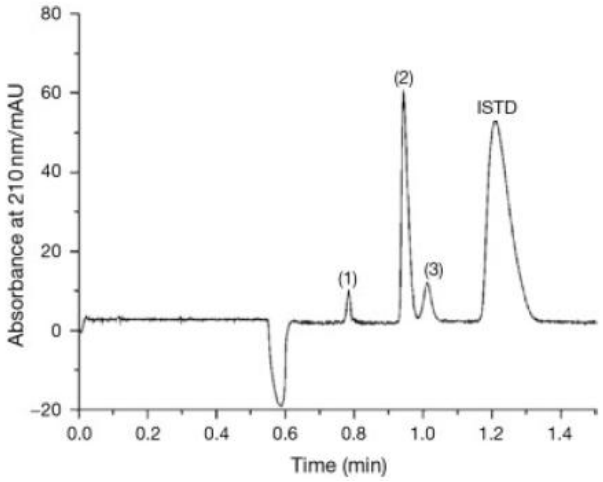


Fig. 10: Representative Electropherogram showing the separation of a seized heroin sample byusing MEKC with short end injection (1) morphine, (2) heroin, (3) acetylcodeine, ISTD = internal standard (N, N-dimethyl-5-methoxytryptamine). UV absorbance at 210 nm, uncoated fused silica capillary 50 cm × 50 μm I.D. ×360μm O.D., effective separation length 8 cm, back- ground electrolyte: 15 mM sodium borate, 25 mM sodium dodecylsulfate, 15% (v/v) acetonitrile, pH 9.5, 25 °C.–25 kV, hydrodynamicinjection.

Application:

1. Capillary electrophoresis (CE) is the primary methodology used for separating and detecting short tandem repeat (STR) alleles in forensic DNA laboratories.
2. Capillary electrophoresis may be used for the simultaneous determination of the ions NH^{+4}, Na^{+}, K^{+}, Mg^{+2} and Ca^{+2} in saliva.
3. Illicit Drug Analysis: Applications of capillary electrophoresis to illicit drugs in seizures and toxicology samples

Analytes	Matrix	Method
Coca alkaloids and sugars	Illicit cocaine	MEKC (Micellar electrokinetic chromatography) with indirect UV detection
Heroin, morphine, acetylcodeine,caffeine, paracetamol	Heroin seizures	MEKC with short-end injection,detection by UV absorbance
Methamphetamine, amphetamine,dimethylamphetamine, and p-hydroxymethamphetamine	Urine from subjects using methamphetamine and dimethylamphetamine	CZE using cyclodextrins for separationof enantiomers with MSdetection

The analytical separations by capillary electrophoresis are done in many ways which are called modes. There are four modes are: (i) capillary zone electrophoresis (CZE) (ii) capillary gel electrophoresis (CGE) (iii) capillary isoelectric focusing(CIEF) and (iv) capillary isotachophoresis(CITP).

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