

CHROMATOGRAPHY

Introduction :

Chromatography is a nondestructive analytical technique used for resolving a multicomponent mixture of true, minor or major constituents into individual fractions. It is distinguished from other physical and chemical methods of analysis by the method of separation. The components are separated by means of bringing into contact with two immiscible phases, one is stationary and the other is mobile phase.

A sample is introduced into a mobile phase and is carried along a column containing distributed stationary phase. The components in the sample undergo repeated reactions between the mobile phase and stationary phase. By proper choice of both phases, the components of the sample are gradually separated into bands in the column. The separated components emerge in order of increasing interaction with the stationary phase. The component with least affinity for stationary phase emerges first and the most strongly affinity component elutes last.

The separation column is the heart of the chromatography. It provides wide choice of materials for stationary and mobile phases and makes it possible for separation of molecules that differ slightly in their physical and chemical properties. After separation of compounds, the final identification of components requires confirmation by some other analytical methods, such as, spectroscopic methods. Quantitative analysis can be carried out by measuring the area of chromatographic peak. Hence, chromatography can be used for both qualitative and quantitative analysis.

Classification :

In chromatography, the mobile phase can be a gas or liquid and the stationary phase may be a solid or liquid. Chromatography can be classified based on nature of phases, principle and modes of separation.

(a) **Based on the Nature of Stationary and Mobile Phase :** The different types of chromatography based on the type of stationary and mobile phase used are :

- (i) Gas-solid chromatography in which liquid is the stationary phase and gas is the mobile phase.
- (ii) Gas-liquid chromatography in which liquid is the stationary phase and gas is the mobile phase.
- (iii) Solid-liquid chromatography in which solid is the stationary phase and liquid is the mobile phase.

Example : Column chromatography, HPLC, thin layer chromatography.

- (iv) **Liquid-liquid chromatography** in which liquid is both stationary and mobile phase.

Example : Column partition chromatography, paper partition chromatography.

- (b) **Based on the Principle of Separation :** The principle of separation involved in chromatography is adsorption or partition of components between mobile phase and stationary phase. They are classified as :

- (i) **Adsorption Chromatography :** The principle involved in this type of chromatography is adsorption of the components onto stationary phase when the sample solution is dissolved in mobile phase is passed through a column of stationary phase.

Example : Gas-solid chromatography, thin layer chromatography, column chromatography.

- (ii) **Partition Chromatography :** The principle involved in this type of chromatography is partition of the components into stationary phase when sample solution is dissolved in mobile phase is passed through a column of stationary phase.

Example : Gas-liquid chromatography, paper partition chromatography.

- (c) **Based on modes of Chromatography :** There are two modes based on the polarity of the stationary and mobile phase used.

- (i) **Normal Phase Chromatography :** In this mode, the stationary phase is polar and the mobile phase is non-polar.

- (ii) **Reverse Phase Chromatography :** In this mode, the stationary phase is non-polar and the mobile phase is polar. This is most widely used in pharmaceutical analysis.

Other types of chromatography used for separation of components are :

- (i) **Ion exchange chromatography** in which the components are separated by exchange of ions between ion-exchange resin and charged ions.
- (ii) **Gel permeation chromatography or size exclusion chromatography** in which the components of a sample are separated according to their molecular sizes by using different gels.
- (iii) **Chiral Chromatography** in which optical isomers are separated by using chiral stationary phases.

5.1 COLUMN CHROMATOGRAPHY

5.1.1 Introduction

Column chromatography is a separation technique in which a column of stationary phase is used. The stationary phase used in column chromatography may be a solid or liquid. Based on the type of stationary phase used column chromatography may be divided into two types.

1. Adsorption column chromatography in which solid is the stationary phase.
2. Partition column chromatography in which liquid is the stationary phase.

5.1.2 Adsorption Column Chromatography

In adsorption column chromatography, the solid stationary phase is packed in a vertical glass column and the liquid mobile phase is allowed to run down the column.

Principle :

The principle involved in the adsorption column chromatography is adsorption at the solid-liquid interface.

When a mixture of components dissolved in the mobile phase is introduced into the column, the stationary phase competes for the solute. The components of mixture must show different degrees of affinity towards the solid stationary phase and interaction between the adsorbent and components must be reversible.

As the components in the solute show different affinities towards the solid phase, the components with least affinity move down the column in a faster rate than that of components with strong affinity and are arranged in order of their affinity for the adsorbent.

5.1.3 Development Techniques

When the mixture of components is dissolved in a mobile phase and is introduced into the column, the components get adsorbed onto the stationary phase. The technique used to recover the components is called **elution**. The components are called as **elute** and the solvent is called as **eluent**. The process of removing the components from the column is called as **elution**. The individual components are separated out from the column by means of development techniques. There are mainly three types of Development Techniques. They are :

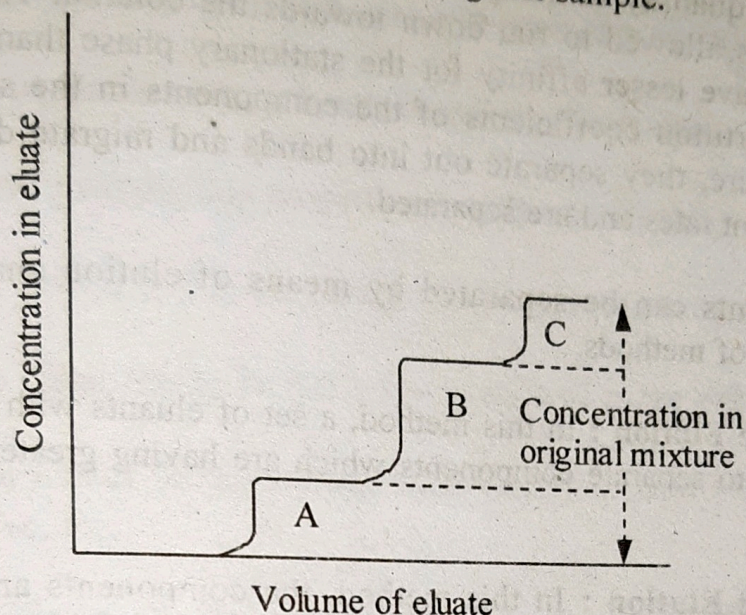
1. Frontal Analysis
2. Displacement Analysis
3. Elution Analysis

1. **Frontal Analysis** : This technique was developed by Tiselius. In this method, when a sample solution which is a mixture of components and mobile phase is passed continuously through the column, the less strongly adsorbed components are adsorbed down the column or collect in the solvent front. The solvent first emerges out from the column which is followed by least adsorbed component. Other components emerge one after another depending upon their degree of adsorptivity.

When the column is filled with a known amount of adsorbent and the sample mixture is continuously added to the column, after certain time, the adsorbent gets saturated with the components. Then, if any mixture is added, the less adsorbed component leaves the column in a pure form. Hence, this method can

be used for separation of readily eluted component from others with greater affinity and is also termed as preparative method. This method is used to remove relatively smaller quantities of unwanted substances, which are strongly adsorbed than that of desired components from a given sample.

Figure :
Frontal
Analysis:
Curve for a
mixture of three
substances, A,
B & C

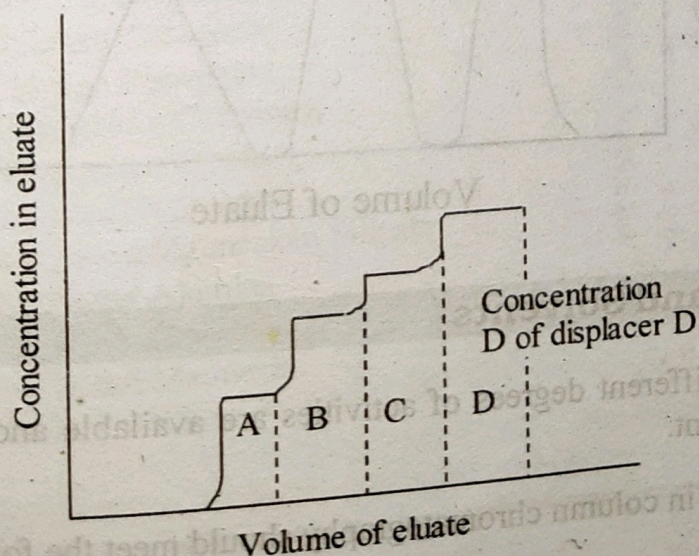


- Displacement Analysis :** In this method, the sample mixture is introduced first into the column. Then, the components in the mixture are separated by running a solution which is consisting of a substance which is more strongly adsorbed than any of the components of the sample mixture. The substance which is employed to separate components is called as displacing agent.

The displacing agent is adsorbed by the column strongly and is adsorbed at the top of the column and spreads downwards forcing out the components of the sample mixture from adsorption sites. The least adsorbed component leaves the column first followed by others depending upon the degree of the adsorptivities.

In this process, the components are separated basing on their differences in properties of adsorption. The separation is not sharp and may contain some fractions of mixtures which can be collected and treated further.

Figure :
Displacement
analysis : curve
for a mixture of
three
substances, A,
B & C
displaced by a
fourth, D



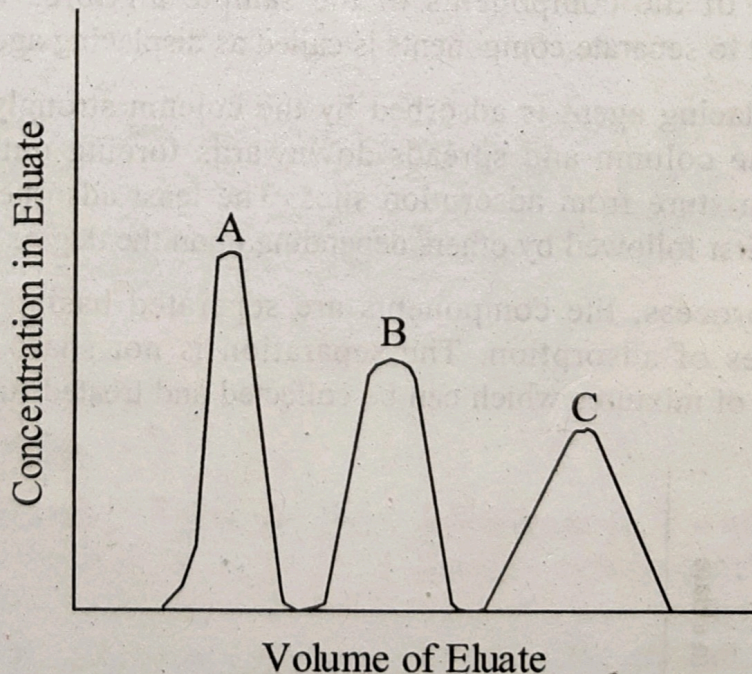
3. **Elution Analysis :** In this method, the individual components of the sample mixture are separated by means of eluting the column with fresh solvent.

A small quantity of sample mixture is introduced into the column. Then, fresh solvent is allowed to run down towards the column. The fresh solvent or eluant should have lesser affinity for the stationary phase than the sample components. If the partition coefficients of the components in the sample are different from the mixture, they separate out into bands and migrate down towards the column at different rates and are separated.

The components can be separated by means of elution analysis by employing two different types of methods.

- (a) **Step-wise Elution :** In this method, a set of eluants with increasing eluting powers are used to separate components which are having greater affinity to the stationary phase.
- (b) **Gradient Elution :** In this method, the components are separated by means of varying the composition of the eluant. The ratio of the eluting components are altered to obtain a range of eluting powers and are introduced into the column.

Figure :
Elution analysis : The separate elution of three substances A, B & C



5.1.4 Adsorbents and Solvents

Adsorbents of different degrees of activities are available and can be chosen according to our requirement.

Adsorbents used in column chromatography should meet the following criteria :

1. Should be chemically inert
2. Should allow suitable flow of mobile phases.
3. Should have uniform size distribution and preferably spherical in shape.
4. Should be insoluble in the solvents or mobile phases used.
5. Should have reproducible properties from batch to batch.
6. Should be active, but, not so active that no movement of components occurs.
7. Should be colourless to facilitate observation of zones and recovery of components.
8. Should be useful for separating wide variety of compounds and freely available, inexpensive, etc.,

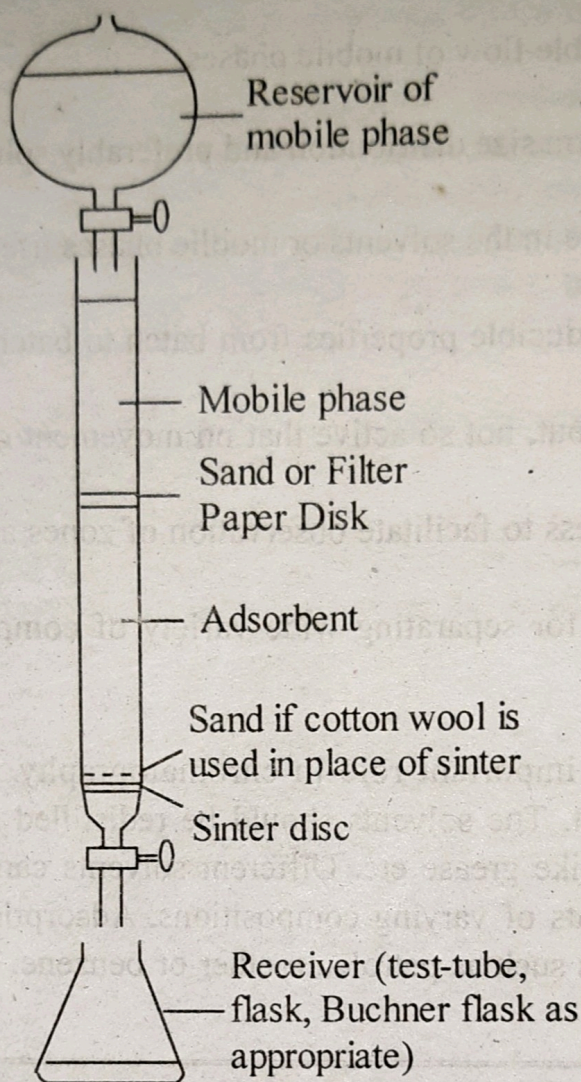
Solvents also play an important role in chromatography. They act as mobile phase, developer and as eluant. The solvents should be redistilled before use to remove traces of non-volatile matter like grease etc. Different solvents can be used in their pure form or as mixture of solvents of varying compositions. Adsorption is usually most powerful from non-polar solvents such as petroleum-ether or benzene.

Adsorbents and Solvents

Adsorbent		Solvent
Weak	Sucrose	Petroleum ether
	Starch	Carbon tetrachloride
	Inulin	Cyclohexane
	Talc	Carbon disulphide
	Sodium Carbonate	Ether (ethanol-free)
Medium	Calcium Carbonate	Acetone
	Calcium Phosphate	Benzene
	Magnesium Carbonate	Toluene
	Magnesium Oxide	Esters
	Calcium Hydroxide	Chloroform
Strong	Activated Magnesium Silicate	Acetonitrile
	Activated Alumina	Alcohols
	Activated Charcoal	Water
	Activated Magnesia	Pyridine
	Activated Silica	Organic Acids
		Mixtures of acids or bases with ethanol or pyridine

Increasing eluting power
↓

Figure :
Apparatus for
Column
Chromatography



5.1.5 Preparation of Column

Columns are mostly made of good quality neutral glass. They should not be affected by solvents, acids or alkalis. The dimensions of the column are important for effective separation. The length : diameter ratio ranges from 10 : 1 to 30 : 1.

The bottom portion of the column is packed with glass wool or cotton plug which acts as a support for the column. Then, the adsorbent is introduced into the column and is packed uniformly. After the adsorbent has settled, a filter paper disc or sand is kept on the top of adsorbent, so that the adsorbent layer is not disturbed when a fresh mobile phase or sample is introduced into the column. In the column, the level of solvent must not be below the level of adsorbent, as cracks may develop between particles of the adsorbent.

The adsorbent may be introduced into the column by means of two techniques. They are :

1. **Dry Packing Technique :** In this technique, the required amount of adsorbent is introduced into the column in dry powder form and the solvent is allowed to flow directly into the tube by tapping frequently stirring as it removes air bubbles and helps in even packing.

2. **Wet Packing Technique** : In this technique, the adsorbent is made into slurry with mobile phase and is introduced into column with stirring to prevent entrapment of air bubbles. The adsorbent settles down uniformly without any air bubbles.

Introduction of Sample :

The sample which is a mixture of components is dissolved in a minimum quantity of mobile phase and is introduced into the column. The individual components can be separated by means of development techniques.

Detection of Components :

The separated components can be detected visually if they are coloured. The coloured bands are observed in the column moving down separately and can be collected separately.

To detect the components which are colourless, several techniques depending on the properties like absorption of radiations, refractive index, fluorescence etc.

Recovery of Components :

The components from the column can be recovered by means of elution techniques. The zones can also be conveniently isolated by means of cutting the column if plastic columns are used instead of glass tubes.

5.1.6 Column Efficiency

The success of column chromatography is measured by means of its ability and efficiency to separate the components from a mixture.

The column is considered as being made up of theoretical plates which are parallel layers placed one above the other along the length of the tube. When a sample mixture is introduced into the column, the components distribute in the column between the stationary phase and mobile phase according to their partition coefficients. The rate of movement of mobile phase is assumed to be in dynamic equilibrium with stationary phase and the components move down the column at a rate depending on rate of movement of mobile phase which is given as :

$$R = \frac{\text{Rate of Movement of Component}}{\text{Rate of Movement of Mobile Phase}}$$

$$= \frac{\text{Distance Moved by Component}}{\text{Distance Moved by Mobile Phase}}$$

The relationship between partition coefficients K and ' R ' is given as

$$R = \frac{A_m}{A_m + KA_s}$$

where,

A_m - Average area of cross-section of mobile phase.

A_s - Average area of cross-section of stationary phase.

$$K = \frac{\text{Concentration of Component in Stationary Phase}}{\text{Concentration of Component in Mobile Phase}}$$

The thickness of the theoretical plate is called the height equivalent to a theoretical plate (HETP).

$$\text{HETP} = \frac{\text{Length of Column}}{\text{Number of Theoretical Plates}}$$

The smaller that the HETP, the greater is the number of theoretical plates and greater is the efficiency of the column. Thus, the efficiency of a column is measured by means of its number of theoretical plates.

Column Characteristics :

Adsorbent/adsorbate Weight Ratio	30 : 1
Length/diameter Ratio	10 - 15 : 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

Factors affecting Column Efficiency :

Various factors that are affecting column efficiency are :

- Nature of Solvents :** As the rate of flow of a solvent is inversely proportional to its viscosity, the solvents used must have less viscosity for effective separation at faster rate and also for proper elution strength.
- Dimensions of Column :** The efficiency of the column can be increased by means of increasing the length/width ratio. The range is observed to be 20 : 1 to 100 : 1 depending upon the efficiency required.
- Particle size of adsorbent :** The adsorbent activity is proportional to the surface area of adsorbent. The efficiency of the column can be increased by decreasing the particle size. The particle size ranges from 100 to 200 mesh.
- Temperature of Column :** The rate of elution can be increased by increasing the temperature of column, but, it decreases the adsorbent power of the column. Hence, difficult soluble samples are generally separated at high temperatures while others are separated normally at room temperatures.
- Pressure :** By applying high pressure above the column and lower pressure below the column, the efficiency of the column can be increased.

5.1.7 Applications

Column chromatography has limited applications when compared to other analytical techniques. They are :

1. It can be used for separation of a mixture of compounds.
2. It can be used for removal of impurities from a compound.
3. It can be used for isolation of active constituents from crude extracts of plants, animals etc.,
4. It can be used for isolation of metabolites from biological fluids like blood, serum or plasma etc.
5. It can be used in the estimation of drugs present in the formulations of crude extracts.
6. It can be used in separation of geometrical isomers, diastereomers, Tautomeric isomers or racemates.

5.1.8 Partition Chromatography

Introduction :

Partition chromatography is a separation technique which is based on the differences in partition coefficients of the individual components of a mixture between a liquid stationary phase and a gaseous or liquid mobile phase. In column partition chromatography, liquid is used as mobile phase.

Principle :

Some substances, when shaken with a mixture of two immiscible liquids distribute themselves between the immiscible liquids such that the concentration in each liquid is proportional to the partition coefficient. By using this principle, the solvents which are having large differences in their partition coefficients can be employed to separate the required component from a mixture.

Theory :

In partition chromatography, the liquid stationary phase is supported in the column by means of a solid material which is usually polar in character. Silica gel and cellulose powder are frequently used as solid supports. The liquid mobile phase should be chosen such that it is immiscible with the liquid stationary phase.

The technique is similar to column adsorption chromatography except the stationary phase used. In partition chromatography, water is usually used as stationary phase and organic solvent as mobile phase.

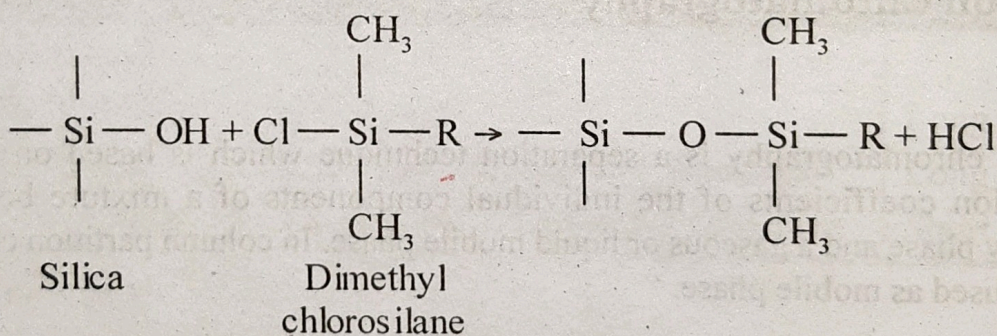
Application :

1. It is used for separation of wide range of biological compounds.
2. It is used for separation of closely related substances such as amino acids formed in the hydrolysis of proteins.
3. It is also used in separation of closely related aliphatic alcohols, sugar derivatives and carboxylic acids.

5.1.9 Bonded Phase Chromatography (BPC)

The problems associated with partition chromatography such as loss of stationary phase from support material can be overcome by using bonded phase supports. In this technique, the stationary phase may be bonded with the support material. This form of partition chromatography in which both monomeric as well as polymeric phases have been bonded to a large number of support materials is called as bonded phase chromatography.

The bonded stationary phases can be prepared by using silylation reactions, where silanol groups (Si – OH) at the surface of silica gel are allowed to react with substituent chlorosilanes.



Polymeric bonded stationary phases can be produced by using di- or tri- chlorosilanes in the presence of moisture as a result of which a polymeric layer is formed at silica surface.

The main advantage of these bonded stationary phase is to prevent the loss of stationary phase from the separation column. The bonded phase liquid chromatography is mainly useful for the separation of bioactive molecules. By careful choice of ionic or ionisable phase, the bonded phases can be used for separation of a wide range of components.

5.2 THIN LAYER CHROMATOGRAPHY

5.2.1 Introduction

The technique of thin-layer chromatography was first introduced by Izmailov and Shraiber. They separated plant extracts using 2 mm thick and firm layer of alumina set on glass plate. Kirchner identified temperes on impregnated filter paper and glass-fiber paper coated with alumina or silica. Stahl developed standard equipment for thin layer chromatography.

5.2.2 Principle

The underlying principle of thin-layer chromatography is adsorption. When a mixture containing one or more components are spotted on a thin-layer of adsorbent coated on a chromatographic plate, and introduced into a development tank containing mobile phase, the mobile phase flows by capillary action. The components move across the layer according to their affinities. The component with more affinity travels slower and that of lesser affinity travels faster. Thus, the components are separated on the thin layer based on their affinities towards the stationary phase.

5.2.3 Technique

The components of a mixture are separated by using thin-layer chromatography by selecting a suitable adsorbent and mobile phase, developing the chromatogram and finally, the components are detected and recovered.

- (a) **Stationary Phases :** A large number of adsorbents can be used as stationary phases. These adsorbents do not adhere directly to the glass plates satisfactorily. So, binders like gypsum or starch are added to the adsorbent. In some cases, fluorescent indicators like zinc silicate are added to the adsorbent to facilitate easy detection of components. In selecting, the adsorbent as a stationary phase, various properties of the adsorbent such as solubility, acidic, basic or amphoteric nature is to be identified. A chemical reaction should not occur between the adsorbents or binders.

Coating Materials Used in TLC :

Adsorbent	Acidic or Basic	Activity	Separatory Mechanism	Components to be separated
Silica gel	Acidic	Active	Adsorption partition	Acidic and neutral substances
Alumina	Basic	Active	Adsorption partition	Basic and neutral
Kiesleguhr	Neutral	Inactive	Partition	Strongly hydrophilic substances
Cellulose Powder	Neutral	None	Partition	Water soluble compounds

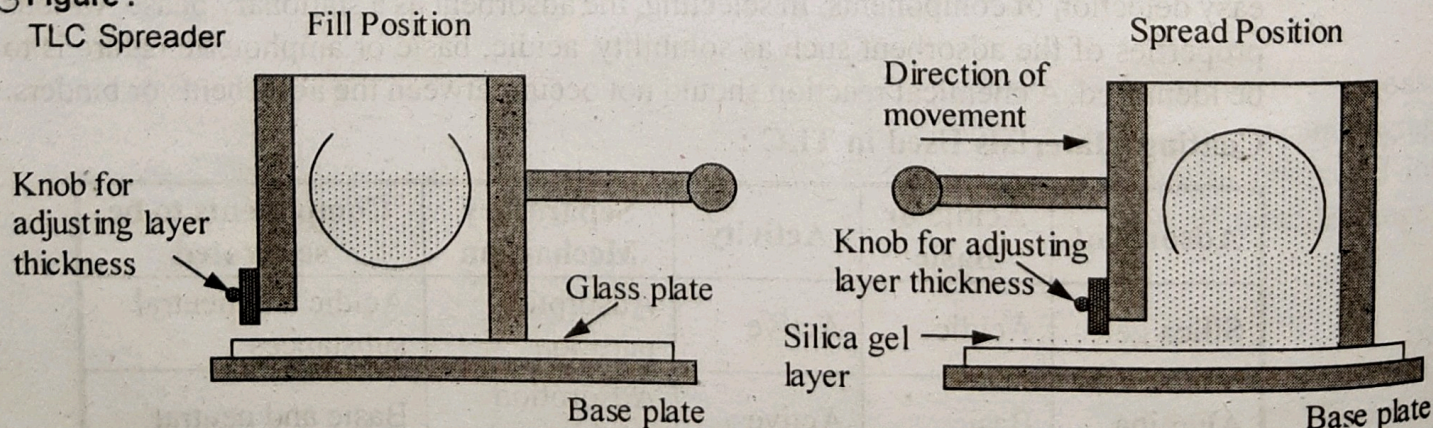
- (b) **Preparation of thin Layer Chromatographic Plates :** In preparation of the plates, glass plates with specific dimensions like 20cm × 20cm (full plate), 20cm × 10cm (half plate), 20cm × 5cm (quarter plate) are normally used. The glass plates should be of good quality and should withstand high temperatures.

The glass plates are coated with the adsorbent by various methods by using the coating material in the form of slurry suspension or in some liquid.

The various methods of coating the plates are :

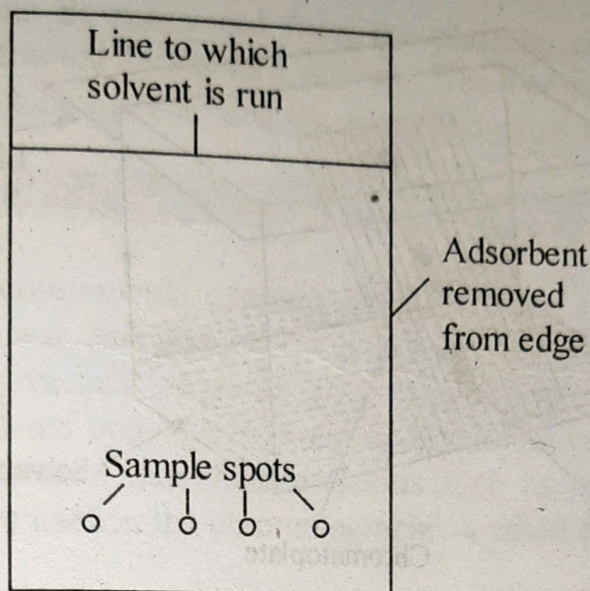
1. **Pouring :** In this method, the adsorbent is made into an slurry and is poured onto a glass plate which is kept on a level surface. The slurry is spread uniformly over the surface and is dried in an oven. This method does not ensure uniformity in thickness and so it is not used to great extent though it is very simple.
2. **Dipping :** In this method, two plates are dipped back to back in to the slurry and are separated and dried. This method requires a large quantity of slurry even for few plates and is also not of much use.
3. **Spraying :** In this method, the adsorbent is made into a suspension and is sprayed on a glass plate using a sprayer. In this method, there is possibility of variations in uniformity of thickness from plate to plate or all over the plate.
4. **Spreading :** In this method, the adsorbent is made into slurry and is placed in an applicator and is spreaded on a plate by means of moving the applicator or plates. By this method, plates of uniform layers can be obtained. The thickness of the layer can be adjusted from 0.0 to 2.0mm.

Figure :



5. **Precoated plates :** TLC plates are also available, which are already precoated on glass or plastic sheets. These are expensive, but we can save time. The thickness of precoated plates varies from 0.1 to 0.2 mm.

Figure :
Preparation of
the plate for
thin-layer
chromatography



Activation of Plates :

After coating the TLC plates, the moisture or any liquid present with the adsorbent should be removed. This can be done by drying the plates for about 30 minutes in air and then in an oven at a temperature of about 110°C for 30 minutes. This drying moves the stationary phase active. To obtain very active layers, these plates can be dried for about 4 hours.

Application of Sample :

The sample mixture is dissolved in a solvent which is volatile or as non-polar as possible. The solution is applied as spots on the plate by using capillary tube or microsyringe. The spots are placed at random or at equal distances. But, the spotted area should not get immersed in the mobile phase in development tank. Usually, 2 - 5 μl of a 10 mg/ml solution is applied as each spot on the plate. The size of the spot applied should be uniform to obtain consistent results.

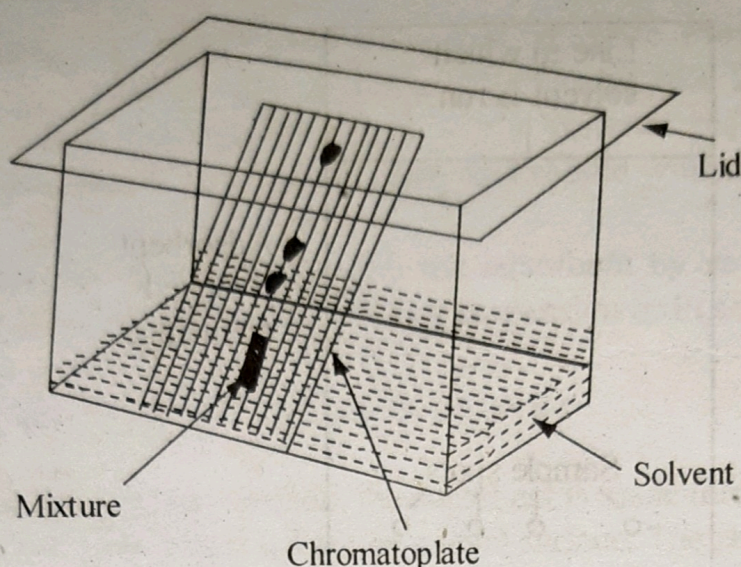
Development of Chromatogram :

The chromatogram is developed in a specially designed equipment called development tank. A development tank is made of chambers to hold TLC plates. The tank is lined inside with filter paper dipped in the solvent, to maintain a saturated environment inside the tank. This lining of filter paper prevents "edge effect" which occurs when the solvent front in the edge of TLC plate moves faster than that of central part of the plate.

After the application of sample spots on the TLC plate, the solvent in the sample solution is allowed to evaporate and the plate is transferred to the development tank. The TLC plate is placed in the development tank at an angle of 45° . The mobile phase is taken in the development tank upto a height of 1mm. The top of the tank is covered with lid to prevent disturbances in the atmosphere of the development tank. The mobile phase used in the TLC can be pure solvents or mixture of solvents. Solvents such as petroleum ether, carbon tetrachloride, cyclohexane, ether, benzene, toluene, chloroform, alcohols and organic acids are used in TLC.

5.16 [Unit - V]

Figure :
Apparatus for
Thin layer
Chromatography



The mobile phase is allowed to rise about a distance of one-half to two-thirds, the length of the plate and the plate is removed from the development tank and dried and is observed for the components.

Detection of Components :

The detection of coloured components can be done visually on a TLC plate. However, the colourless components can be detected by several methods :

- (a) **Destructive Technique :** In this method, the TLC plates are treated with visualising agents by spraying them on a plate where the components are destroyed for detection.

Examples :

- (i) By spraying 70 - 80% of sulphuric acid, black spots are seen due to changing of components.
- (ii) Ferric chloride is used for detection of phenolic compounds and tannins.
- (iii) Ninhydrin reagent is used for detection of amino acids.

- (b) **Non-Destructive Technique :** In this method, the components are identified by examining the plate containing fluorescent indicator under ultraviolet light. The components are observed as dark zone on a yellow-green back ground.

Recovery of Components :

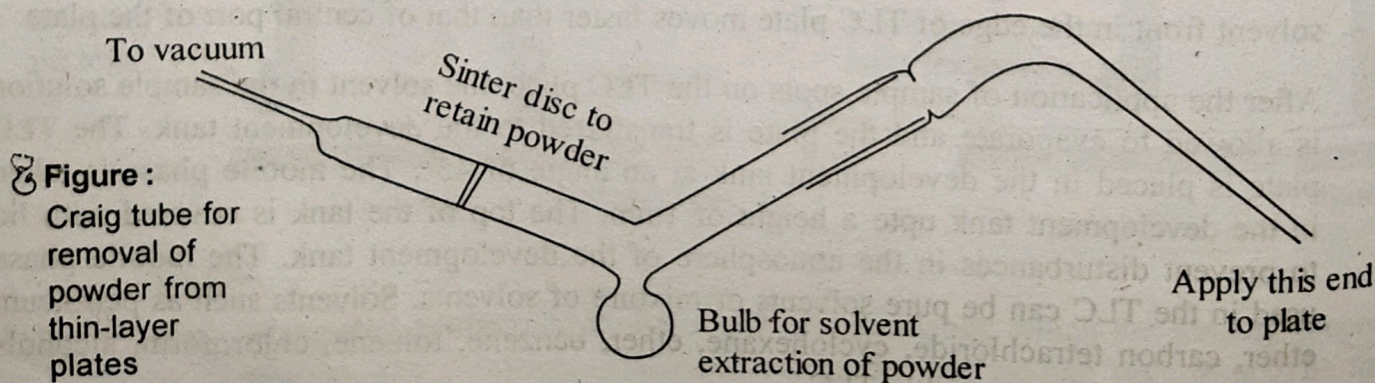


Figure :
Craig tube for
removal of
powder from
thin-layer
plates

The components can be recovered from the plate by removing the zones by using spatula and arc extracted by using a solvent. The components can also be collected by means of a craig tube by removing the zones and extracted with solvents.

5.2.4 Qualitative Analysis

The movement of components depends on the amount and nature of both stationary phase and mobile phase and also on the partition coefficient. Different parameters such as R_F , R_X and R_M values which is a function of partition coefficient is used to identify the components both qualitatively and quantitatively. These values are constant for a substance provided that the conditions such as temperature, stationary phase and mobile phase are used in the chromatographic method are kept constant.

The R_F value (Retardation factor) is defined as the ratio of distance travelled by component from centre to distance travelled by solvent front.

$$R_F = \frac{\text{Distance Travelled by Centre of Component}}{\text{Distance Travelled by Solvent Front}}$$

The value of R_F ranges from 0 to 1. The ideal values of R_F are 0.3 to 0.8. The R_F values are constant for every component in specific combination of stationary and mobile phases. By comparing the R_F values of sample and reference substances, the component can be identified by its standard.

The R_X vlaue is given as

$$R_X = \frac{\text{Distance Travelled by the Component from Origin Line}}{\text{Distance Travelled by Reference Substance from Origin Line}}$$

R_X – Values are always closer to 1.

R_F – Values are determined when the solvent front runs off at the end of the plate.

R_M – Values are used in qualitative analysis. The R_m values are calculated by

$$R_m = \log\left(\frac{1}{R_F} - 1\right)$$

R_m – Values are used to identify whether the compounds belong to a homologous series. if they belong to a homologous series, the ΔR_m values are constant.

5.2.5 Applications

Thin layer chromatography is extensively used for both quantitative and qualitative analysis. The various applications of TLC are :

1. It is used for separation of mixtures of drugs of both chemical or biological origin.
2. It is used for identification of substances by using R_F or R_X values.
3. It is used for identifying the impurities present in a sample.
4. It is used for identifying the related compounds in drugs.
5. It is used for detection of the decomposition products in drugs.
6. It is also used in the examination of a chemical reaction and purification processes.

5.2.6 High Performance Thin Layer Chromatography (HPTLC)

HPTLC is a sophisticated form of TLC. The HPTLC plates are prepared from optimized absorbent layers and even surfaces. The adsorbent used consists of extremely small particles size and very uniform in size. These plates offer high separation efficiency and reproducibility. The development time is much shorter and the detection limits can be attained for upto a nanogram range. The components can be detected by ultraviolet light adsorption in nanogram range and fluorimetric detection in picogram range. The substances can be accurately and precisely assayed using high performance grades of silica.

5.3 PAPER CHROMATOGRAPHY

5.3.1 Introduction

Paper Chromatography is an analytical technique in which paper is used as support for adsorbent or as adsorbent itself. Initially, paper chromatography is used for separation of amino acids. Later, by modifying the characteristics of paper, the technique is applied for separation of a wide variety of components.

5.3.2 Principle

The underlying principle of paper chromatography is mainly partition rather than adsorption. The cellulose layers which are present in paper contains moisture which acts as stationary phase. By suitable modification of paper organic solvents can also be used as stationary phases.

5.3.3 Technique

Paper Chromatography usually refers to paper partition chromatography. This technique is used mainly for separations based on partition only. To separate the components by applying principle of adsorption, the paper is impregnated with silica or alumina which acts as adsorbent.

Selection of Stationary and Mobile Phases :

The choice of filter paper plays an important role in the success of paper chromatography. The paper is to be selected on the basis of whether the paper is used for

- (a) Qualitative or Quantitative Analysis.
- (b) Analytical or Preparative Chromatography.
- (c) substances like hydrophilic, lipophilic, neutral or charged species.

Various types of Whatmann papers like Whatmann 31, Whatmann 20 are used. Cellulose paper is used for support for adsorbents like alumina, silica etc. Various modified papers like carboxyl papers, Acetylated papers and ion-exchange papers are also used in paper chromatography.

Pure solvents, mixture of solvents or buffer solutions can be used as mobile phase. Various solvents used in paper chromatography are chloroform, benzene, isopropanol, ammonia, cyclohexane, etc.,

Table :
Suitable
Solvent
Systems for
Paper
Chromato-
graphy

Stationary Phase	Mobile Phase
Water	Isopropanol-ammonia-water (9 : 1 : 2)
Water	<i>n</i> -butanol-acetic acid-water (4 : 1 : 5)
Water	Phenol saturated with water
Formamide	Chloroform
Formamide	Benzene
Formamide	Benzene-cyclohexane (from 1 : 9 to 9 : 1)
Dimethyl Formamide	Cyclohexane
Phenoxy Ethanol	Heptane
Kerosene	70% iso-propanol
Liquid Paraffin	Dimethyl formamide-methanol-water (10 : 10 : 1)

Application of Sample :

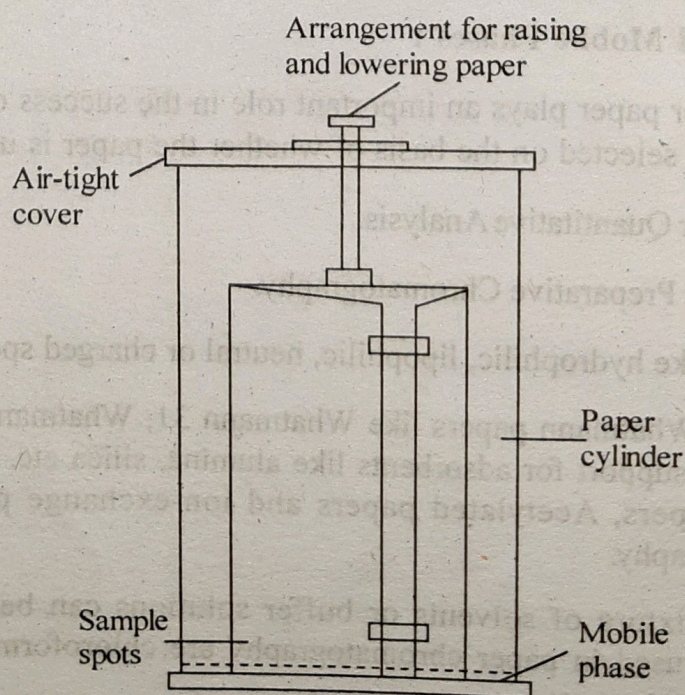
The sample mixture is dissolved in a suitable solvent and is applied as a spot on the filter paper by using capillary tube or microsyringe. The spot should be applied with low concentrations of sample solution to avoid larger zones on the paper.

Development of Chromatogram :

The development of Chromatogram can be done by using different techniques, such as :

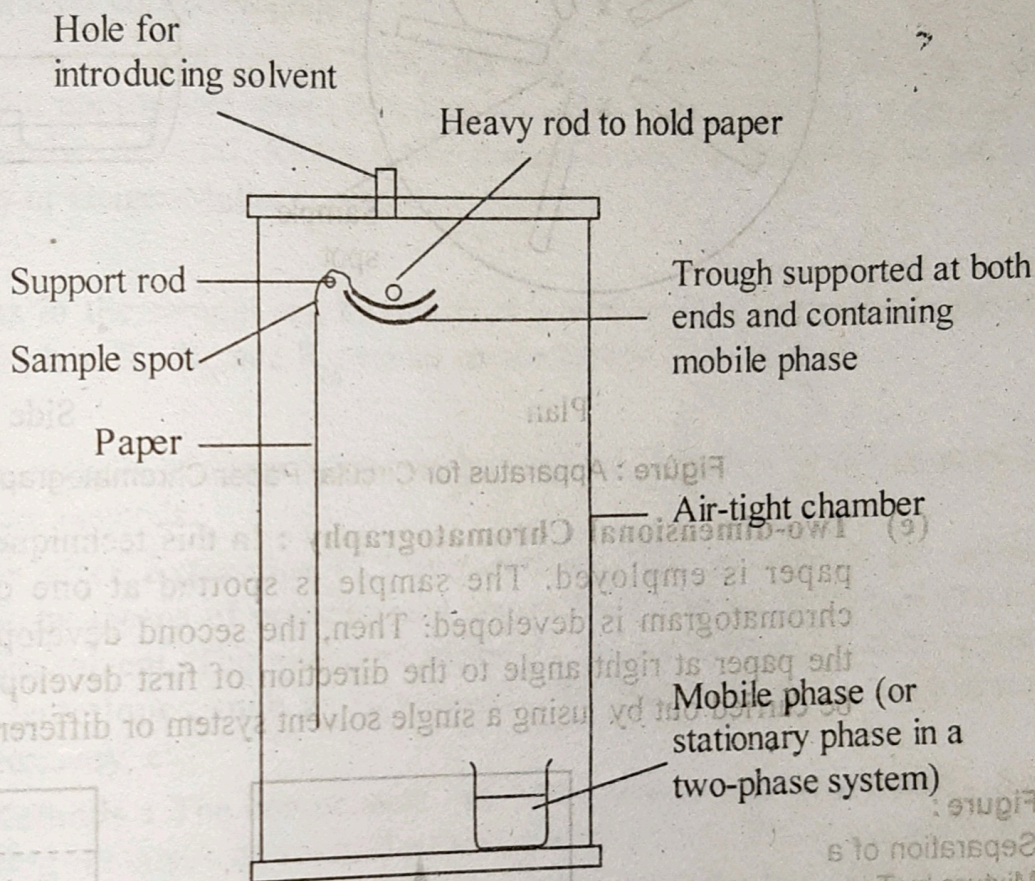
- (a) **Ascending Chromatography :** In this technique, the solvent (mobile phase) is allowed to travel in the upward direction in the paper. The mobile phase is placed in bottom of the container and the filter paper is dipped in the mobile phase. The solvent travels against gravity and the chromatogram is developed in upward direction.

Figure :
Apparatus for
Ascending
Paper
Chromatography



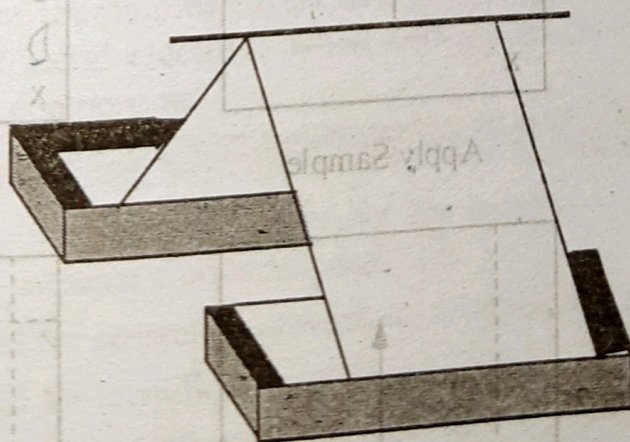
- (b) **Descending Chromatography** : In this technique, the development of chromatogram is done by allowing the solvent to travel down the paper. This is done by placing a solvent holder at the top of the developing tank and the paper is inserted with the spotted sample at the upper end in the trough containing mobile phase. As the movement of solvent is assisted by gravity, the development takes at faster rate and also continuously even if the solvent runs at the other end of the paper.

Figure :
Apparatus for
Descending
Paper
Chromatography



- (c) **Ascending-Descending Chromatography** : In this technique, the chromatogram is developed by ascending technique followed by descending technique.

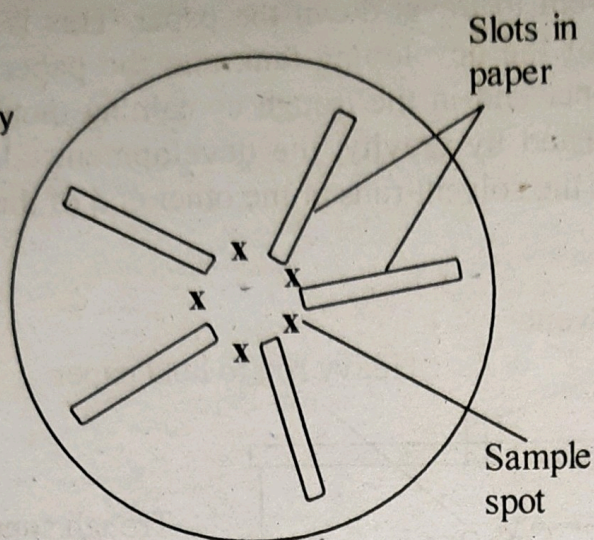
Figure :
Ascending-
Descending
Chromatography



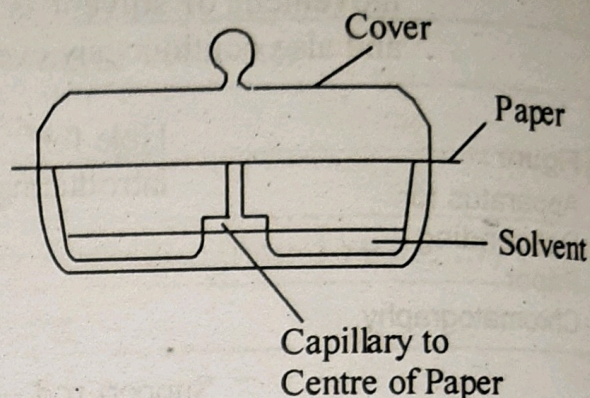
- (d) **Radial Paper Chromatography** : This technique is also called as circular paper chromatography. In this technique, the sample is applied at the centre of a circular paper and the paper is fixed horizontally and the solvent flows through a wick at the centre of the paper. The solvent spreads uniformly in all directions and the chromatogram appears in the form of separated concentric circular zones.

5.22 [Unit - V]

Figure :
Apparatus for
Circular Paper
Chromatography



Plan

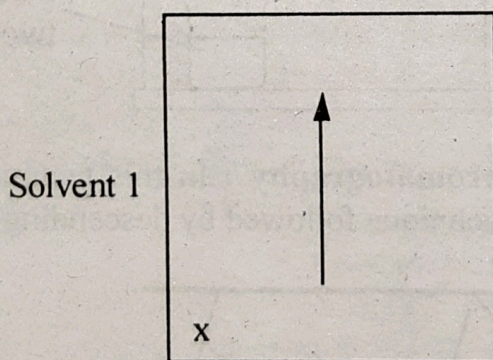


Side View

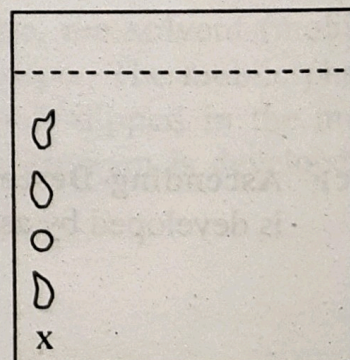
Figure : Apparatus for Circular Paper Chromatography

- (e) **Two-dimensional Chromatography :** In this technique, a rectangular or square paper is employed. The sample is spotted at one corner of the paper and chromatogram is developed. Then, the second development is done by placing the paper at right angle to the direction of first development. This technique can be carried out by using a single solvent system or different solvent systems.

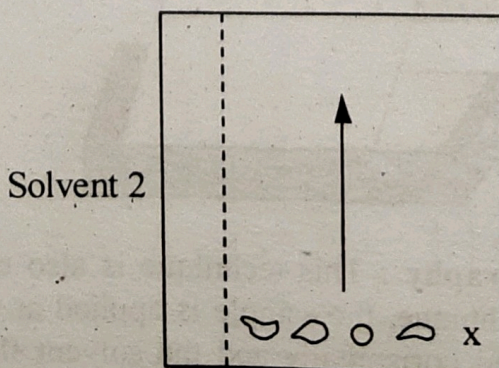
Figure :
Separation of a
Mixture by Two-
dimensional
Paper
Chromatography



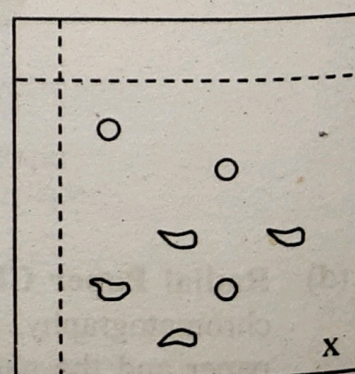
Apply Sample



Result



Turn 90°



Final Separation

Drying the Chromatogram :

The wet chromatogram after development is dried in special cabinets under controlled temperatures.

Detection of Components :

After developing the chromatogram, the colour components can be detected visually. Various methods are available to detect colourless components are similar to that of employed in thin layer chromatography.

The antibiotics can be identified by laying the chromatogram on nutrient agar which is inoculated with appropriate strain of micro-organism. The inhibition zones indicate the regions of active material. Radio active materials can be identified by means of autoradiography or Geiger-Muller counters.

Qualitative Analysis :

The components in the sample can be analysed qualitatively by means of examining the parameters such as R_F , R_X and R_M values for the sample and standard.

Quantitative Analysis :

The quantitative analysis of components can be done by extraction of components from paper or by *in-situ* methods.

- (a) **Estimation after Extraction of Components :** The components are extracted from paper by means of eluting and separating different zones and extracting with suitable solvent. The quantity present in the extract can be determined by different techniques such as Gravimetry, UV-Spectrometry, Colorimetry, Flame Photometry, etc.
- (b) **In Situ Methods :** The components can be quantitatively measured by means of comparing the areas of zones developed from a spot. The linear relationship between area of spot and area of zone developed is established. The components can also be quantitatively estimated by means of Densitometry and Potentiometry. By using Densitometer, the intensity of a coloured substance is measured directly from the chromatogram and by using potentiometer, the changes in the potential of metal electrode when kept in contact with filter paper is used to estimate the components.

5.3.4 Applications

Paper Chromatography is mainly used for the separation of Amino acids. The various other applications of paper chromatography are :

1. It can be used for separation of mixtures of plant, animal origin.
2. It can be used for identification of drugs.
3. It can be used for identification and separation of impurities.
4. It can be used for analysis of metabolites of drugs in body fluids.
5. It can be used for identification of foreign substances and decomposed products in a substance.
6. It is used for analysis of mixture of sugars, vitamins, antibiotics, etc.

Chapter - 11

5.4 ION EXCHANGE CHROMATOGRAPHY

5.4.1 Introduction

Mainly, drugs and pharmaceutical substances are usually weakly or strongly acidic or basic in nature. As they contain ionisable groups, they can be separated by means of employing a technique called ion exchange chromatography. In this technique, a mixture of similarly charged ions can be separated by using an ion exchange resin which exchanges ions according to their relative affinities.

Ion exchange can be defined as a reversible reaction in which the exchange of ions takes place between different ions of similar charge.

5.4.2 Principle

The principle involved in this technique is the reversible exchange of ions between ions present in sample and ion-exchange resin.

5.4.3 Theory

Ion-exchange separations are usually carried out in columns consisting of ion-exchange resin. There are mainly two types of ion-exchangers. They are :

1. **Cation Exchangers** : These exchangers consists of negatively charged groups and attract positive charged groups. These are also called as acidic ion-exchangers as the negative charge is result of protolysis of acidic groups.
2. **Anion Exchangers** : These exchangers consists of positively charged groups and attract negatively charged groups. These are also called as basic-ion exchangers as the positive charge is the result of association of protons with basic groups.

5.4.4 Ion Exchange Materials

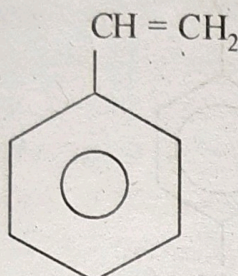
Ion exchange materials are available naturally and they also can be synthesised.

- (a) **Natural Ion-Exchangers** : The naturally obtained ion-exchangers are of different forms. They are :
 1. Cationic exchangers like zeolyte, etc.
 2. Anionic exchangers like dolomite, etc.
- (b) **Synthetic Ion-Exchangers** : The synthetic ion exchange resins are based on cross linked polystyrene prepared by copolymerisation of styrene and Divinyl benzene. Divinyl benzene acts as cross linking agent and provides structural rigidity and mechanical stability to the resin.

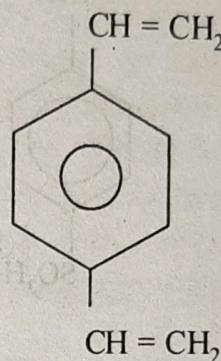
Other synthetic ion-exchange resins used are :

- Copolymers of acrylic acid derivatives and divinyl benzene.
- Cross linked dextran (sephadex)
- Cellulose fibres with introduced charged groups.

Figure :
Strong Cationic
Exchange
Resin



Styrene



Divinyl benzene

Properties of Ion-exchangers :

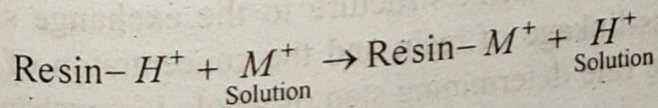
The common properties of all ion exchangers are as follows :

- They are insoluble in water and organic solvents like benzene, ether, carbontetrachloride etc.,
- They are complex in nature. Usually they are polymeric in nature.
- They possess active or counter ions that are easily exchangeable reversibly with other ions in surrounding solution without any change in the material.

Cation Exchangers :

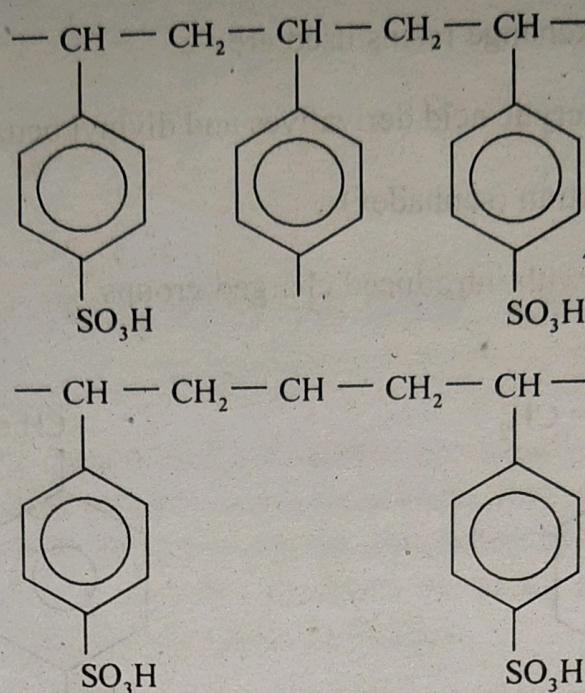
Cation exchangers are usually high molecular weight cross-linked polymers having sulphonic, carboxylic, phenolic groups etc. with equal amount of cations. In cationic exchangers hydrogen ions are mobile and are exchangeable with other cations and the anions (COO^- , SO_3^{2-}) remain attracted to resin.

When a salt solution is passed through cation exchanger, the hydrogen ions enter the solution and the cations of the salt get attached to the resin.



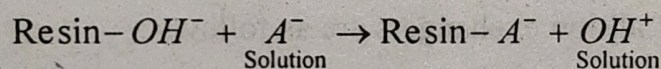
Strong cation exchange resins consists of SO_3H^+ group and weak cation exchange resins consists of groups like COOH^- , OH^- , SH^- , PO_3H_2^- .

Figure :
Strong Anionic
Exchange
Resin

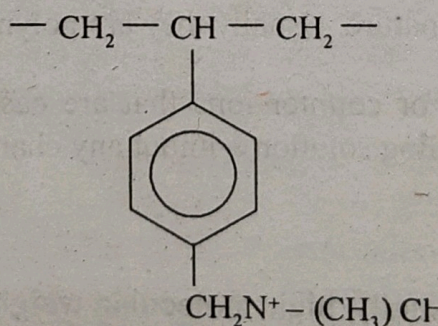


Anion Exchangers :

Anionic exchangers are usually resins consisting of amine or quarternary ammonium groups as integral part and equal amount of Cl^- , SO_4^{2-} , OH^- ions which are mobile and exchangeable.



Strong anion exchange resins consists of groups such as N^+R_3 , NR_2 etc. and weak anion exchange resins consists of NHR , NH_2 groups etc.



5.4.5 Mechanism of Ion-exchange Process

The mechanism of ion-exchange takes place in the following process :

1. The ion diffuses to the exchanger surface. This occurs very quickly in homogenous solutions.
2. The ion diffuses from the matrix structure to the exchange site which is based on the degree of cross-linkage of resin and the concentraton of solution. This step is considered to be the rate determining step of whole ion-exchange process.
3. The exchange of ions takes place at the exchange site which occurs instantaneously till equilibrium is attained.

4. The exchanged ion diffused through the exchanger to the surface.
5. The molecules are separated by altering the pH or ionic concentrations or by affinity elution in which the ion with greater affinity for the exchanger their the bound molecule is employed.

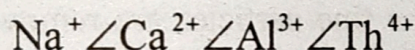
5.4.6 Factors Affecting Ion-exchange

The various factors affecting ion-exchange are :

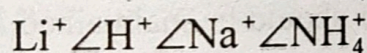
1. **Nature of Exchange Resins :** The cross-linking and swelling properties of exchange plays an important role in exchange of ions. When more cross-linking agent is present, the resins become rigid and swell less and when less cross-linking agent is present, the resin swell more. When the swelling is less, separation of ions of different sizes become difficult as they cannot pass through the pores and if the swelling is more, the separation is not effective as the pores are wide. Hence, an optimum amount of cross-linking agent should be used for effective separation.

2. **Nature of Exchanging Ions :**

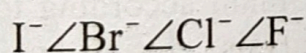
- (a) The exchange increases with increase in valency at low concentrations and ordinary temperatures.



- (b) For similar charged ions, exchange increases with decrease in the size of hydrated ion.



3. The greater polarizable ion has great exchange capacity.

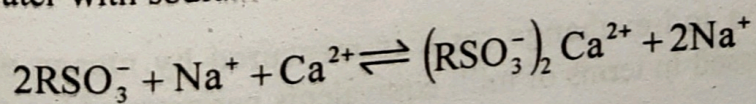


4. Polyvalent anions are generally absorbed preferentially in dilute solutions.
5. If the resin has higher positive charge and solution has lower positive charge, exchange is favoured at higher concentration and vice versa.

5.4.7 Ion-Exchange Technique

The required components can be separated generally by two types of techniques. They are :

1. **Batch Method :** This is a single step method in which the resin and the solution are taken into a single container and is mixed till equilibrium is attained. Then, the solution is filtered. This method is usually used for softening of water. The hardness of water is removed by exchange of calcium and magnesium ions present in water with sodium ions of resin.

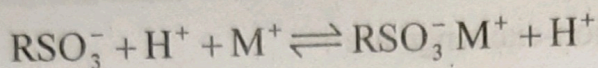


5.28 [Unit - V]

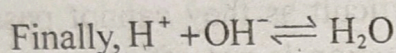
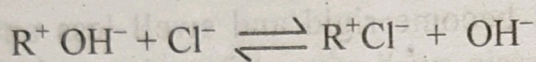
The exhausted resin is treated with 15% solution of sodium chloride to regain exchange capacity.

Deionised or demineralised water can also be prepared by this method as follows :

1. Cations of electrolyte are replaced by hydrogen ions.



2. Water is then treated with anion exchangers.



Demineralised water is prepared as above which is widely used in conductance experiments and also in biological studies.

2. **Column Method :** In this method, the resin is made into a slurry by using distilled water and is placed in column as in column Chromatography. The resin in dry form should not be used to pack the column. Care should be taken to prevent the entrapment of air bubbles. The fine particles are removed from the column by back washing in which water is runned into the column both from top and bottom also. This back washing is also useful in removal of air bubbles. The particle size of resin used should be small to provide large surface area for efficient exchange. The level of water should be maintained above the level of resin, to prevent drying of resin.

The components are separated in the column according to their selectivity from the resin by techniques like frontal analysis, elution analysis and displacement development etc.,

Regeneration of the Ion-exchange Resin :

After separation of components by using Ion-exchange resins, the resin is not useful for another separation, as it loses its exchangeable functional groups. The resin should be regenerated to its original form for effective separation. The regeneration is done by replacing the exchangeable functional groups.

Regeneration of anion-exchange resin is usually done by using strong alkalis like sodium hydroxide and potassium hydroxide and cation-exchange resin by strong acid like hydrochloric acid.

Ion-exchange Capacity :

The efficiency of a ion-exchange resin is measured by means of its ion-exchange capacity. It is expressed in terms of milli equivalents per gram of Ion-exchange resin.

The exchange capacity of cation exchange resin is calculated by determining the number of milliequivalents of sodium ions adsorbed by one gram of resin in hydrogen form. It is done by passing a solution of sodium chloride of known concentration into column containing a known weight of resin and the acid eluted from the column is collected and titrated with a standard solution of alkali. If V ml of an alkali solution of strength N in milliequivalents per litre is required to titrate the acid eluted from resin weighing W gms.

Then,

$$\text{Ion-exchange Capacity} = \frac{V \times N}{W}$$

Similarly, the exchange capacity of an anion exchanger is determined by passing sodium nitrate solution of known concentration through column and the hydroxide ions eluted from the column is titrated with standard solution of silver nitrate or acid respectively.

5.4.8 Applications

Ion-exchange chromatography has a wide range of applications. Strong ion-exchange resins are more frequently used as they have wide range of applications because they give better resolution of substances. Various applications of ion-exchange chromatography are :

1. It can be used for softening of water.
2. It is used for demineralisation of water.
3. It is used for separation of similar ions from one another in a sample.
4. It is used for separation of alkaloids and other nitrogenous substances from complex samples such as crude extracts.
5. It can be used for separation of metals from mixture.
6. It can be used for separation of organic or inorganic ions.
7. It can be used for separation of amino acids and in preparation of pure reagents.

Chapter - 12

5.5. GAS CHROMATOGRAPHY

5.5.1 Introduction

Gas chromatography is an analytical technique used for separation of thermally stable and volatile substances. In gas chromatography, the mobile phase used is gas and the stationary phase used may be a solid or liquid. It is most widely used technique now-a-days, because of its speed resolving power and sensitivity.

5.5.2 Principles

The principle involved in gas chromatography is adsorption or partition which is dependent on the stationary phase used.

If the stationary phase used is liquid, it is called as Gas Liquid Chromatography (GLC) and the separation takes place by partition. When solid is used as stationary plate, it is called as Gas Solid Chromatography (GSC) and the components are separated by adsorption. The component which is having lesser affinity towards stationary phase travels faster and of greater affinity travels slowly. Hence, the components are separated based on their affinities towards stationary phase.

5.5.3 Theory

In this technique, the sample is to be vapourised and is carried along a prepared column of suitable temperature by means of a carrier gas which acts as mobile phase. When the vapour of the sample is passed through the column, the components are separated by adsorption or partition basing on the stationary phase used.

The sample to be analysed by gas chromatography should be stable when vapourised and when passed through prepared or packed column in order to avoid decomposition of components and complex chromatograms. If unstable or non-volatile components are to be separated by means of gas chromatography by preparing their derivatives.

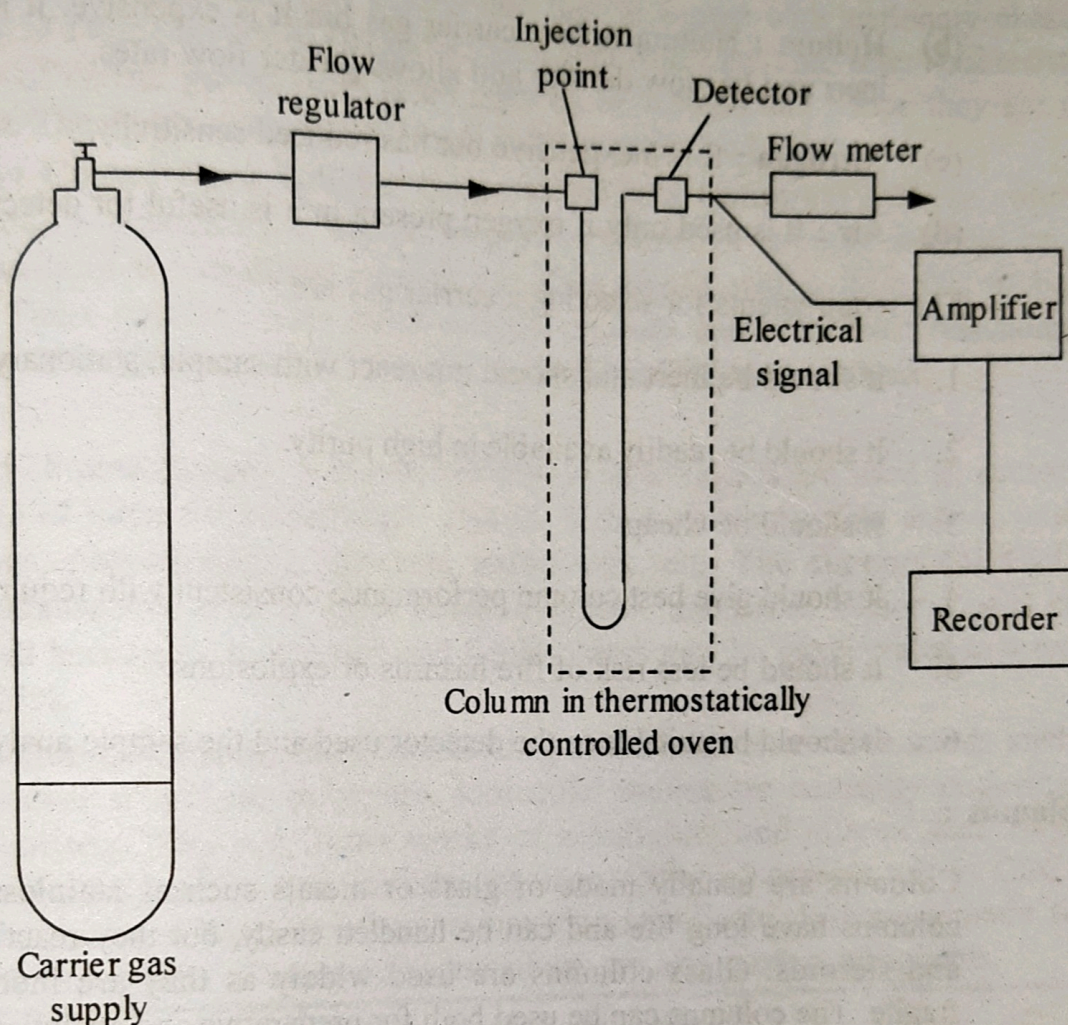
Example : Trimethyl silyl derivatives of carbohydrates.

Due to limited availability of solid adsorbents available gas solid chromatography is not widely used. In gas-liquid chromatography, the stationary liquid-phase is coated on a solid support (diatomaceous earth) or the wall of capillary tube. The stationary phase used in GLC should have low vapour pressure at the column temperature and almost non-volatile. After developing the chromatogram, the sample components are eluted and detected by means of a suitable detector.

5.5.4 Instrumentation

The apparatus used in gas chromatography is shown diagrammatically below.

Figure :
Basic Gas
Chromatography
Apparatus
(Diagrammatic)



The separation of components is carried out in a column made of glass or metal. The solid adsorbents are packed in the form of fine size graded powder and the liquid stationary phase may be coated as fine film on the column or coated over the solid support and is packed in the column.

The carrier gas is heated to the temperature of column and is passed through the injection point of a suitable pressure. When, the sample is injected, it vapourises instantaneously and is carried along with the mobile phase and passes down the column. Various components of sample are separated out depending upon their distribution coefficients. The detector which is present at the outlet of the columns produces electrical output proportional to amount of compound emerging from the column. The response produced by the detector is amplified and recorded to provide a chromatogram. The flow of gas should be controlled by measuring the gas flow at the outlet of the detector.

Various components used in gas chromatography are described below :

Carrier Gas :

Carrier gas is nothing but mobile phase in the gas chromatography. The most widely used carrier gases are hydrogen, helium, nitrogen and air.

5.32 [Unit – V]

- (a) **Hydrogen** : It is more advantageous than other gases but is dangerous to use. It has better thermal conductivity and lower density but it may react with unsaturated compounds and causes explosion.
- (b) **Helium** : Helium is best carrier gas but it is expensive. It is thermally conductive, inert and has low density and allows greater flow rates.
- (c) **Nitrogen** : It is inexpensive but has reduced sensitivity.
- (d) **Air** : It is used only if oxygen present in it is useful for detection or separation.

The requirements for selecting a carrier gas are :

1. It should be inert and should not react with sample, stationary phase of equipment.
2. It should be readily available in high purity.
3. It should be cheap.
4. It should give best column performance consistent with required speed of the analyte.
5. It should be less risk of fire hazards or explosions.
6. It should be suitable to the detector used and the sample analysed.

Columns :

Columns are usually made of glass or metals such as stainless steel. Stainless steel columns have long life and can be handled easily, but they react with some compounds and steroids. Glass columns are used widely as they are inert, but they are highly fragile. The columns can be used both for preparative and analytical purposes.

Three types of analytical columns are used in gas chromatography. They are :

- (a) **Packed Columns** : These are prepared by packing the column with granular stationary phase. The GSC columns are packed with graded adsorbent or porous polymers, whereas GLC columns are packed with liquid phase coated graded inert solid support.

The advantages of packed columns are :

- (i) There is no bleed of column and the polymers are stable upto 250°C and cause no base line drift and therefore, allows the use of highly sensitive detector.
- (ii) The adsorption of polar compounds such as water, alcohol or acid does not take place, as they are eluted rapidly as sharp symmetrical peaks.
- (iii) The recovery of sample overload is easy without tailing.
- (iv) Porus beds are mechanically strong and can be easily packed.
- (v) The separation is unique and the retention date is highly reproducible.

- (b) **Open Tubular Columns** : These are also called as Capillary or Golay columns. These are made up of long capillary tubing of 30 – 90 meters in length and have uniform and narrow internal diameter of 0.025 – 0.075 cm. They are made up of stainless steel. The inner wall of capillary tube is coated with stationary phase in the form of a thin film (0.5 to 1 μ) and uniform film. These columns offer least resistance to flow of carrier gas as there is no packing in column and hence they are more efficient. The disadvantage is that, more sample cannot be loaded.
- (c) **Support Coated open Tubular Columns (SCOT Columns)** : These columns are made by depositing a micron size porous layer of support material on the inner walls of the capillary column and then coated with a thin film of liquid phase. These columns have more sample capacity and offer least resistance to the flow of carrier gas. SCOT columns are preferred for trace analysis.

Stationary Phases :

In gas-liquid chromatography, a number of non-volatile liquids are used as stationary phases. Some of them are polyethylene glycols, high molecular weight esters, amides, hydrocarbons, polysiloxanes, apiezon, carbo wax, etc. The support for GLC is generally diatomaceous earth or glass beads of suitable mesh size. Diatomaceous earth is mostly used because of its porosity and liquid phases may be incorporated to an extent of 0.5% to 25%.

In gas-solid chromatography, the columns are packed with interactive solids such as molecular sieves or porous polymers. Molecular sieves are naturally occurring or synthetic zeolites. They are frame works of aluminium and silicon with oxygen with interconnected cavities and uniform openings. Other adsorbents used in GSC are sodium and calcium analogues of potassium aluminosilicate. Polymers made from acrylic esters, copolymers of aromatic hydrocarbons are also used as adsorbents.

Table :
Preferred
Stationary
Phases for
Gas-liquid
Chromatography

Stationary phase	Solvent for stationary phase	Temperature maximum ($^{\circ}\text{C}$)	Uses
Apiezon L 1/2%	Light petroleum	245	Hydrocarbons, steroids and esters
Carbowax 1000 20%	Chloroform	150	Alcohols, chloroform, camphor, essential oils
Carbowax 20M 10% with KOH 5%	Methanol	225	Volatile bases, e.g., amphetamines
Diethylene glycol succinate (DEGS) 10%	Chloroform	190	Methyl esters of fatty acids, nitriles, essential oils
SE-30 2.5%	Chloroform	350	Hydrocarbons, methyl derivatives of barbiturates, general purpose
OV-17 3%	Chloroform	375	General purpose

Sample Injection System :

Liquid samples are injected by a hypodermic syringe through a self-sealing silicone rubber septum into a chamber, where it is heated at fixed temperature sufficiently to convert into a plug of vapour and is introduced in to the following carrier gas stream by means of a valve. Solid samples must be dissolved in volatile solvents or may be introduced directly, if they can be liquified. Gas samples require special valves for introduction into carrier gas stream.

Head-space sampling is a very effective technique for analysis of complex volatile components or viscous mixtures containing high proportion of non-volatile material.

In this technique, the sample is heated in a closed container such as glass vial with aluminium cap and PTFE coated silicone rubber septum. The temperature and heating time are selected to produce an equilibrium of the volatile components between the sample and vapour space above the sample. The head space vapour is then injected into column. It not only prevents contamination of the injection port and column with non-volatile components, but also increases sensitivity of the analysis at lower concentrations.

Detectors :

The detector located at the exit of the separation column, senses the presence of components as they leave the column. When a carrier gas alone is passed through the column, no ~~signal~~ is produced. When a component is eluted, it is detected and a signal proportional to its concentration is produced. The detector volume must be small to prevent the remixing of components eluted from the column.

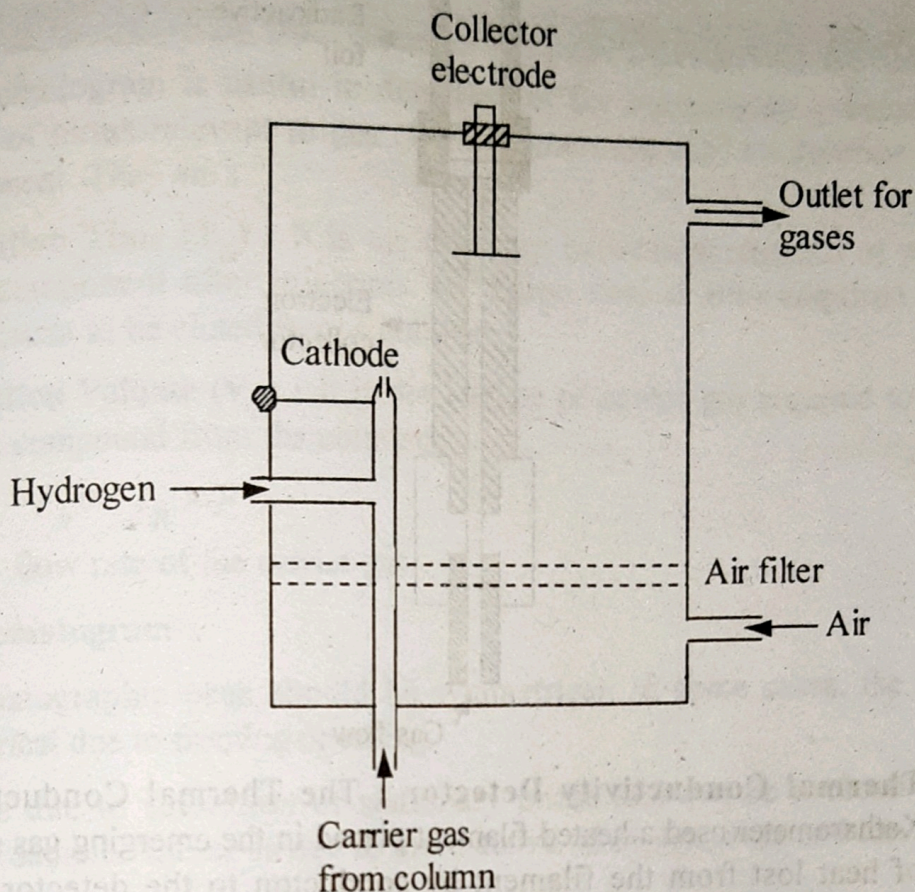
The electrical signal produced in the detector is amplified and recorded on a strip chart or conversed to a digital signal and sent to a microcomputer system.

Different types of detectors available for gas chromatography are :

1. **Flame Ionisation Detector :** In flame ionisation detector, the effluent from the column is mixed with hydrogen and is passed through a jet where it is buried in presence of external air. Twin electrodes are positioned 0.5 to 1.0 cm above the tip of the flame. A potential of about 400 V is applied across the two electrodes, which lowers the resistance between electrodes and allows the flow of current. The ions and free electrons generated in a pure hydrogen flame produces current. When an ionisable material enters the flame, the current increases markedly. The produced current flows through an external resistor, sensed as voltage drop, amplified and finally sent to an output device or recorder. The base line is to be adjusted by passing pure carrier gas.

The detector is insensitive to water vapour of air in carrier gas and the background current is low, so that small quantities can be measured with amplification. The main draw back of the detector is that, the sample is destroyed.

Figure:
Flame
Ionisation
Detector (FID)



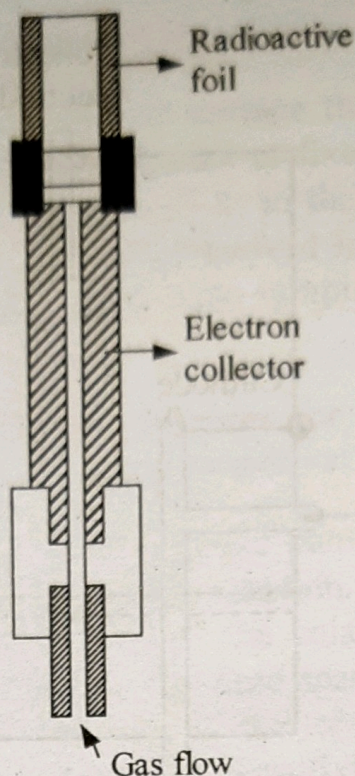
2. **Electron Capture Detector :** In electron capture detector, the effluent from the column is passed between two electrodes. One of the electrode consists of radio isotope that emits high energy electrons (beta particles) as it decays. These electrons bombard with the carrier gas resulting in formation of positive ions, radicals and thermal electrons by a series of collisions. This process is very rapid. The application of a potential difference to the electron capture cell allows the collection of electrons that constitute detector current, or base line signal when only carrier gas is passed.

The electron absorbing components in the carrier gas stream react with thermal electrons to produce negative ions of large mass. The rate of recombination between negative and positive ions is many times faster than between thermal electrons and positive ions. The decrease in detector current due to removal of thermal electrons by recombination in presence of electron capturing compounds is the basis for quantitative estimation.

The linearity depends on cell design, composition of carrier gas and method of applying potential difference to collect thermal electrons. The carrier gas used in this detector depends upon electron affinity of the compounds. For compounds with high electron affinity argon is used as carrier gas and for those of lower affinity, nitrogen, hydrogen or helium is used.

The advantage of this detector is that, it is highly sensitive and can be useful for detecting nanogram quantities also. But, the drawback is that, it can only be used for compounds with electron affinity.

Figure :
Electron
Capture
Detector

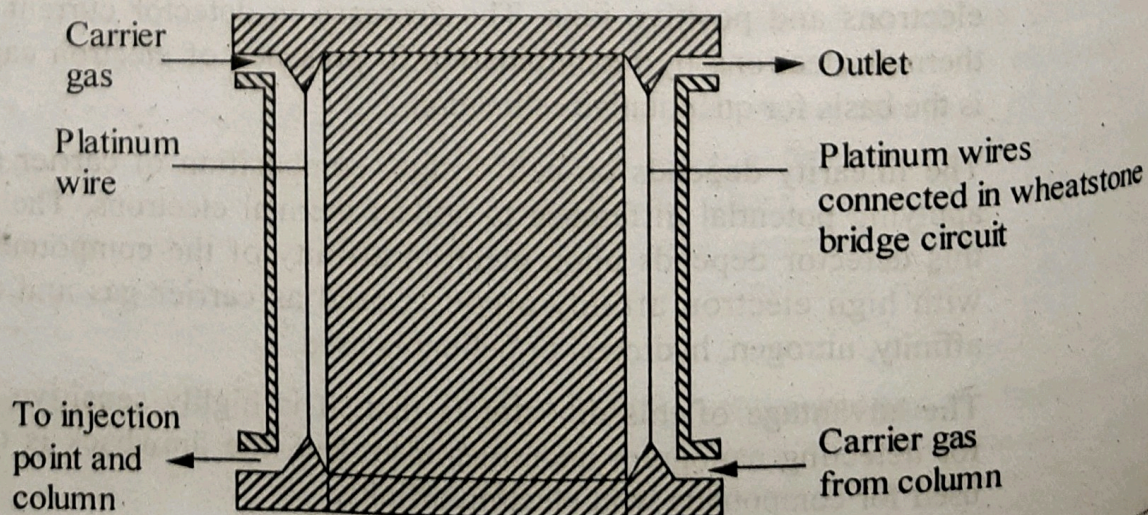


3. **Thermal Conductivity Detector :** The Thermal Conductivity Detector or Katharometer used a heated filament placed in the emerging gas stream. The amount of heat lost from the filament by conduction to the detector walls depends on the thermal conductivity of the effluent gas stream. The filament is heated to a constant temperature regulated by DC current supply. The heat lost from filament to walls of detector is constant when the carrier gas flows.

The standard detector consists of four identical filaments making the arms of a wheat stone bridge. The effluent is passed through one pair of filament and the second pair is placed in gas stream ahead of injection point. Any imbalance produced between the filament pairs is recorded. The base line is drawn by passing carrier gas through both pair of filaments.

Hydrogen and helium have high thermal conductivities and can be used as carrier gas. It is applicable to many compounds and the linearity is also good, but it is less sensitive and is affected by fluctuations in temperature and flow rate.

Figure :
Thermal
Conductivity
Detector
Katharometer



5.5.5 Gas Chromatogram

A gas chromatogram is useful in detection of the components present in a sample. A number of terms relevant to gas chromatogram are used to describe the behaviour of a component. They are :

1. **Retention Time (T_R)** : It is the time gap between emergence of peak maximum of a component after injection. Retention time is time required for 50% of a component to be eluted from a column.
2. **Retention Volume (V_R)** : It is the volume of carrier gas required to elute one half of the compound from the column.

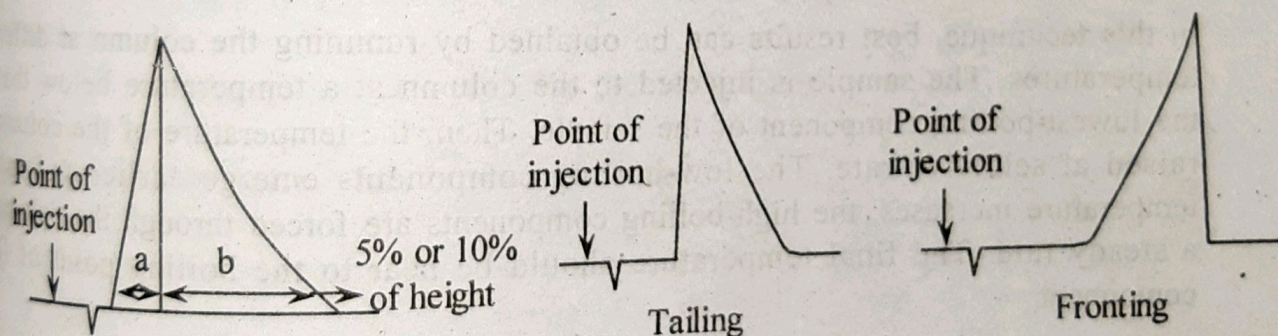
$$V_R = T_R \times F$$

where, F is flow rate of the carrier gas.

Asymmetry in Chromatogram :

The chromatographic peak should be symmetrical. In some cases, the peak may not be symmetrical due to fronting or tailing.

Fronting is due to saturation of stationary phase and can be avoided by using less quantity of sample. Tailing is due to more active adsorption sites and can be eliminated by pretreatment.

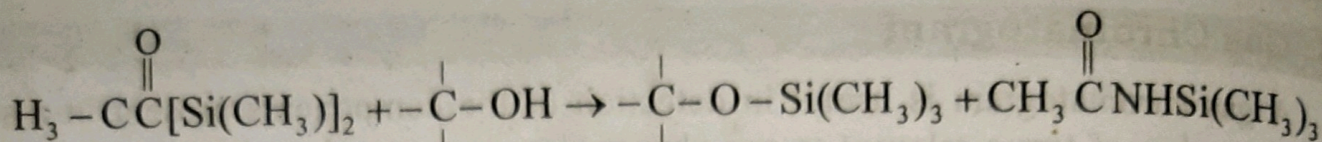


5.5.6 Derivatisation Technique

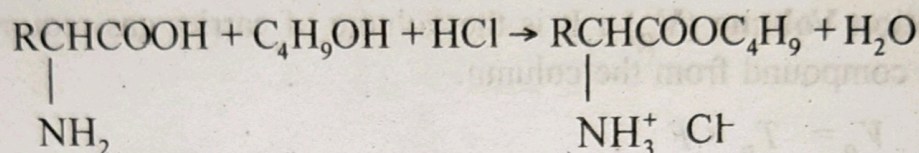
Some classes of drugs such as barbiturates, phenols and basic nitrogen compounds exhibit non-linearity and tailing of peaks even when examined under different conditions. This problem can be solved by derivatisation technique. This technique is used to

1. Improve the thermal stability of compounds, particularly compounds containing polar functional groups.
2. Change the separation properties of compounds by adjusting their volatility, and
3. Introduce a tag to detect molecule easily.

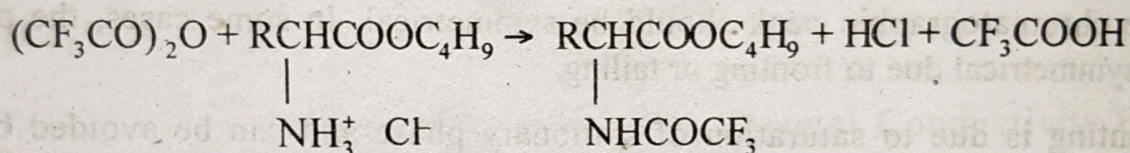
Silylating agents such as NO, O - bis (trimethyl silyl) acetamide or trifluoro analogue. N, O-bis (trimethyl silyl) trifluoro acetamide convert the active hydrogen in polar groups such as $-\text{OH}$, $-\text{COOH}$, $-\text{NH}_2$ and $-\text{SH}$ to $-\text{O}-\text{Si}(\text{CH}_3)_3$ group. A typical silylation reaction with alcohol is as below :



Amino acids are estimated both qualitatively and quantitatively by esterification. Amino acids are converted to butylester hydrochlorides by treatment with 1-butanol and hydrogen chloride.



The butyl esters are then converted to volatile N-trifluoro acetyl butyl esters.



5.5.7 Programmed Temperature Gas Chromatography

In this technique, best results can be obtained by running the column at different temperatures. The sample is injected to the column at a temperature below that of the lowest-boiling component of the sample. Then, the temperature of the column is raised at selective rate. The low-boiling components emerge earlier and as the temperature increases, the high-boiling components are forced through the column at a steady rate. The final temperature should be near to the boiling point of final component.

This technique has extended the use of gas chromatography to the analysis of mixtures containing components with a wide range of molecular weights. When, the column temperature is increased linearly, the members of homologous series are eluted at equally spaced intervals. The column should be leaved uniformly during the analysis, otherwise, the sample components will be diffused and the efficiency of the column is decreased.

5.5.8 Applications

Gas chromatography can be used for qualitative and quantitative analysis of liquids, gases and vapours and particularly organic compounds.

In qualitative analysis, the individual components can be identified by comparing the retention times or retention volumes of unknown with standard or by collecting the individual components after separation and identifying them by other methods.

In quantitative analysis, the area under a single component elution peak is proportional to the quantity of the detected component. Various methods of quantitative analysis are :

1. **Response Factor** : Since no detector responds to the same degree for equal mass of different components, a response factor is determined for each pure component under a set of conditions and is used for quantitative evaluation of an unknown mixture under same set of conditions.
2. **Graphs and Calibration** : Instead of calculating response factor, a standard graph is plotted by concentration against peak height under a set of conditions by using pure components and the concentration of the unknown can be determined.
3. **Internal Standard** : In this method, a known weight of an internal standard is added to a series of samples of known weights. The internal standard should be similar structure to the sample component and the retention values must be close to each other. A linear calibration graph is prepared by plotting the ratio of peak areas of samples to area of the unknown against weight ratio of the added internal standards. The weight of the unknown can be determined from graph.

Gas chromatography can also be used :

1. To isolate and identify metabolites in body fluids.
2. In multicomponent analysis of mixture of drugs.
3. To determine purity of the compounds.

Gas chromatography can be interfaced with Mass Spectroscopy and Infrared Spectroscopy for getting best results of analysis of components in less time.

5.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

5.6.1 Introduction

HPLC was developed in early 1970's from a knowledge of already established chromatographic techniques and the advances made in the chromatographic equipment and packing materials. Originally, HPLC was referred to as High Pressure Liquid Chromatography because, high pressures were used to increase the efficiency of separation, but, now-a-days, the term High Performance Liquid Chromatography is preferred as it describes the characteristics of the chromatography.

5.6.2 Theory

Earlier, the separation of components by liquid chromatography was dependent on gravity and it took a lot of time to separate components. This problem was overcome by HPLC in which the separation is assisted by means of increasing the force of mobile phase at higher rate. HPLC is not limited to sample volatility or thermal stability. It is able to separate, macromolecules and ionic species, labile natural products, polymeric materials and a wide variety of other high molecular weight polyfunctional groups. The separated components in HPLC can be easily collected and can be estimated both qualitatively and quantitatively.

The advantages of HPLC over other chromatographic techniques are improved resolution, faster separation times and increased accuracy, precision and sensitivity with which the separated substances may be estimated.

5.6.3 Principle

As the resolving power of a column increases with increase in column length as number of theoretical plates per unit length increases, it may sometimes lead to broadening of peak due to increase in length of column. The number of theoretical plates can be increased by decreasing the particle size of stationary phase. As the particle size decreases, the resistance to flow of mobile phase increases. This can be overcome by increasing the flow rates by means of employing pressure pumping systems.

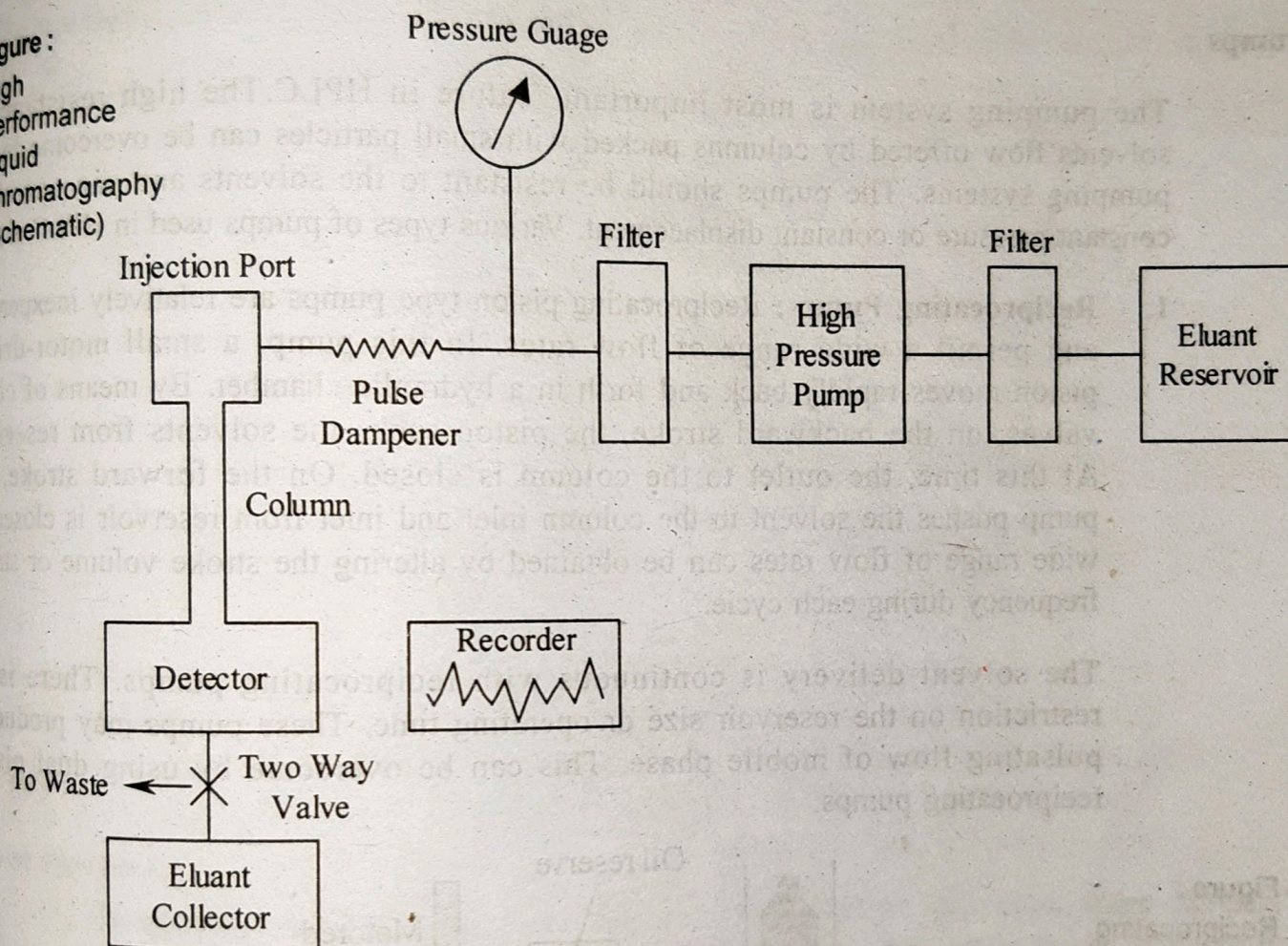
Hence, by decreasing the particle size and employing pressure pumping system to increase the flow rates of mobile phase increases the efficiency of the column. This is the basic principle involved in HPLC.

5.6.4 Instrumentation

The general instrumentation employed in HPLC is illustrated below :

The mobile phase from the reservoir is pumped into the column by means of a pumping system without any pulses. The sample is injected into the column at injection port. The separation of components takes place in the column and as the effluent flows out of the column it is detected and recorded.

Figure :
High
Performance
Liquid
Chromatography
(Schematic)



Mobile-Phase Delivery System :

The mobile phase must be delivered to the column at high pressure over a wide range of flow rates. To use the delivery systems for a wide variety of compounds, the components of the system must be made of materials which are chemically resistant to mobile phase.

All the solvents used as mobile phase in HPLC must be specially purified and should not contain any traces of impurities as it affect the column and interfere with the detection system.

Solvent Treatment Systems :

Solvents of purified form are commercially available for HPLC. But even with these solvents, a 1-5 μm microfilter is introduced into system prior to the pump. All solvents must be degassed before introduction into pump as it can alter the resolution of column and interfere with the monitoring of the column effluent. Degassing (removal of dissolved gases in solvent) is done in many ways like :

1. Warming the solvents.
2. By stirring vigorously with a magnetic stirrer.
3. By subjecting to vacuum filtration.
4. By ultrasonication.
5. By bubbling helium gas through the solvent reservoir.

The solvents are delivered at high pressures into the column by means of pumps.

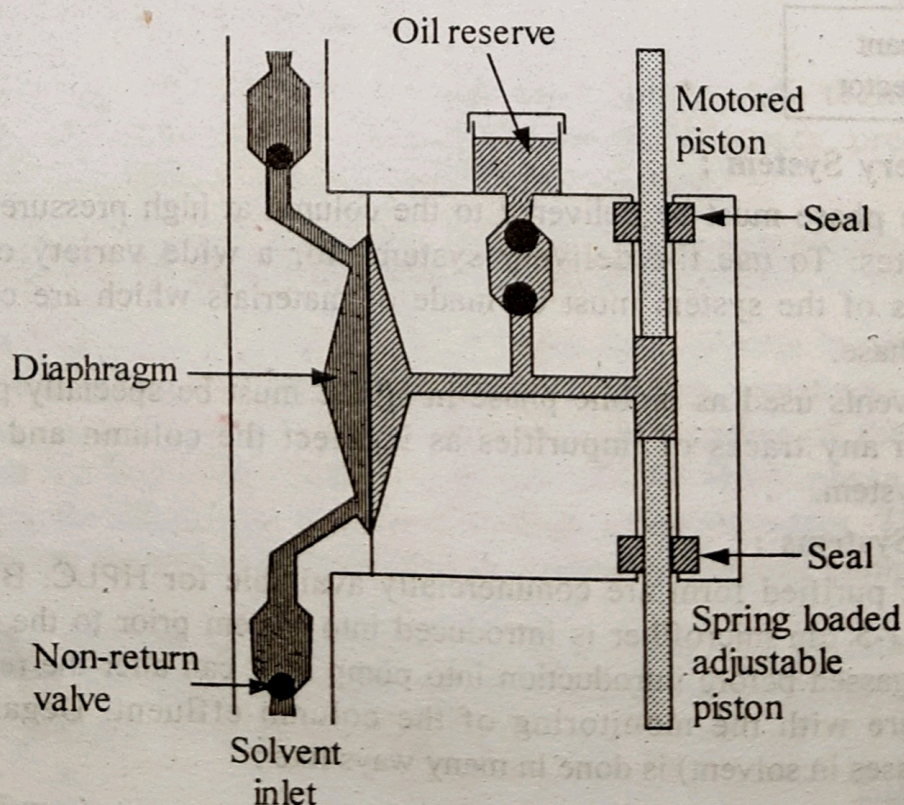
Pumps :

The pumping system is most important feature in HPLC. The high resistance to solvents flow offered by columns packed with small particles can be overcome by the pumping systems. The pumps should be resistant to the solvents and can operate at constant pressure or constant displacement. Various types of pumps used in HPLC are :

1. **Reciprocating Pump :** Reciprocating piston type pumps are relatively inexpensive and permit a wide range of flow rates. In this pump, a small motor-driven piston moves rapidly back and forth in a hydraulic chamber. By means of check valves, on the backward stroke, the piston sucks the solvents from reservoir. At this time, the outlet to the column is closed. On the forward stroke, the pump pushes the solvent to the column inlet and inlet from reservoir is closed. A wide range of flow rates can be obtained by altering the stroke volume or stroke frequency during each cycle.

The solvent delivery is continuous with reciprocating pumps. There is no restriction on the reservoir size or operating time. These pumps may produce a pulsating flow of mobile phase. This can be overcome by using dual piston reciprocating pumps.

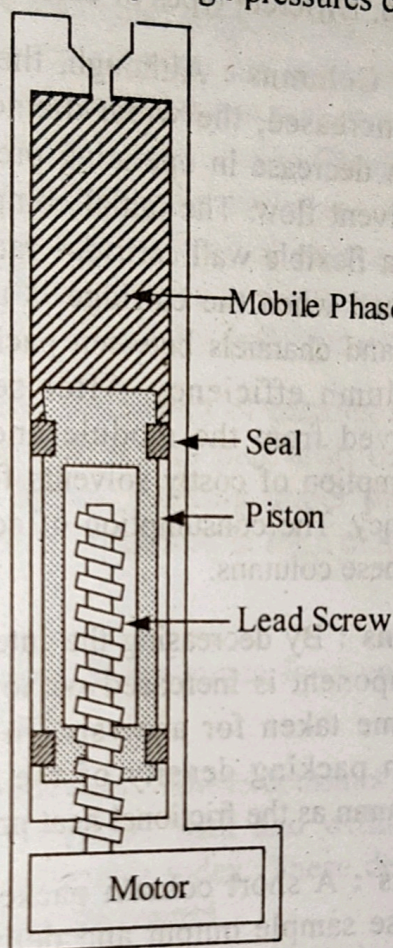
Figure :
Reciprocating Pump



Displacement Pumps :

These pumps work through positive solvent displacement by a piston mechanically driven at a constant rate. The piston is driven by a screw-feed drive through a gear box the voltage of the motor. The rate of flow of mobile phase is controlled by changing the voltage of the motor. The solvent chamber has only a finite capacity and must be refilled. By employing these pumps high-pressures can be achieved without pulses.

Figure :
Syringe-type
Pump

**Pulse Pumpers :**

The detectors used in HPLC are sensitive to variations in flow rates. Pulse dampers are used to dampen the pulses and achieve a constant flow rate. Pulse dampers use a compressible fluid separated from mobile phase by a flexible inert diaphragm. In this type, the compressed fluid expands when the pump piston retracts and maintain system pressure and constant solvent flow.

Injection of Sample :

Sample is injected into the pressurized column as a narrow plug. The injection system should not contain any void volume.

Microsampling injectors are the mostly used sampling devices. The calibrated sample loop is filled with sample solution by means of an ordinary syringe. A rotation of valve rotor places the sample filled loop into the mobile-phase stream without interruption of flow.

Stopped-Flow Injection :

In stopped-flow injection, the flow of mobile phase is stopped and the sample is injected and the pump is turned on to allow the flow of a mobile phase.

Columns :

Columns are constructed of gas-lined metal tubing or high quality stainless steel, polished internally to a mirror finish. Straight columns are preferred and are operated in vertical position. Standard columns are 4-5 mm in diameter and 10 - 30 mm in length and provide good compromise between efficacy, sample capacity and the amount of packing and solvent required. Different types of columns used are :

- (a) **Radial Compression Columns :** Although, the response is decreased when the column diameter is increased, the wider-diameter radial compression columns have some benefits. A decrease in operating pressure allows to decrease analysis time by increasing solvent flow. The radial compression module applies hydraulic pressure to compress a flexible wall cartridge radially held within a plastic holder. Separation is performed when the cartridge is under compression. Compression diminishes the voids and channels between packing particles and cartridge walls and increase the column efficiency. After separation, the cartridge can be decompressed, removed from the module and can be reused without losing efficiency. The consumption of costly solvents for column purging can be reused without losing efficiency. The consumption of costly solvents for column purging can be eliminated by these columns.
- (b) **Narrow-Bore Columns :** By decreasing the internal diameter of the column, the signal of sample component is increased without change in column velocity of mobile phase and time taken for analysis. In narrow-bore columns, there is better homogeneity in packing density of the column and small temperature gradient across the column as the frictional heat produced is dissipated better.
- (c) **Short, Fast Columns :** A short column packed with 3- μm particles can save solvent costs, increase sample output and deliver higher sensitivity rates than conventional length columns. These columns are used when the analysis is to be done in a short time as in quality control work.
- (d) **Guard Columns and In-line Filters :** The life of analytical columns can be increased by insertion of guard columns a head of analytical column. These act as both chemical and physical filters. These columns are relatively short and contain stationary phase similar to analytical column. They protect the analytical column from particular contamination that comes from mobile phase or from degrading sample injection valves. These extend the life time of expensive columns by preventing the contamination of upper layers of analytical column. These can be repacked, replaced or reconditioned.

Column Packing :

Three forms of packing material are generally used for HPLC basing on their rigid solid structure. They are :

1. Microporous supports where microporous ramify through the particles of about 5 - 10 μm in diameter.

2. Pellicular supports where porous particles are coated onto an inert solid core such as glass bead.
3. Bonded phases where the stationary phase is chemically bonded onto an inert support.

For adsorption chromatography, silica or alumina in microporous forms with a range of particle size are used. Pellicular supports have high efficiency but low sample capacity and therefore, microporous supports are preferred. Packing material used in HPLC is characterised by regular spherical shape to pack most efficiently and for good flow properties. In partition systems, microporous and pellicular supports are used for coating the stationary phase. In the process, the stationary phase coated may be gradually washed off. To overcome this problem, bonded phases can be used as supporting material. Silicone polymer bonded phases are generally used as they are not eluted by developing solvent and are chemically, hydrolytically and thermally stable.

For packing the column, suspension of packing material is made and pumped at high pressure onto a column with porous plug at its outlet. When hard gels are packed, they are allowed to swell before packing under pressure. Soft gels cannot be packed under pressure. They are to be packed in form of slurry under gravitational force only.

Detectors :

The separated components in the eluate form can be detected by means of their bulk property or solute property.

- (a) **Bulk Property Detectors :** These detectors compare an overall change in physical property of mobile phase with and without an eluting solute. The physical property used is refractive index. These detectors are relatively insensitive and require good temperature control.
- (b) **Solute Property Detectors :** These detectors respond to a physical property of the solute that is not exhibited by the mobile phase. Properties such as absorbance, fluorescence and electrochemical property are used. These detectors are highly sensitive even in nanograms of sample or less. Precolumn and postcolumn derivatization expand their applicability.

UV Detectors :

These detectors operate in the ultraviolet region spectrum. Commercial spectrophotometers serve as excellent detectors and the only modification required is the installation of small volume flow cell in the cell compartment. Detector cell volumes on the order of 8 μ l per centimeter of optical path length are acceptable. A quartz collimating lens focusses the radiation on the sample and reference cells. Energy that passes through sample and reference cells are compared by electronic signal processing circuits to produce output which is linear with solute concentration.

Cell optics must prevent false absorbance arising due to failure of recognising sample peaks and pseudo peaks due to refraction effects and wall reflectance. The mobile phase chosen should absorb radiations only weakly or not at all.

UV detectors of Various types used are :

- (a) **Fixed-Wavelength Detector** : A medium-pressure mercury lamp which produces maximum light intensity at one or discrete wavelengths produced is used and the wavelength of 254 nm is used by using filter.
- (b) **Variable-Wavelength Detector** : It is a relatively wide-bandpass UV-Visible spectrophotometer coupled to chromatographic system. It offers selection between UV and visible wavelengths. To obtain a complete spectrum, the flow of eluent is stopped to trap the component of interest in the detector cell and the UV-Visible spectral region is scanned.
- (c) **Scanning-Wavelength Detector** : Diode array detectors are used to obtain a real-time spectrum. The diode arrays work in parallel, simultaneously monitoring all wavelengths.
- (d) **Simultaneous Monitoring** : Instead of scanning the complete spectrum, some instruments can be configured to monitor signals at wavelength intervals permitting multicomponent detection in complex samples.

Fluorimetric Detectors :

For substances that are fluorescent, these detectors are more sensitive than photometric detectors. The fluorescence is collected at right angles to the excitation beam. A concave mirror is placed around the flow cell to collect fluorescence. By using microflow cells, scattered radiation from excitation source is selectively removed with cutoff or band pass filters, components are detected by photomultiplier tube.

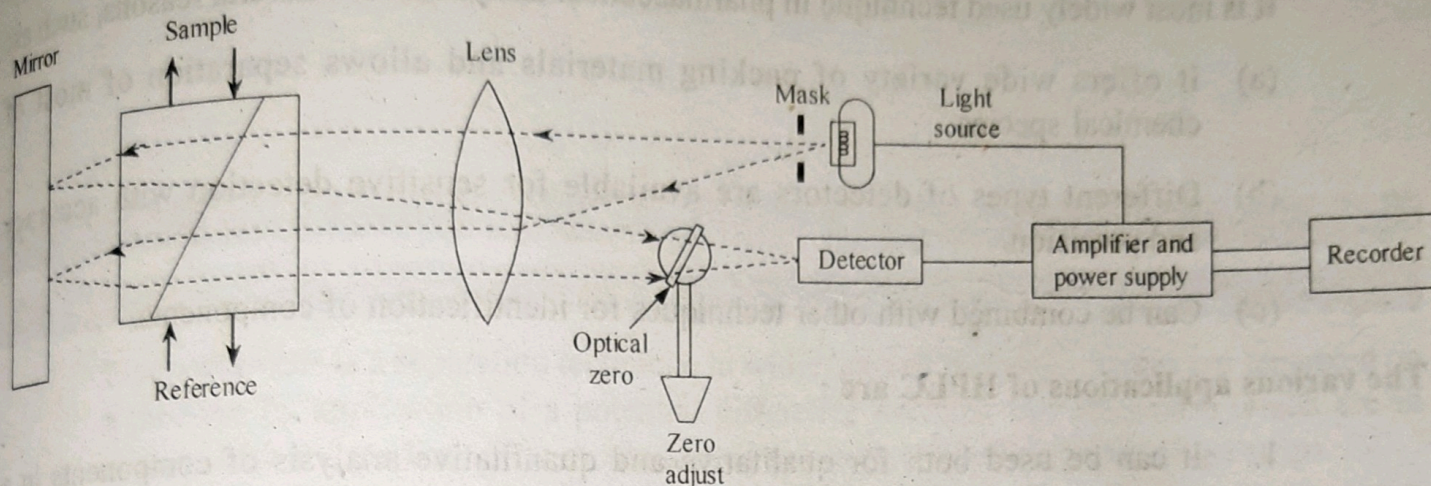
Refractive Index Detectors :

A differential refractometer which responds to the change in bulk property of the refractive index between the mobile phase and the common eluent used in these detectors. These detectors operate on one of the two principles.

- (a) In deflection type, the deflection of a beam of monochromatic light by a double prism is measured. Eluent is passed through one half of the prism and the mobile phase passes through other half. An optical mask confines a beam of light from an incandescent tungsten lamp to the face of reference and standard compartments. The beam is collimated by a lens is passed through the compartments and is reflected back by the mirror through the compartments again. The beam is focussed on a beam splitter before passing into twin photodetectors. The reference and sample compartments are separated by a diagonal glass divider.

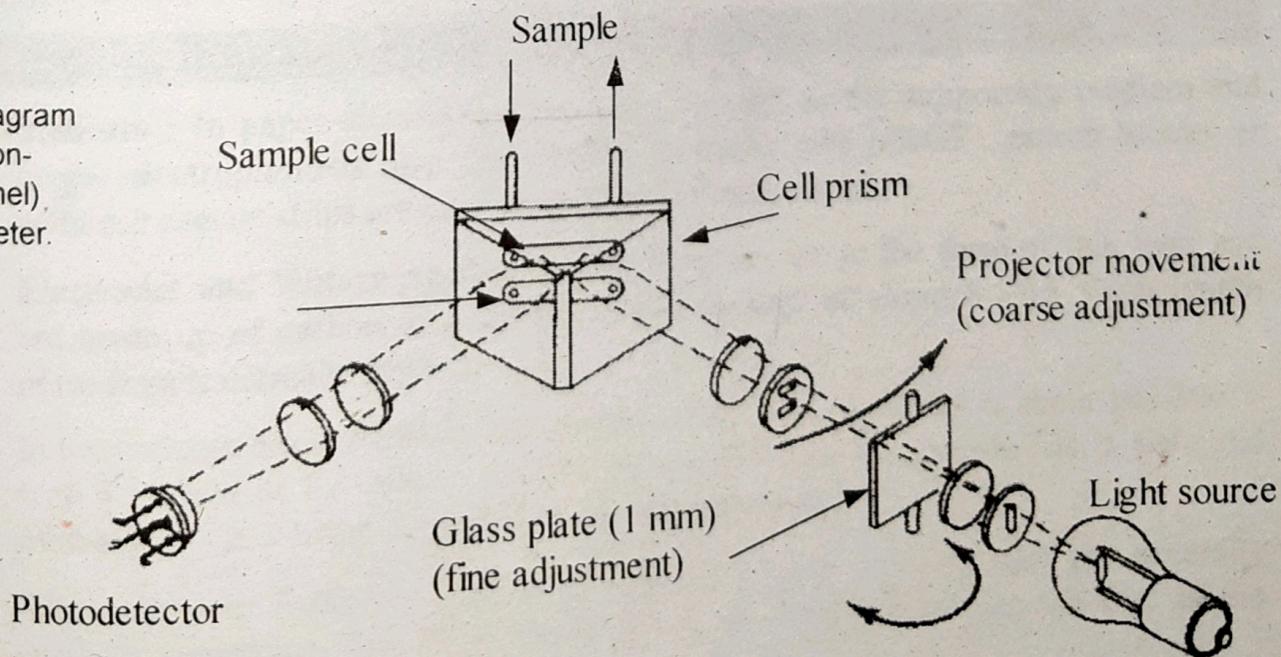
If the refractive index of the mobile phase changes due to presence of a solute, the beam from the sample compartment is slightly deflected. As the beam changes location on the detector, a signal is generated which is proportional to the concentration of solute. A zero output signal is produced by passing mobile phase in both prism compartments. These detectors have wide range of linearity and one cell covers entire refractive index range.

Figure : Deflection-type Refractometer



- (b) In reflection-type refractometer, the change in percentage of reflected light at a glass liquid interface is measured as the refractive index of the liquid changes. In the optical path, two collimated beams from the projector illuminate the reference and sample cells. The cells are made with a teflon gasket, which is clamped between the cell prism and a stainless steel reflecting backplate. As the light beam is transmitted through the cell interfaces, it passes through the flowing liquid and impinges on the surface of reflecting back plate. This diffuse, reflected light appears as two spots of light that are imaged by lenses onto dual photodetectors. As the ratio of reflected light to transmitted light is function of refractive index of two liquids, the illumination of the cell back plate is direct measure of the refractive index of the liquid in each chamber. This has a limited range. Two different prisms must be used to cover the useful refractive index.

Figure :
Optical Diagram
of Reflection-
type (Fresnel)
Refractometer.



Although, the sensitivity of the detectors is much less when compared to solute property detectors, they are useful for detection of substances which do not exhibit other properties.

5.6.5 Applications

It is most widely used technique in pharmaceutical analysis due to several reasons, such as:

- (a) It offers wide variety of packing materials and allows separation of most of chemical species.
- (b) Different types of detectors are available for sensitive detection with accuracy and precision.
- (c) Can be combined with other techniques for identification of components.

The various applications of HPLC are :

1. It can be used both for qualitative and quantitative analysis of components in a sample.
2. It can be used for checking purity of components and presence of impurities.
3. It can be used for isolation and identification of mixture of components of both natural and synthetic origin.
4. It can be used in Biopharmaceutical, Pharmacokinetic and stability studies.
5. It can be used for analysis of drugs and metabolites present in body fluids.
6. It can be used to separate closely related compounds efficiently.

5.7 ELECTROPHORESIS

5.7.1 Introduction

Electrophoresis is a separation technique in which ions of different charges are separated on a medium by application of a potential difference between two electrodes which are in contact with the medium. The medium used in electrophoresis is paper or gel strips.

5.7.2 Principle of Separation

A mixture of ions or ionisable substances is applied on the centre of the medium which is previously immersed in a buffer of known ionic strength. The medium is placed across two trays filled with buffer into which electrodes are immersed. When voltage is applied across the two electrodes, the ions or ionisable substances migrate towards the electrode based on their charge and other factors. Neutral or non-ionisable substances do not migrate. Anionic and Cationic substances move towards anode and cathode respectively. Finally anionic, cationic, non-ionic or zwitter ionic substances are separated. The spots/bands formed by migration can be detected by using visualising agents or by other methods. This technique can be used for both qualitative and quantitative analysis.

5.7.3 Equipment for Paper and Gel Electrophoresis

- Medium :** In paper electrophoresis, paper is used as the supporting medium and in gel electrophoresis agar gels, polyacrylamide gels (PAGE), starch blocks or cellulose acetate strips are used as medium for separation.
- Electrodes and Voltage Applied :** The electrodes are in the form of thin wire and are made up of carbon or platinum. A DC voltage of about 8 - 15 V/cm length of medium is normally applied.

In low-voltage electrophoresis, the voltage across two electrodes is about 100-300 V with a current of 0.4 mAmp per cm. In high voltage electrophoresis, a potential of about 50 - 215 V/cm is applied across the electrodes.

- Electrolytes or Buffers :** Buffers of different P^H and ionic strength are generally used for separation. The P^H of the buffer to be used depends on the type of the compounds to be separated.

The ionic strength of the buffer used affects the migration velocity of compounds. Migration of compounds is inversely proportional to ionic strength. A low ionic strength, migration is faster, but the bands appear diffused. Usually ionic strengths of 0.05 - 0.5 is used in most separations.

Some of the buffers used in electrophoresis are :

- (i) Barbitone buffer (veronal buffer) (0.07 mol/litre, PH 8.6, Ionic strength 0.05).
- (ii) Tris-acetate buffer (0.07 mol./litre, PH - 7.6).
- (iii) Citrate buffer (0.07 mol/litre, PH 3.0 or 6.8).

5.7.4 Factors Governing Electrophoresis

The following are the factors affecting migration of ions in electrophoresis.

- (a) **Charge of Ions** : The mobility of ions is directly proportional to charge of the ions.
- (b) **Size of Ions** : The mobility of ions is inversely proportional to the size of the ions.
- (c) **Viscosity of Medium** : The mobility of ions is inversely proportional to the viscosity of the medium. As the viscosity depends on temperature, the mobility of ions also depends on temperature of the medium.
- (d) **Voltage Applied** : The separation is faster when higher voltages are applied and sharp bands can also be obtained. But, steps should be taken to prevent evaporation of solvent/buffer due to heat generated by high voltage.
- (e) **P^H of buffer and Ionic Strength** : Migration of ions is inversely proportional to ionic strength.

5.7.5 Electrophoretic Methods

Electrophoretic methods are mainly divided basing on whether the separation is carried out in the absence or presence of a supporting or stabilizing medium. The separation carried out in the absence of a supporting medium is called as free solution medium and in the presence of a stabilizing medium such as paper is called as Electrochromatography or zone electrophoresis. The technique based on electrophoresis in a stabilizing medium is also known as electromigration or ionophoresis.

Free Solution Electrophoresis :

In free solution electrophoresis, the samples are usually injected into the bottom of the U tube filled with unstabilized buffer solution by means of a capillary tube. The electric field is applied at the electrodes present at the ends of the tube. The components are separated according to the difference in mobilities. The mobility of particle is approximately proportional to its charge to mass ratio.

Moving Boundary Electrophoresis :

In free solution electrophoresis, the volume of the sample can be large, so long as the boundary, or leading edge, between the sample and the buffer is kept sharply defined. When such a sample is subjected to electrophoresis, the boundary of each component retains its identity as the components move at rates proportional to their mobilities. This results in the overlap of components to make sharply defined step like contribution to the total solute concentration. The components in the tube are visualized as a separate cylinder of solute migrating in an independent manner. As electrophoresis proceeds, the cylinders move at different rates. Each fraction of the component remains as a well defined cylinder and sharp changes in concentration occur at the points where

successive fraction overlap. The boundaries of the fractions can be detected by means of scanning with refractometer as the refractive index of a solution is directly proportional to the concentration change in the refractive index. This type of electrophoresis is hence called as moving boundary electrophoresis because measurements depend upon maintaining sharply defined boundaries while the fractions migrate.

Density Gradient Electrophoresis :

In this method, the fractionation is carried out in a vertical cylinder filled with electrolyte containing an electrophoretically inert solute such as dextrose, sucrose or dextran. The density of the solution is graded such that the specific gravity decreases towards the top of the cylinder. The sample is layered on the solution and the components are forced to migrate against density gradient. This results in reduction of convection currents and complete separation of sample components.

Zone Electrophoresis or Electrochromatography :

In this method, a stabilizing medium such as paper is used to separate components of a sample without distortion of the electrophoretic patterns by convection currents. The separations depend mainly upon the properties of the medium and may result primarily from the electrophoretic effect or from a combination of electrophoresis and adsorption, ion exchange or other distribution equilibria.

5.7.6 Types of Electrophoresis

Electrophoresis can be divided into low voltage and high voltage electrophoresis based on the voltage applied between electrodes.

The other types of electrophoresis based on design of instrument are :

- Horizontal Electrophoresis.
- Vertical Electrophoresis.
- Continuous Electrophoresis.

Figure : Paper Electrophoresis (Horizontal Type)

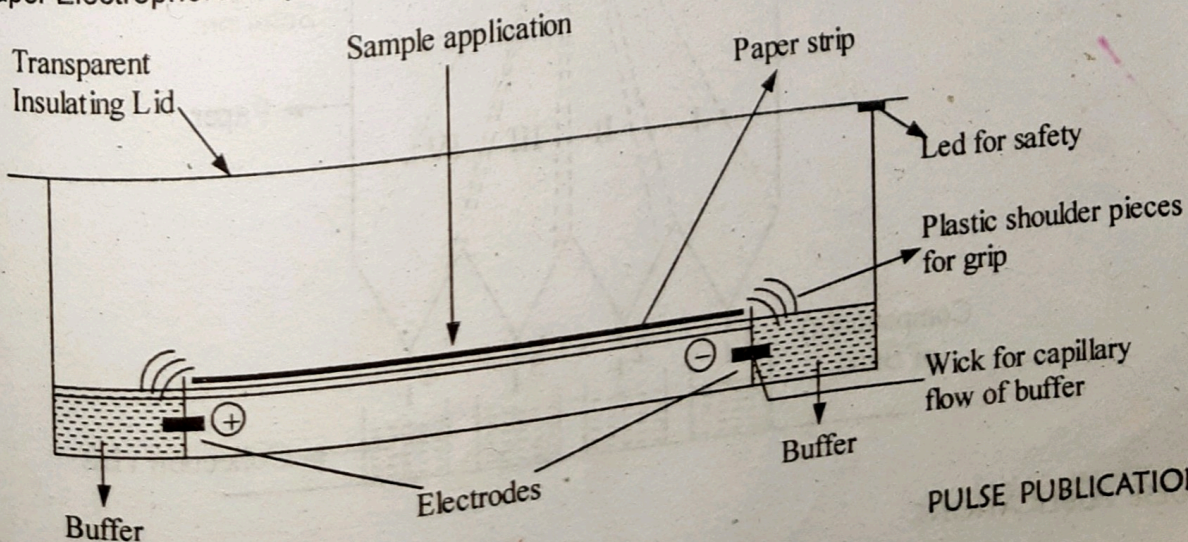


Figure :
Paper
Electrophoresis
(Vertical Type)

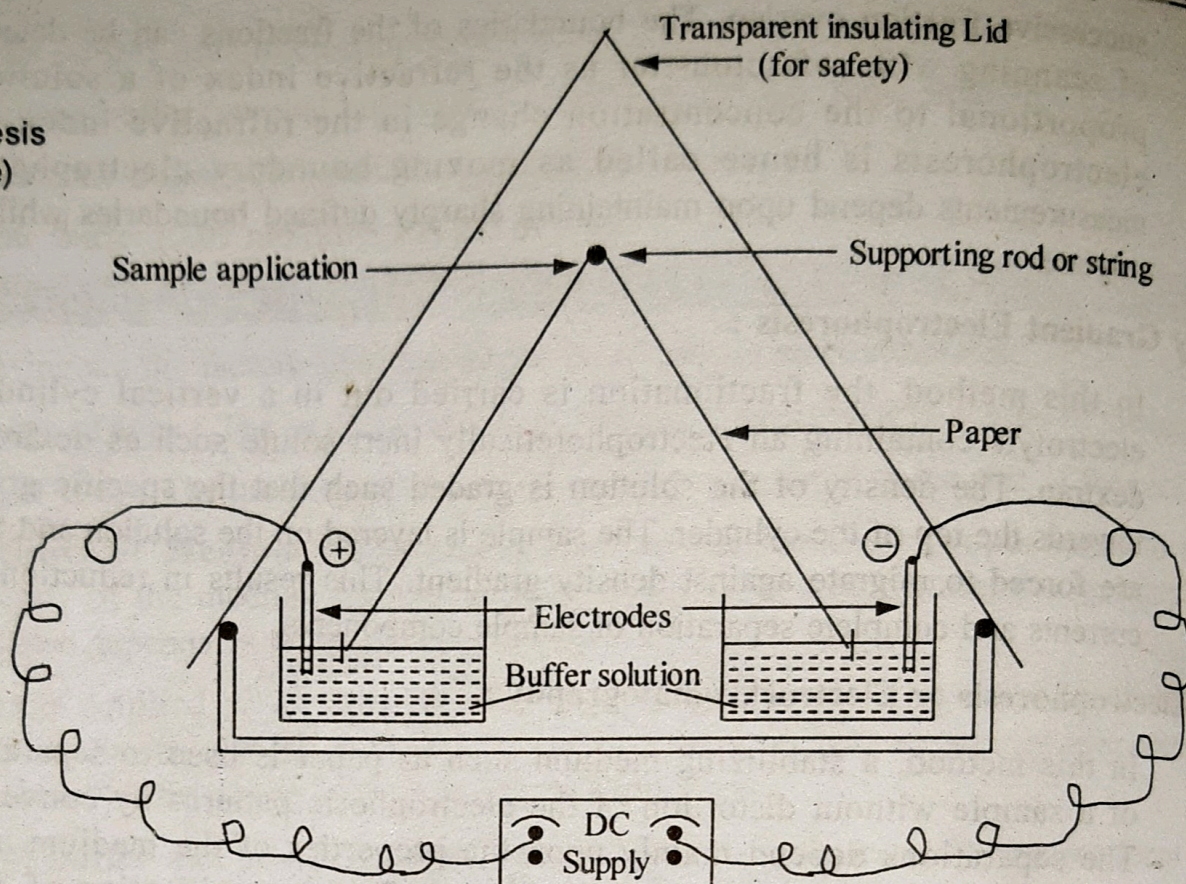
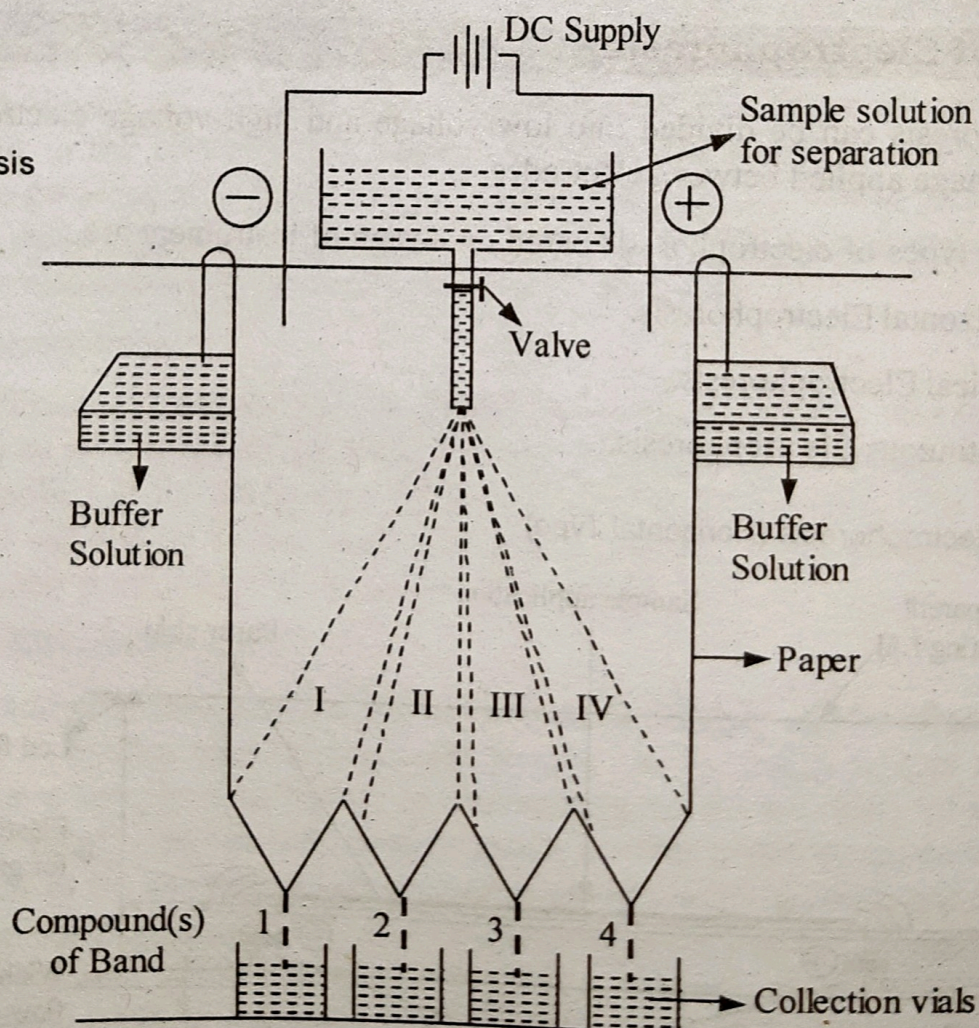


Figure :
Continuous
Electrophoresis
(Preparative
scale)



Horizontal and vertical types are used for analytical purposes whereas continuous electrophoresis is used for preparative purposes.

In vertical and horizontal types filter paper of suitable grade and dimensions is immersed in a buffer solution and the sample is applied at the centre of paper and is fixed in position. A suitable potential is applied across the electrodes.

In vertical mode, the migration of ions is assisted by gravity and separation takes place in about 6 - 8 hours, whereas, in horizontal mode, the time taken is about 12 - 14 hours.

In continuous electrophoresis, the sample is applied continuously on the centre of a paper. The application of voltage causes migration of samples and they separate as bands. Each band is allowed to fall down and pure components are collected in separate containers.

5.7.7 Applications

Electrophoresis is mainly used for the separation of ionizable substances by using buffers of different P^H and ionic strength.

Some of the applications of electrophoresis are :

1. Separation of amino acids.
2. Separation of proteins and lipoproteins in serum.
3. Separation of enzymes in blood.
4. Separation of alkaloids and antibiotics in different samples.
5. Separation of inorganic substances.

FREQUENTLY ASKED QUESTIONS

Q1. Give a detailed account of Principle, Classification and Chemistry involved with application of ion-exchange process in pharmaceutical analysis.

[2004, (5 M)]

[November, 2003, (5 M)]

[2003, (5 M)]

[2001, (5 M)]

Answer :

Refer Unit – V, Page No. 5.24, Section Nos. 5.4.2 and 5.4.4.

Q2. Explain with a diagram, any two detectors used in gas chromatography.

[October, 2005, (5 M)]

[2003, (5 M)]

[2001, (5 M)]

Answer :

Refer Unit – V, Page Nos. 5.31 and 5.34, Section No. 5.5.4.

Q3. Explain with a neat diagram, any three detectors used in Gas Chromatography.

[April, 2005, (10 M)]

[2004, (10 M)]

[November, 2003, (2 M)]

[2001, (10 M)]

Answer :

Refer Unit – V, Page Nos. 5.31 and 5.34, Section No. 5.5.4.

Q4. Discuss the stationary phases used in gas chromatography.

[April, 2005]

[2004, (5 M)]

[November, 2003]

[2001, (5 M)]

Answer :

Refer Unit – V, Page Nos. 5.33, Section No. 5.5.4.

Q5. What are “step wise” and “gradient” elutions ?

[2001, (2 M)]

Answer :

Refer Unit – V, Page No. 5.6, Section No. 5.1.3.

Q6. What is “retention time” ? Mention its significance.

[2001, (2 M)]

Answer :

Refer Unit – V, Page No. 5.37, Section No. 5.5.5.

Q7. Compare the principle, technique, limitations and applications of paper chromatography with paper electrophoresis?

[Unit – V] 5.55

[2003, (10 M)]

Answer :

Refer Unit – V, Page No. 5.19, Section No. 5.3.

Q8. What are “Bonded Phase Supports”? List their advantages and applications in liquid chromatography?

[2003, (5 M)]

Answer :

Refer Unit – V, Page No. 5.12, Section No. 5.1.9.

Q9. What are “bonded phase supports”? List their advantages and applications in liquid chromatography?

[2003, (5 M)]

Answer :

Refer Unit – V, Page No. 5.12, Section No. 5.1.9.

Q10. Explain the concept of “programmed temperature gas chromatography” with relevant examples?

[2003, (5 M)]

Answer :

Refer Unit – V, Page No. 5.38, Section No. 5.5.7.

Q11. Define and classify “Chromatography”?

[2003, (2 M)]

Answer :

Refer Unit – V, Page No. 5.2, Section No.

Q12. What is the importance of a “Guard Column” in HPLC ?

[April, 2006, (2 M)]

[2004, (2 M)]

[2003, (2 M)]

Answer :

Refer Unit – V, Page No. 5.44, Section No. 5.6.4.

Q13. Explain the terms :

(a) HETP

(b) Theoretical plate

(c) Retention Volume

(d) Retention Time

[April, 2006]

[October, 2005]

[2004]

[2001]

Answer :

Refer Unit – V, Page Nos. 5.37 and 5.9, Section Nos. 5.5.5 and 5.1.6.

Q14. Enumerate detectors of HPLC?

[April, 2005, (2 M)]

Answer :

Refer Unit – V, Page No. 5.45, Section No. 5.6.4.

Q15. Define chromatography and R_f ?

[April, 2005]

Answer :

Refer Unit – V, Page Nos. 5.17 and 5.23, Section No. 5.2.4.

Q16. What are the merits of TLC?

[April, 2005, (2 M)]

Answer :

Refer Unit – V, Page No. 5.18, Section No. 5.2.5.

Q17. Classify adsorbents used in column chromatography with examples.

[April, 2006, (2 M)]

[2004, (5 M)]

Answer :

Refer Unit – V, Page No. 5.6, Section No. 5.1.4.

Q18. Describe the instrumentation and applications of H.P.L.C.

[October, 2005, (10 M)]

[November, 2003, (5 M)]

[2001, (10 M)]

Answer :

Refer Unit – V, Page No. 5.41 and 5.48, Section Nos. 5.6.4 and 5.6.5.

Q19. What is meant by “reverse phase chromatography” ? Give an example of stationary and mobile phase ?

[2001, (2 M)]

Answer :

Refer Unit – V, Page No. 5.3, Section No. .

Q20. What is R_f value?

[October, 2007, (2 M)]

[October, 2005, (2 M)]

Answer :

Refer Unit – V, Page No. 5.17, Section No. 5.2.4.

Q21. Write a note on paper electrophoresis?

[October, 2005, (5 M)]

Answer :

Refer Unit – V, Page Nos. 5.49 and 5.51, Section Nos. 5.7.3 and 5.7.6.

Q22. Advantages of radial chromatography?

[October, 2005, (2 M)]

Answer:

Refer Unit – V, Page No. 5.21, Section No. 5.3.3.

Q23. Describe the construction and working of a 'Gas Chromatograph'? Emphasize on the ideal characteristics of stationary phases and mobile phases used in gas liquid chromatography?

[April, 2006, (10 M)]

Answer:

Refer Unit – V, Page No. 5.31, Section No. 5.5.4.

Q24. Write a note on detectors present in HPLC?

[November, 2003, (5 M)]

Answer:

Refer Unit – V, No. 5.45, Section No. 5.6.4.

Q25. Discuss the principle and development techniques used in column chromatography? Add a note on the adsorbents, mobile phases and detection systems used in column chromatography?

[2004, (10 M)]

[November, 2002, (10 M)]

Answer:

Refer Unit – V, Page No. 5.3, Section Nos. 5.1.1 to 5.1.4

Q26. What is Electrophoresis? Mention their types.

[2004, (2 M)]

Answer:

Refer Unit – V, Page Nos. 5.49 and 5.50, Section Nos. 5.7.2 and 5.7.5.

Q27. Mention the various methods of preparing plates in TLC?

[2004, (2 M)]

Answer:

Refer Unit – V, Page No. 5.14, Section No. 5.2.3.

Q28. Enumerate the applications of paper chromatography?

[2004, (2 M)]

Answer:

Refer Unit – V, Page No. 5.23, Section No. 5.3.4.

Q39. How development is carried out in column chromatography, TLC and paper chromatography.

[2004, (5 M)]

Answer:

Refer Unit – V, Page Nos. 5.4, 5.15, 5.20, Section Nos. 5.1.3 and 5.2.3, 5.3.3.

5.58 [Unit – V]

Q30. Enumerate four adsorbents used in TLC.

FAQ's

[2004, (2 M)]

Answer :

Refer Unit – V, Page No. 5.13, Section No. 5.2.3.

Q31. Describe the principle and applications of electrophoresis?

[2002, (10 M)]

Answer :

Refer Unit – V, Page Nos. 5.49 and 5.53, Section No. 5.7.2 and 5.7.7.

Q32. Classify the chromatographic methods. Describe the types of column, stationary phase and detectors employed in gas chromatograph.

[November, 2002, (10 M)]

Answer :

Refer Unit – V, Page No. 5.31, Section No. 5.5.4.

Q33. Explain 'two dimensional' and 'reverse phase' paper chromatography?

[November, 2002, (5 M)]

Answer :

Refer Unit – V, Page No. 5.22, Section No. 5.3.3.

Q34. What are the various development techniques used in paper chromatography?

[October, 2007, (5 M)]

Answer :

Refer Unit – V, Page No. 5.19, Section No. 5.3.3.

Q35. Describe the preparation of plates and adsorbents used in TLC?

[October, 2007, (5 M)]

Answer :

Refer Unit – V, Page No. 5.13, Section No. 5.2.3.