CHROMATOGRAPHY

Introduction to chromatography

Chromatography is a non-destructive procedure for resolving a multi-component mixture of trace, minor, or major constituents into its individual fractions. Different variations may be applied to solids, liquids, and gases. While chromatography may be applied both quantitatively, it is primarily a separation tools.

Chromatography is relatively a new technique which was first invented by M. Tswett, a botanist in 1906 in Warsaw. In that year, he was successful in doing the separation of chlorophyll, xanthophyii and several other colored substances by percolating vegetable extracts through a column of calcium carbonate. the calcium carbonate column acted as an adsorbent and the different substances got adsorbed to different extent and this gives rise to coloured bands at different positions, ion the column. Tswett termed this system of coloured bands as the *chromatogram* and the method as *chromatography* after the Greek words chroma and graphs meaning "colour" and "writing" respectively. However, in the majority of chromatographic procedures no coloured products are formed and the term is a misnomer.

Considerable advances have since been made and the method is used to separate coloured as well as colourless substances. The column of calcium carbonate, used in Tswett's method, remains stationary and is therefore termed as the stationary phase. The solution of vegetable extracts moves or flows down the column and is therefore termed as the mobile phase. Chromatography may be regarded as a method of separation in which separation of solutes occur between a stationary phase and a mobile phase.

Definition of chromatography

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases.

Essentially, the technique of chromatography is based on the differences in the rate at which the components of a mixture move through a porous medium (called stationary phase) under the influence of some solvent or gas (called moving phase).

The chromatography method of separation, in general, involves the following steps :

1. Adsorption or retention of a substance or separation, in general involves the following steps:
2. Separation of the adsorbed substances by the mobile phase.
3. Recovery of the separated substances by a continuous flow of the mobile phase, the method being called elution.
4. Qualitative and quantities analysis of the eluted substances.

Types of chromatography

In chromatography, the stationary phase may be a solid or a liquid and the mobile phase may be liquid or a gas. Depending on the stationary and the mobile phase used, separation occurs because of a combination of two or more factors such as rates of migration, capillary action, extent of adsorption etc., chromatographic methods can be classified on the basis of the stationary and the mobile phase used.

The various types are tabulated in table

Technique	Stationary Phase	Mobile phase
1. Column chromatography or adsorption chromatography	Solid	Liquid
2. partition chromatography	Liquid	Liquid
3. paper chromatography	Liquid	Liquid
4. thin layer chromatography (TLC)	or solid	Gas
5. Gas-liquid chromatography (GLC)	Liquid	Gas
6. Gas-solid chromatography (GSC)	Solid	Liquid
7. Ion exchange chromatography	Solid	

While the above classification is based on the phases involved there are a great number of combinations of phases and processes giving risse to a large number of methods with individual names (table)

Table : classification of chromatographic methods

Stationary phase	Mobile phase	Name
Solid	Liquid	Plane chromatography Paper chromatography (PC) Thin layer chromatography (TLC) Adsorption column chromatography High performance liquid chromatography (HPLC)
Solid (ion exchange resin)	Liquid	Ion exchange chromatography (IEC)
Solid	Gas	Gas-solid chromatography (GSC)
Solid matrix	Liquid	Gel Permecation chromatography (Exclusion chromatography) (GPC)
Liquid	Gas	Gas-liquid chromatography (GLC)
Liquid	Liquid	Liquid –liquid chromatography (LLC)

The method to be discussed in the following chapters will be in terms of the terminology commonly employed and will come under one or the other of the categories listed in the table.

A more recent development in liquid-liquid chromatography is Countercurrent Chromatography which entirely eliminated the use of a solid matrix support. Another form of chromatography where the stationary phase is a porous gel and the separation is according to the size of the molecule is Gel (exclusion) chromatography. Chromatography using gels modified to develop highly specific biochemical reactions for separations is termed as Affinity chromatography (also called bioaffinity chromatography). Other modifications of this technique are methal–Chealte Chromatography, Ligand Exchange Chromatography, and Dye-Ligand Affinity Chromatography. A newer technique which makes use of all the above principles permits very rapied separations is High Performance Liquid Chromatography (HPLC).

I. PAPER CHROMATOGRAPHY

It is defined as a techniques in which the analysis of an unknown substance is carried out mainly by the flow of solvent on specially designed fitter paper one of the two solvents is immiscible or partially miscible with other solvent. The separation is effected by differential migration of the mixture of substances, which takes place due to difference in partition coefficients.

PRINCIPLE:

This technique is a type of partition Chromatography in which the substance are distributed between two liquids i.e. one is stationary liquid which is held in the fibres of the paper and called stationary phase and other is the moving liquid or developing solvent and called the moving phase. The components of the mixture to be separated migrate at different rates and appear as sports at different point on the paper.

In this technique a drop of the test solution is applied as a small spot on a filter paper and the post is dried. The paper is kept in close chamber and the edge of paper is dipped into a solvent called developing solvent. As the filter paper gets the liquid through its capillary axis and when it reaches the spot of the test solution, the various substances are moved by solvent system at various appends. When reached or travelled to a suitable length the paper is dried and spot are visualised by suitable reagents called visualising reagents. The movement of substance relative to the solvent is expressed in terms of RF values i.e. migration parameters.

MIGRATION PARAMETERS

The positions of migrated spots on the Chromatogram are indicated by terms such as RF, Rx, RM.

RF : The R is related to the migrations of solute front from solvent front.

RF = distance travelled by the solute from the origin line distance travelled by the solvent from the origin line.

R is a function of partition coefficient. It is constant for a given substance provided the conditions of the Chromatographic system are kept constant, with respect to temperature,

type of paper, duration and direction of development, amount of liquid in the reservoir, humidity etc.

i.e. quality of paper I this case

- Nature of mixture.
- Temperature and,
- The size of the vessel in which the operation is carried.

Rx : In some cases the solvent front runs off the end of filter paper, the movement of substance is expresses as Rx.

Rx = Distance travelled by the substance from the origin line/distance travelled by the standard substance from the origin line.

R_M: The term RM is additive and is composed of the partial RM values of the individual functional groups of atoms in molecule.

RM = Log [1/ RF – 1]

TYPES OF CHROMATOGRAPH

1. Descending Chromatography:

   When the development of the paper is done by allowing the solvent to travel down the paper it is known descending technique. The advantage of descending technique is that the development can be continued indefinitely even though the solvent runs off at the other end of paper.

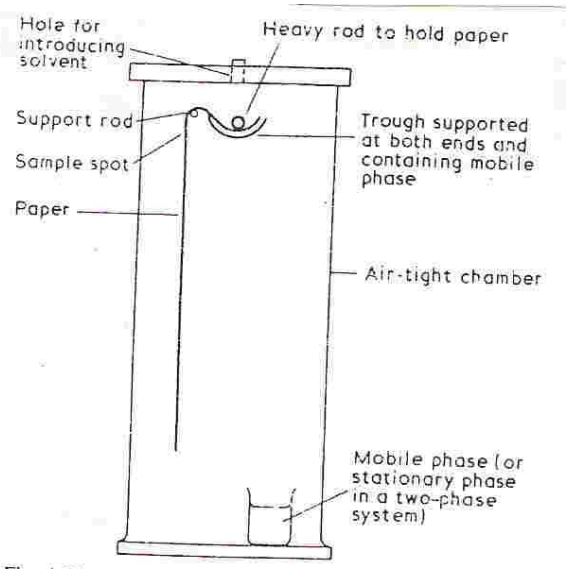

Fig.1 Descending Chromatography

2. Ascending Chromatography:

When the development of the paper is done by allowing the solvent to travel up the paper it is known ascending technique.

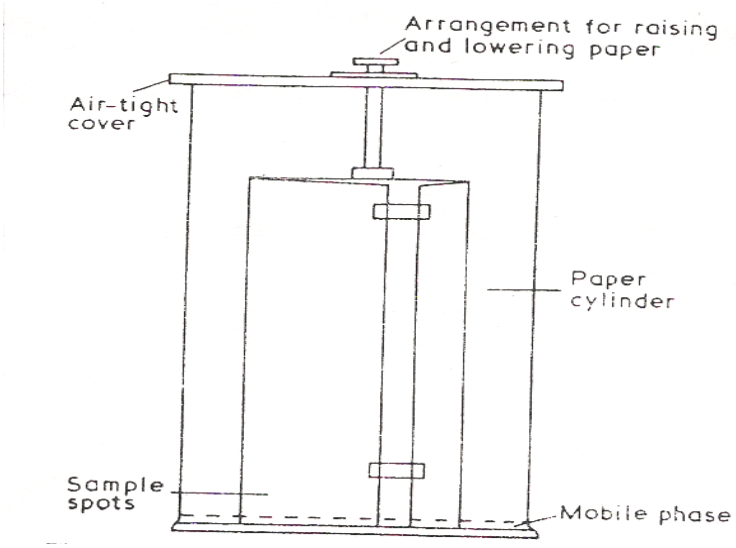

Fig.2 Ascending Chromatography

Both ascending and descending technique have been employed for separation but descending technique is preferred if the RF values of various constitutions are almost same.

3. Ascending - Descending Chromatography:

It is a hybrid of two techniques. The upper part of ascending chromatography can be folded over a glass rod allowing the descending development to change over into the descending after crossing the glass rod.

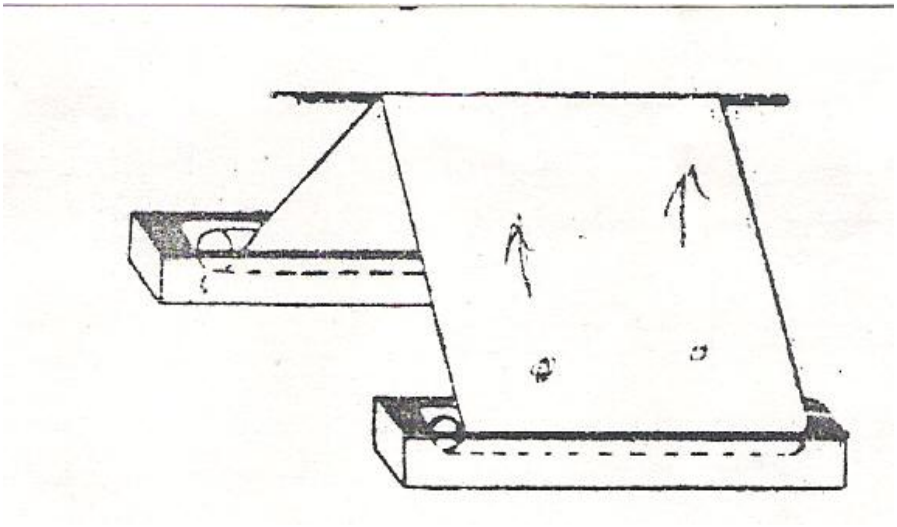

Fig.-3 Ascending - Descending Chromatography

4. Radial Paper Chromatography:

This is also known as circular paper chromatography. In this technique a circular filter paper is employed, various materials to be analysed are placed in a centre. After drying the spot the filter paper is fixed horizontally on pet dish possessing solvent, so that tongue of paper dips in solvent when solvent front has moved through a sufficient large distance the components gets separated. Forming concentric circular spots.

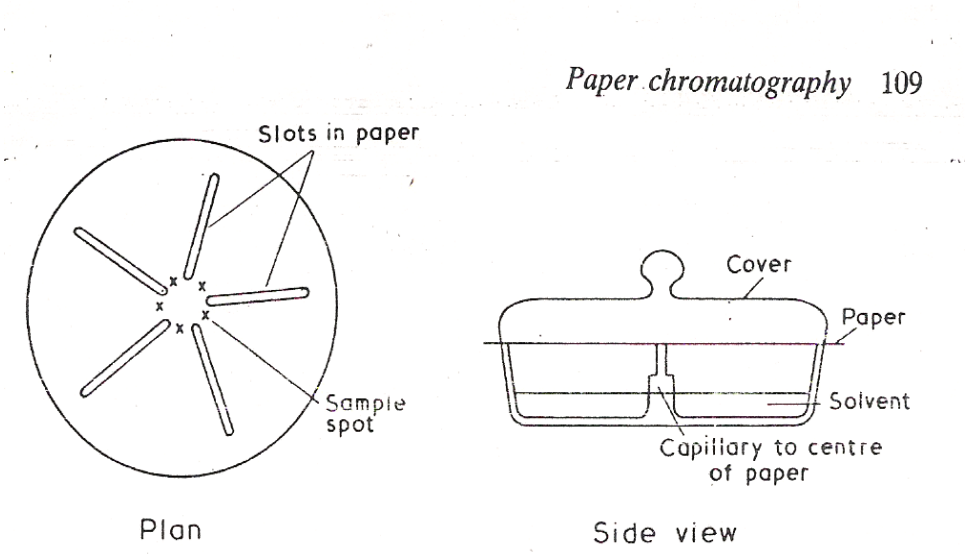

Fig.4 Radial Paper Chromatography

5. Two dimensional chromatography:

In this a square rectangular paper is used. The sample applied on one of the corners. The second development is performed at right angle of the direction of first run. This type of chromatography can be carried out with identical solvent system in both directions or by two solvent systems.

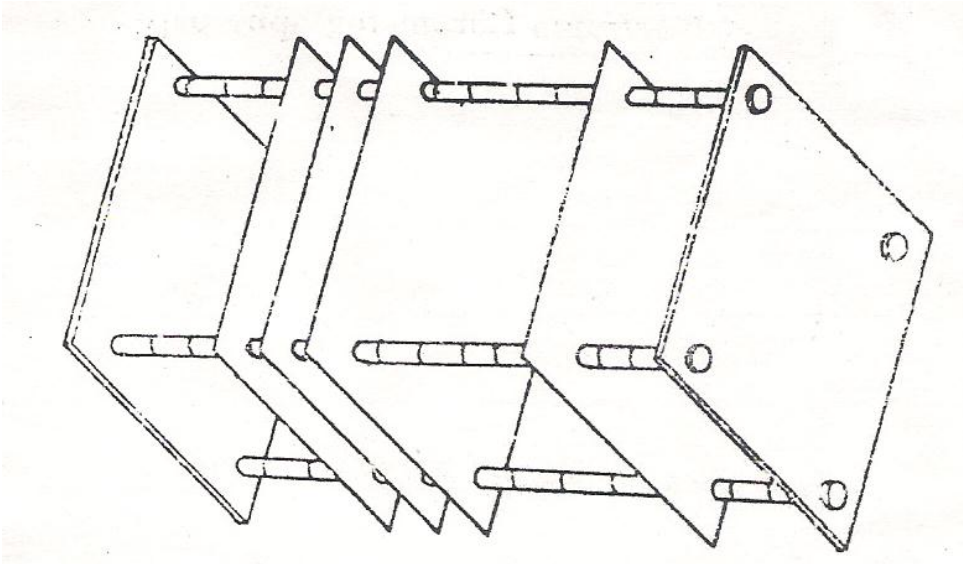

Fig.5 Two dimensional chromatography

EXPERIMENTAL DETAILS:

1. Choice of the proper chromatographic technique:

   The choice of technique is depends upon the nature of substances to be separated.

2. Choice of filter paper:

   - Whether the paper is being used for quantitative or qualitative analysis.
   - Whether it is used for analytical or preparative chromatography.
   - Whether substance used are hydrophilic or lipophilic neutral or charged species.

3. Proper developing solvent:

   The choice of this depends upon the simple fact that R_F values should be different for different constituents present in mixture. A solvent or mixture of solvent, which gives a RF 0.2 – 0.8 for sample, should be selected. The solvent are listed in order of increasing polarity:

SOLVENT LISTED IN ORDER TO INCREASING POLARITY

Solvent	T20	T26
n-hexane	1.89	-
Cyclohexane	2.02	-

Carbon tetrachloride	2.24	-
Benzene	2.29	-
Toluene	2.44	-
Trichroroethylene	3.40	-
Diethyl ether	4.43	-
Chloroform	4.91	-
Ethyl acetate	-	6.02
n-butanol	17.80	17.10
n-propanol	-	20.10
Acetone	-	20.10
Ethanol	-	24.30
Methanol	-	32.60
Water	80.40	78.50

4. Preparation of samples:

It is not possible to give any standard procedure for preparation of samples because this problem resolves around several factors of given samples. However sample volume of 10-220 having as many as Ng of substance is the ideal quantity to be spotted.

5. Spotting:

A horizontal line is drawn on the filter paper by a pencil. This is origin line (For ascending chromatography). The test solution are applied above the line and dried cautiously by a stream OI hot or cold air.

6. Drying the chromatograms:

The wet chromatograms after development are dried in drying cabinets, which are being heated electrically with temperature controls.

7. Visualisation:

It can be done by two ways:

- Chemical means.
- Physical means.

1.) Chemical Detection:

Chemical treatment can develop the colour of colourless solvents on paper. The reagents used for visualising spots are Chromographic reagents. The regents applied by dipping or spraying their aqueous solution.

2.) Physical Detection:

Some colourless spots when held under a UV lamp fluorescent occur.

Applications:

Paper chromatography is widely used for qualitative analysis of inorganic, organic and biochemical interests. It is also useful is analysis of mixture of amino acid and mixture of sugars.

II. THIN LAYER CHROMATOGRAPHY

The technique of thin layer chromatography closely resemble to those of column and paper chromatography. In thin layer chromatography, partition however occurs on a layer of finely divided adsorbent, which is supported on a glass plate. This chromatography using thin layers of an adsorbent held on a glass plate or other supporting medium is known or thin layer chromatography.

ADVANTAGES OF TLC:

1) It is an elegantly simple procedure for chromatography in all kinds of solid-liquid & liquid system.
2) It is performed in analytical & operative in large preparation scale.
3) Applicable to almost all chemical compounds.
4) Because of its rapid speed it can be employed for checking the course of chemical reaction; in laboratory as wall as industrial scale.
5) It has great resolving power, and so can be used for uncovering adulteration of foods and drugs caused by improper storage or incorrect use.
6) TLC can readily detect compounds, which are encountered in trace amount, due to high sensitivity. (e.g. Narcotics; Air pollutant; pesticides etc.)

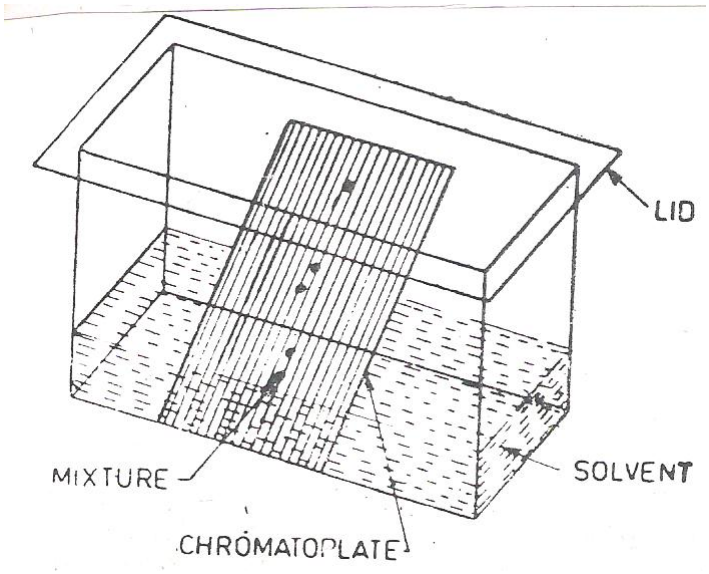

Fig.6- Apparatus for Thin layer chromatography

Basic operations involved in TLC:

1) Methods for production of thin layer plates:

Spreading, pouring, spraying or dipping can achieve coating of glass plates with adsorbent layer. Most uniform layers are obtained by spreading.

Layers are classified in to two types:

a) Solid layers.
b) Loose layer.

i. For solid layers a uniform layer of the adsorbent material is applied to a clean glass plate with help of applicator.
ii. For loose layer may be prepared by.
    I. Pouring of suspension on plate.
    II. Dipping of plates in suspension.
    III. Spraying with thin suspension.

2) Application of sample on chromatoplates:

In analytical TLC, 0.1% solution of sample is applied to the plates with the help of capillaries, micropipettes. The solvent in which the substances are dissolved is allowed to evaporate. Solutions are applied as single spots in a row along one side of the plate about 2 cm from edge. It is desirable to chromatograph a sample to be analysed in different

amounts e.g. 1,5,10 and 60 Ng on one plate. The amount of sample that can be applied in one spot depends upon:

- Thickness of layer:

  In absorption TLC, whore 25 mm thick layer is used 70-500 Ng of mixture of lipophilic substances can be fractioned.
- Principle of chromatography employed.

3) Choice of adsorbent:

The common adsorbents used in TLC are silicagel alumnae, Kieselguhr and powdered cellulose, coating materials used in TLC, depends upon their acidity or basicity, Activity and separating mechanism. So it will depend and change according to nature of compound.

Normally a 0.25 mm thick layer can be prepared by spreading aqueous slurry of adsorbent with applicator on glass plates. Thick layers (1-2 mm) of silica gel can be prepared by slurring silica gel G with water in ratio 25:40. The layers are air dried for about leman and then activated by heating in an over at about 11 for 2 hrs.

A binding a gent usually plasters of Paris is after incorporated to hold adsorbent firmly.

Preparation of thin layers in plates:

A large number of applicators are commercially available which are used for coating the glass plates with different adsorbent layers of uniform thickness. The various methods of preparing layers are:

I. Pouring:

A measured amount of slurry is put on a given size of plate, which is kept on a level surface. The plate is then tipped back and forth to spread the slurry uniformly over the surface.

II. Dipping:

In this method plates are prepared by dipping then two at a time back to back in $CHCl_3$ slurries of adsorbent.

III. Spraying:

A small point sprayer for distribution of the slurry on glass plate. This is not used because it is difficult to obtain uniform layers on a single plate and also there may be a variation from plate to plate.

IV. Spreading:

The slurry is an applicator. This is either moved over the stationary plate or it is tied stationary and the plate is posted or pulled through. The apparatus developed by state for getting actsorpont layers insists of aligning tray in which the plates are sot in a line and spreader, which takes up the spreading mixture and applies it uniformly on thin layer.

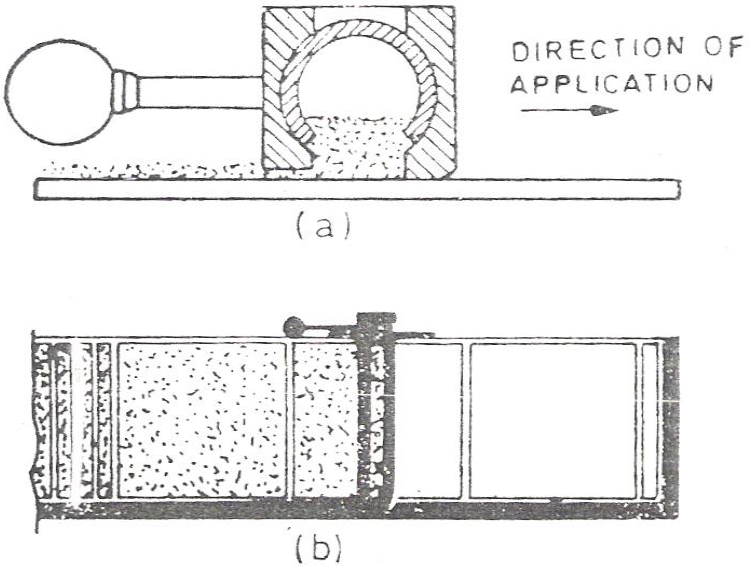

Fig.7 (a) Operation of thin-layer spreader.

V. Precoated plates:

Ready to use thin layers of the common aolsortmts are now available pirouetted on glass or plastic stets. These plates are quite expensive. The thickness of precoated plastic sheets usually varies from 0.1 to 0.2 mm.

4) Choice of solvent:

If one does not know about the nature of communists of the mixture to be separated the best lucent is found by trial and error using small very rapid running TLC plates. If the nature of component is known then it is possible to know a suitable solvent by using original stain's triangle, which has inter-relating adsorbent activity, nature of solute and nature of solvent. If the triangle is rotated so that the corner M points to the type of mixture to be separated, this specifies at corners S and E respectively, the necessary activity of the adsorbent and the optimum polarity of the event with mixtures of solvents, it is possible to obtain, intermediate elution behaviour. Mixtures of two or more solvent of different polarity often give better operations than chemically homogenous solvents.

Sine polar solvents produce the greater migration, a better operations is effected in their presence.

5) Detecting reagents:

Compounds separated by thin layer chromatography are colourless their positions are thus located or detected with help of some reagents known as locating or detecting reagents. Iodine vapour and sulphuric acid (mixed with aromatic aldehydes or oxidising agents like KMNO4; 1 + NO 3; chromic acid etc.) are common locating agents. Iodine forms a number of coloured loose complexes with a variety of compounds. Sulphuric acid also forms coloured complexes, which are visible in daylight and ultraviolet light.

The process of detecting the sports on the sheet after completion of the development is called visualisation. Where over there is a sport, the fluorescence of the sport is quenched with respect to the background upon scanning with a U.V lamp. Amino acids are detected by spraying ninhydrin on the sheep, to get fluorescent derivatives.

6) Developing chamber:

In the TLC plates are usually developed by placing them on edge in jar containing a 0.5 – 10 cm layer of solvent. It is a type of chromate jar or a common tank, is used which is closed form all sides. According to requirements the jar is jacketed and connected to a thermostat in order to develop a chromatogram at constant temperature.

Usually it is possible to develop micro chromatoplatos in body food jars or in glass beaker covered with aluminium foil. The jar is saturated with solvent vapour. by lining the inside of jar with filter paper, as it has following advantage

- It yields straight solvent fronts
- Developing time is reduced to one third.
- RF values are must less than in unsaturated tanks.

7) Development & Detection:

Chromatoplates are usually developed once with a single solvent by either horizontal, ascending or descending elution. Some techniques are as:

1. Ascending or vertical development:

The sample is spotted at one end of the plate and then developed by the ascending technique used in paper chromatography. The plates are placed vertically in a container saturated with developer vapour and the solvents as ends from bottom to top.

2. Horizontal development:

It is useful with adhering thin layers and loss layers. The sample is placed in the centre of the plate and developed either by slowly dripping solvent on it from micropipette. This procedure is also called circular TLC.

3. Multiple developments:

In this the development is carried repeatedly with same solvent in same direction each time after drying.

4. Stepwise development:

It is carried out consecutively with two different solvents but is same direction. One of the solvents is run to a height of 15 – 18 cm and the other to 10 – 12 cm.

5. Gradient development:

Sometimes it is advantageous especially when fractionating compounds of widely different properties change the composition of the solvent continuously during chromatography. This technique is called gradient elution. In this technique the chrumatoplate is lowered into a jar containing a solvent and then a second more polar solvent is added in chamber with help of burette. The elute is continuously stirred. As a result polarity of the former solvent polarity of the former solvent is modified by second solvent.

6. Continuous development:

When there are small differences in RF values, the development distance is increased in order to achieve complete separation. This is done by continuous development in which a solvent is forced to run over the edges of the chromatoplates. Where it can be collected instead of being left to evaporate.

7. Two dimensional development:

It is also possible to develop the square plates in two dimensions. A sample is spotted in a corner of the plate and then developed consecutively, in two directions either with two same solvent or different solvents.

The location of compound after development is dire by spraying different developing reagents. If substance shows dark spots against a florescent background they can be made visible under a U.V. lamp.

Applications:

- For checking purity and progress of reaction.
- For purification as well as identifying compounds. Such as amino acids; protein; peptides & Antibiotics.

III. COLUMN CHROMATOGRAPHY

The principle for selective adsorption is used in column chromatography.

The mixture to be separated is dissolved in a suitable solvent and allowed to pass through a to be component which has greater adsorbing power is adsorbed in the upper part of the column. The next component is adsorbed in the column, which has lesser adsorbing power than the first component. This process is continued. As a result the materials are partially separated & adsorbed in the various parts of the column. The initial separation of the various components can be improved by passing either the original or some other suitable solvent slowly through the column.

The various bonds in the column become more defined. The banded column of adsorbent is termed a chromatogram & the operation is spoken of as development of chromatogram. The portion of a column, which is occupied by a particular substance, is called its zone.

The zones contain the substance which can be separated by two propellers:

A. After development, the column of adsorbent may be posted out of to be, the various zones are cut with a knife at boundaries and the substances present in zones extracted with a suitable solvent. The process of recovery of constituents form the chromatogram is known as elution.
B. After development, the column may be washed with more solvent, now termed as event and each component is collected separately as it reacts and of the column and is released.

APPARATUS:

A Simple glass tube with a stopper cork at one end is taken; having 20-30 cm length and 2-3 cm diameter long enough to carry 50-100 gm of adsorbent and may retain several grams of adsorbent. The adsorbent is supported by plug of cotton or glass wool. Long and narrow tubes are used for difficult separation.

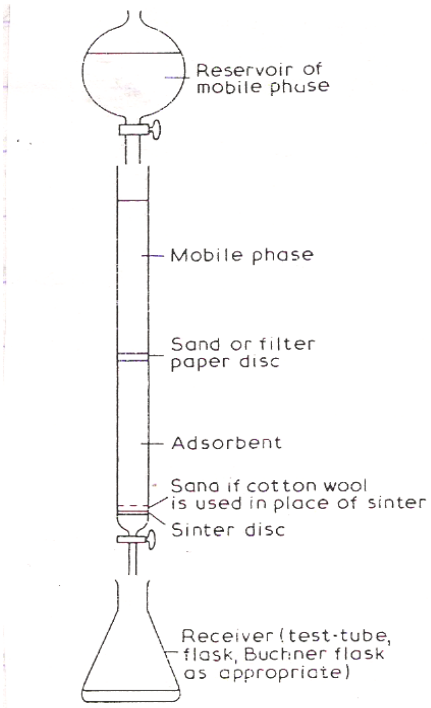

Fig.8- Apparatus for column chromatography.

ADSORBENTS REQUIREMENT:

- Particles should be spherical in shape and uniform in size.
- Their mechanical stability must be great enough to prevent the formation of fine dust which might deposited in the channels of the packing.
- They should not react chemically either with the eluting solvent or with the sample components.
- They should contain as small amount of soluble components as possible.
- They should be catalytically inactive and have neutral surface.

Suppose talc, starch, insulin, sodium carbonate, calcium carbonate, calcium phosphate, magnesium carbonate, lime, activated silicic acid, activated cilicic acid, activated magnesium silicate, activated alumna and fullers earth are the most common adsorbents.

Preparation of Adsorption column:

The glass wool or cotton plug is used as a support for the column. It is first kept in position in the tube. The tube is then clamped vertically. Now the adsorbent is added in portion so that the tube is packed uniformly with the adsorbent. Whenever and portion is added it is pressed from above with a flattened glass rod before next portion is added. This is continued till nearly two third of it is filled. Now either eluting solvent or

petroleum ether is passes and bottom and is connected with section. It is important that the surface of column is ether covered with eluting solvent or the pet either.

=> SOLVENTS:

The choice of solvent will naturally depend in the frits place upon the solubility relations of substance. The solvents generally employed pusses boiling points between 40^0 and 85^0 C. the must widely used is light petroleum and others are cyclohoxane, carbon disulphide, benzene, chloroform, carbon tetra chloride, methylene chloride, ethyl acetate, ethyl alcohol, acetone, ether and acetic acid.

The solvents used in chromatography have three functions.

1) They serve to introduce the mixture of the column.
2) They affect the process of development by which the zone of chromatogram is separated to their full extent.
3) They are also used to remove the required content of each zone from mechanically separated parts of the column or from the column as a whole after it is properly developed.

=> DETECTORS:

These are sued to determine the dissolved substances emerging from the column.

1) Optical Detectors: These are old type of flow analysers, which are small cells, made from glass or quarts and are used for continuous photometric analysis with visible or UV light of appropriate wavelength.
2) Differential Refractometer: This method was employed as the refraction of the emerging power fro detection. This method has been improved to differential refractometer having sensibility. (Limit of detection 10^{-6} gm.)
3) Detectors based heat adsorptions: These detectors are known as micro adsorption detectors. In these, the liquid emerging from a separating column is passed through two cells.
4) Flame ionisation detectors: Organic components are readily pyrolysed cover introduced into a hydrogen oxygen flame. As a result of pyrolysis ions are produced. Theses ions by which current can be carriod through flame. The ions can be collected at a charged electrode and the resulting current pressured by electrometer amplifier.

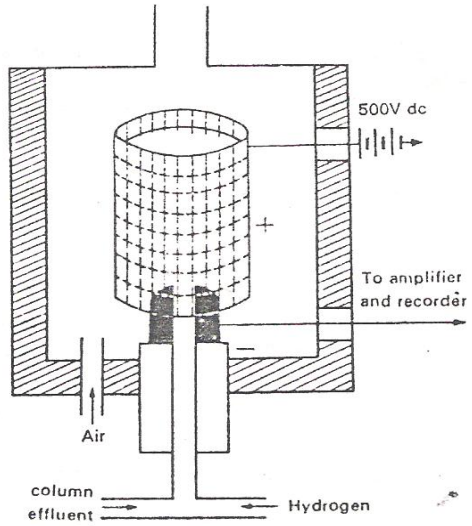

Fig.9- Hydrogen flame ionisation detector.

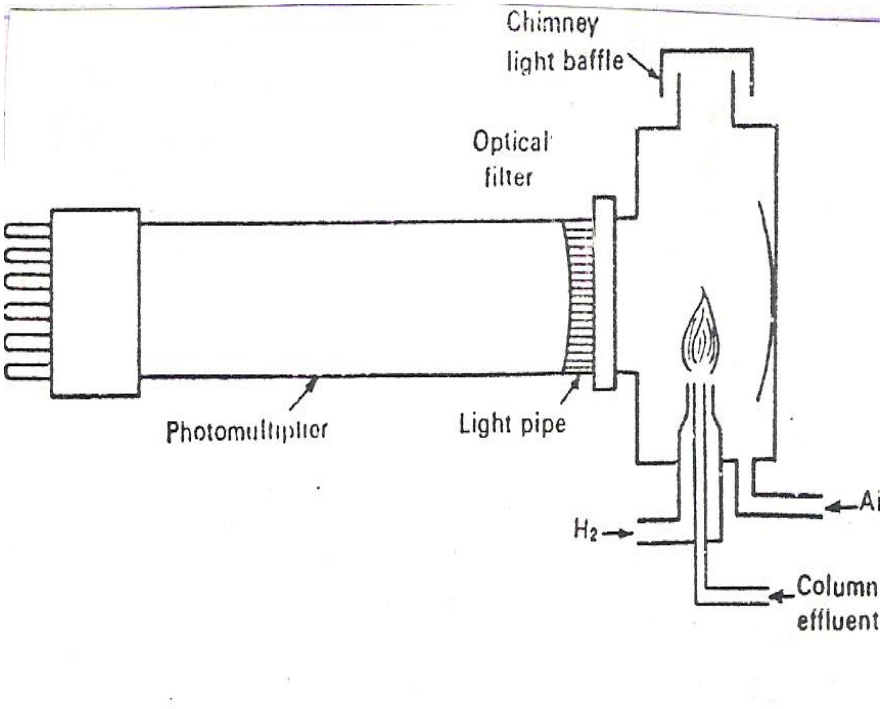

Fig.10- Flame photometric detector.

The carrier gas emerging from the column is mixed with an equal amount of hydrogen and burned at a metal set or burned in an atmosphere of air. The set or a surrounding ring acts as a negative electrode and a loop of cylinder of inert metal surrounding the flame acts as the positive electrodes. As the composition of gas in flame changes the number of ions and electrons will also change. Thus current flow will vary with the change in composition of the gas eluted from gas chromatographic column.

The ionisation of carbon compounds in a flame is roughly proportional to the number reduced carbon atoms in the flame. Oxidised carbon atoms produce fewer ions or nor at all.

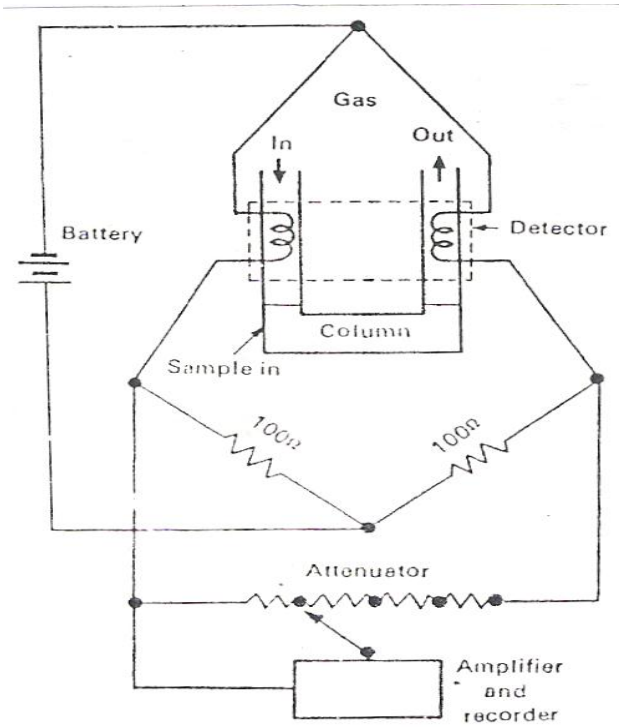

Fig.11 – Thermal conductivity detector.

Located on top of the other while the lower cell is filled with an adsorbent. In the centre of the packing of each of the cell, the glass covered measuring point of a small thermistor is located. The total deflection is proportional to the concentration at least to a range of 102.

4.) Flame ionisation detectors:

In these detectors those is an endless wire, which is passed by the column exit. The decomposition products of the substances, which are transported by the wire, are led to a flame ionisation.

5.) Conductivity Detectors:

These are suitable for ionised substances in aqueous solutions. The effluent is passed through the measuring cell of the detectors, which contains two or three platinum electrodes within a wheat sore bridge circuit and is operated by alternating current.

- **FACTORS AFFECTING COLUMN EFFICIENCY:**

A. Nature of solvents:

Solvents of law viscosities are generally used for high efficiency separations. The reason for this is that rate of flow is inversely proportional to viscosity and hence it becomes necessary to select a solvent of lower viscosity and proper elution strength.

B. Dimensions of column:

It is possible to improve the column efficiency by increasing the length/width ratio of the column. For common preparative separation sample/column packing ratios have found to range from 1:20 to 1:100.

C. Particle size column packing:

It is possible to increase the column efficiency by decreasing the particle size of the adsorbent. The usual particle size ranges from 100 to 200 meshes.

D. Pore diameters of column packing:

Polar adsorbent posses a pore diameter of $\leq$ 20 A^0, A decrease in average pore diameter from 170 – 20 A^0 does not affect efficiency.

E. Temperature of column:

Difficult soluble samples are generally at higher temperatures while other samples are separated at room temperature.

- APPLICATIONS:
- For analytical uses.
- Separation of geometric isomers. I.e. cisltrans.
- Separation of diameters and Tautumeric mixtures.
- Separation of racemates.