13. COLUMN CHROMATOGRAPHY

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INTRODUCTION

When a column of stationary phase is used, the technique is called as column chromatography. Based on the nature of stationary phase, i.e. whether it is solid or liquid, it is called as column adsorption chromatography or column partition chromatography. Most of the discussions in this chapter will be devoted to column adsorption chromatography, since column partition chromatography is not being used widely.

PRINCIPLE

A solid stationary phase and a liquid mobile phase is used and the principle of separation is adsorption. When a mixture of components dissolved in the mobile phase is introduced into the column, the individual components move with different rates depending upon their relative affinities. The compound with lesser affinity towards the stationary phase (adsorbent) moves faster and hence it is eluted out of the column first. The one with greater affinity towards the stationary phase (adsorbent) moves slower down the column and hence it is eluted later. Thus the compounds are separated. The type of interaction between the stationary phase (adsorbent) and the solute is reversible in nature. The rate of movement of a component (R) is given as follows:

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

This equation can be simplified as follows:

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

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

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

where α is the partition co-efficient $= \frac{\text{conc. in stationary phase}}{\text{conc. in mobile phase}}$

A_m is the average cross section of mobile phase

A_s is the average cross section of stationary phase

PRACTICAL REQUIREMENTS

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

1. STATIONARY PHASE (ADSORBENTS)

An adsorbent used in column chromatography should meet the following criteria:

a. **Particle size and geometry:** The particles should have uniform size distribution and have spherical shape. Particle size: 60-200μ.

b. Should have high mechanical stability.

c. Should be Inert and should not react with the solute or other components.

d. Insoluble in the solvents or mobile phases used.

e. It should be colourless to facilitate observation of zones and recovery of components.

f. It should allow free flow of mobile phase.

g. It should be useful for separating for wide variety of compounds.

h. Above all it should be freely available, inexpensive, etc.

Types of adsorbents

Based upon their adsorbent activity, they can be classified as weak, medium, and strong adsorbents. They are

Weak	Medium	Strong
Sucrose	$CaCO_3$	Activated Mg Silicate (Silica gel)
Starch	$Ca_3(PO_4)_2$	Activated Alumina
Inulin	$MgCO_3$	Activated Charcoal
Talc	MgO	Activated Magnesia
Na_2CO_3	$Ca(OH)_2$	Fuller's earth

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

Selection of stationary phase

The success of chromatography depends upon the proper selection of stationary phase. The selection of stationary phase in column chromatography depends on the following:

i. **Removal of impurities:** When a small quantity of impurity is present and there is difference in affinity when compared to the major component, a weak adsorbent is sufficient.

ii. **No. of components to be separated:** When few components are to be separated, weak adsorbent is used. When more components are to be separated, a strong adsorbent is selected.

iii. **Affinity differences between components:** When components have similar affinities, a strong adsorbent will be effective. When there is more difference in affinities, a weak adsorbent is selected.

iv. **Length of the column used:** When a shorter column is used, strong adsorbent has to be used. When a longer column is used, a weak adsorbent can be used.

v. **Quantity of adsorbent used:** 20 or 30 times the weight of the adsorbent is used for effective separation.

Adsorbate : Adsorbent ratio = 1:20 or 1:30

2. MOBILE PHASE

Mobile phase is very important and they serve several functions. They act as solvent, developer and as eluent. The functions of a mobile phase are

To introduce the mixture into the column - As solvent

To develop the zones for separation - As developing agent

To remove pure component out of the column - As eluent

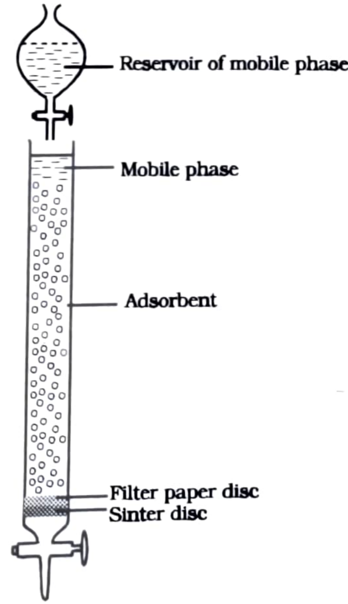
Different mobile phases used: (in increasing order of polarity or elution strength)

Petroleum ether, Carbon tetrachloride, Cyclohexane, Carbondisulphide, Ether, Acetone, Benzene, Toluene, Esters (Ethyl acetate), Chloroform, Alcohols (Methanol, Ethanol, etc), Water, Pyridine, Organic acids (Acetic acid, etc), Mixture of acids or bases with ethanol or pyridine etc.

These solvents can be used in either pure form or as mixture of solvents of varying compositions.

3. COLUMN CHARACTERISTICS

The material of the column is mostly good quality neutral glass since it should not be affected by solvents, acids or alkalies. An ordinary burette can also be used as column for separation. The column dimensions are important for effective column separations. The length : diameter ratio ranges from 10:1 to 30:1. For more efficiency, the length : diameter ratio can be 100:1. The length of the column depends upon:



→ Affinity of compounds towards the adsorbent used.

→ Number of compounds to be separated.

→ Type of adsorbent used.

→ Quantity of the sample.

4. PREPARATION OF THE COLUMN

The bottom portion of the column is packed with cotton wool or glass wool or may contain a asbestos pad, above which the column of adsorbent is packed. A Whatman filter paper disc can also be used. After packing the column with the adsorbent, a similar paper disc is kept on the top, so that the adsorbent layer is not disturbed during the introduction of sample or mobile phase. Disturbance in the layer of adsorbent will lead to irregular bands in separation.

There are two types of preparing the column, which are called as packing techniques. They are

i. **Dry packing technique:** In this technique, the required quantity of adsorbent is packed in the column in dry form and the solvent allowed to flow through the column till equilibrium is reached. The demerit with this technique is that air bubbles are entrapped between the solvent and the stationary phase and the column may not be uniformly packed. Cracks appear in the adsorbent present in the column. Hence uniformity in flow characteristics and clear band of the separated component may not be obtained.

ii. **Wet packing technique:** This is the ideal technique. The required quantity of the adsorbent is mixed with the mobile phase solvent in a beaker and poured into the column. The stationary phase settles uniformly in the column and there is no entrapment of air bubbles. There will not be any crack in the column of adsorbent. The bands eluted from the column will be uniform and ideal for separation.

5. INTRODUCTION OF SAMPLE

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

6. DEVELOPMENT TECHNIQUE (ELUTION)

After the introduction of the sample, by elution techniques, the individual components are separated out from the column. The two techniques are

i. **Isocratic elution technique:** (Iso means same or similar) In this elution technique, the same solvent composition or solvent of same polarity is used throughout the process of separation. eg. Chloroform only, Pet.ether : Benzene = 1:1 only, etc.

ii. **Gradient elution technique:** (Gradient - gradually) In this elution technique, solvents of gradually increasing polarity or increasing elution strength are used during the process of separation. Initially low polar solvent is used followed by gradually increasing the polarity to a more polar solvent. eg. Initially Benzene, then Chloroform, then Ethyl acetate, then to Methanol, etc.

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

7. DETECTION OF COMPONENTS

The detection of coloured components can be done visually. Different coloured bands are seen moving down the column which can be collected separately. But for colourless compounds, the technique depends upon the properties of the components. Different properties which can be used are

i. Absoption of light (UV/Vis) - using UV/Vis detector

ii. Flourescence or light emission characteristics - using flourescence detector

iii. By using flame ionisation detector

iv. Refractive index detector - based on the refractive index difference between the mobile phase and mobile phase+component

v. Evaporation of the solvent and weighing the residue

vi. By monitoring the fractions by thin layer chromatography

Any one of the above techniques can be used for detection of compounds so that it can be used for qualitative analysis and for isolation of compounds.

8. RECOVERY OF COMPONENTS

Earlier, recovery of the components were done by cutting the column into several distinct zones. Later, extrusion of the column into zones were

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

FACTORS AFFECTING COLUMN EFFICIENCY

For any separation, efficiency of the column is important. Unless the factors affecting the column efficiency are known, efficiency cannot be improved. They are:

i. **Dimensions of the column:** A length:diameter ratio of 20:1 or 30:1 are ideal. But for improving the efficiency, 100:1 may be more satisfactory.

ii. **Particle size of the adsorbent:** Adsorbent activity depends on the surface area of adsorbent. For increasing the surface area, particle size can be reduced and hence the adsorbent activity increases.

iii. **Nature of solvent:** The flow rate of solvent is affected by its viscosity. The flow rate is inversely proportional to viscosity. Hence less viscous solvents are better efficient than more viscous solvents.

iv. **Temperature of the column:** Speed of elution is increased at higher temperature. But adsorbent power is decreased at higher temperatures. Hence a compromise is made between speed of elution and adsorbent power.Normally room temperature is used for all samples. Difficult samples are separated at higher temperatures.

v. **Pressure:** High pressure above the column and low pressure below the column increases the efficiency of separation. High pressure above the column is achieved by maintaining a column of liquid on the top of the column (reservoir) or by using pressure devices

(pumps). Pressure below the column is decreased, by applying vacuum, using vacuum pump.

APPLICATIONS

1. **Separation of mixture of compounds:** Column chromatography can be used for the separation of several classes of drugs and constituents like alkaloids, glycosides, amino acids, plant extracts, drugs and formulations, etc.

2. **Removal of impurities or purification process:** Impurities present in a compound can be removed by using appropriate stationary and mobile phase.

3. **Isolation of active constituents:** From plant extracts, from formulations or other crude extracts, active constituents or required constituents can be isolated

4. **Isolation of metabolites from biological fluids:** eg. 17-ketosteroids from urine, cortisol, other drugs etc from biological fluids like blood, plasma or serum, etc.

5. **Estimation of drugs in formulations or crude extracts**

   i. Determination of % w/w of stychnine in syrup of ferrous phosphate with quinine and strychnine.

   ii. Determination of primary and secondary glycoside in digitalis leaf.

   iii. Determination of phytomenadione in injection and tablets.

   iv. Determination of Flucinolone actonide or Betamethasone 17-valerate in formulated products.

   v. Separation of geometrical isomers: Cis and trans forms of bixin and crocetin dimethyl ether using alumina.

   vi. Separation of diastereomers.

   vii. Separation of inorganic ions like copper, cobalt , Nickel, etc.

   viii. Separation of tautomers and racemates.

ADVANTAGES OF COLUMN CHROMATOGRAPHY

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

DISADVANTAGES OF COLUMN CHROMATOGRAPHY

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

PARTITION COLUMN CHROMATOGRAPHY

The technique is similar to column adsorption chromatography except that, the stationary phase is liquid. A solid support like silica gel or cellulose is used to hold the liquid stationary phases like water, aqueous buffer solutions, etc. as thin film on the surface. Mobile phase is similar to that of column chromatography, but gradient elution technique is not used, since the equilibrium will be disturbed. All the other requirements and the technique is similar to column adsorption chromatography. Column partition chromatography is not being used widely.

14. THIN LAYER CHROMATOGRAPHY (TLC)

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- ☞ Principle
- ☞ Advantages of TLC
- ☞ Practical requirements
  - ☆ Stationary Phases
    - Glass plates
    - Preparation and activation of TLC plates
    - Application of sample
    - Development tank
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    - Development technique - One dimensional development
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    - Detecting or visualising agents
      - Specific & Non-specific methods
      - Destructive & Non-destructive methods
- ☞ Qualitative analysis - R_f, R_x, R_M
- ☞ Quantitative analysis
  - ☆ Direct and Indirect methods
- ☞ Applications of TLC
- ☞ HPTLC (High Performance Thin Layer Chromatography)

INTRODUCTION

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

PRINCIPLE

The principle of separation is ***adsorption***. One or more compounds are spotted on a thin layer of adsorbent coated on a chromatographic plate. The mobile phase solvent flows through because of capillary action (against gravitational force). The components move according to their affinities towards the adsorbent. The component with ***more affinity*** towards the stationary phase ***travels slower***. The component with ***lesser affinity*** towards the stationary phase ***travels faster***. Thus the components are separated on a thin layer chromatographic plate based on the affinity of the components towards the stationary phase.

ADVANTAGES OF TLC

1. Simple method and cost of the equipment is low.
2. Rapid technique and not time consuming like column chromatography.
3. Separation of μg of the substances can be achieved.
4. Any type of compound can be analysed.
5. **Efficiency of separation:** Very small particle size can be used which increases the efficiency of separation. Flow rate is not altered because of the particle size since it is not a closed column. It is a planar type having thin layer of adsorbent.
6. Detection is easy and not tedious.
7. Capacity of the thin layer can be altered. Hence analytical and preparative separations can be made.

8. Corrosive spray reagents can be used without damaging the plates.
9. Needs less solvent, stationary phase and time for every separation when compared to column chromatography.

PRACTICAL REQUIREMENTS

1. Stationary phases
2. Glass plates
3. Preparation and activation of TLC plates
4. Application of sample
5. Development tank
6. Mobile Phase
7. Development technique
8. Detecting or visualising agents

1. STATIONARY PHASES

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

Name	Composition	Adsorbent : water ratio
Silicagel H	Silicagel without binder	1 : 1.5
Silicagel G	Silicagel + $CaSO_4$	1 : 2
Silicagel GF	Silicagel + Binder + flourescent indicator	1 : 2
Alumina Neutral Basic	Al_2O_3 without binder	1 : 1.1
Acidic		1 : 2
Al_2O_3 G	Al_2O_3 + binder	1 : 5
Cellulose powder	Cellulose without binder	1 : 6
Cellulose powder	Cellulose with binder	1 : 2
Kieselguhr G	Diatomaceous earth + binder	1 : 9 ($CHCl_3$: CH_3OH = 2 : 3)
Polyamide powder	Polyamide	

2. GLASS PLATES

Glass plates which are specific dimensions like 20cm x 20cm (Full plate), 20cm x 10cm (Half plate), 20 cm x 5cm (Quarter plate) can be used. These dimensions are used since the width of the commercially available TLC spreader is 20cm.

Microscopic slides can also be used for some applications like monitoring the progress of a chemical reaction. The development time is much shorter like 5 minutes.

Glass plates of different dimensions can also be used when the TLC plates are prepared without the use of TLC spreader. In general, the glass plates should be of good quality and should withstand temperatures used for drying the plates.

3. PREPARATION AND ACTIVATION OF TLC PLATES

The slurry, which is a mixture of stationary phase and water is prepared by using the ratio mentioned earlier. After preparing the slurry, the TLC plates can be prepared by using any one of the following techniques: pouring, dipping, spraying and spreading.

In ***pouring*** technique, the slurry is prepared and poured on to a glass plate which is maintained on a levelled surface. The slurry is spread uniformly on the surface of the glass plate. After setting, the plates are dried in an oven. The disadvantage is that uniformity in thickness cannot be ensured.

In ***dipping*** technique, two plates (either of standard dimensions or microscopic slides) are dipped in to the slurry and are separated after removing from slurry and later dried. The disadvantage is that a larger quantity of slurry is required even for preparing fewer plates.

Spraying technique resembles that of using a perfume spray on a cloth. The suspension of adsorbent or slurry is sprayed on a glass plate using a sprayer. The disadvantage is that the layer thickness cannot be maintained uniformly all over the plate.

Spreading is the ***best technique*** where a TLC spreader (Fig 14.1) is used. The glass plates of specific dimensions (20cm x 20cm/10cm/5cm) are stacked on a base plate. The slurry after preparation is poured inside the reservoir of TLC spreader. The thickness of the adsorbent layer is

adjusted by using a knob in the spreader. Normally a thickness of **0.25mm** is used for analytical purpose and 2mm thickness for preparative purpose. Then the spreader is rolled only once on the plates. The plates are allowed for setting (air drying). This is done to avoid cracks on the surface of adsorbent. After setting, the plates are activated by keeping in an oven at 100°C to 120°C for 1 hour.

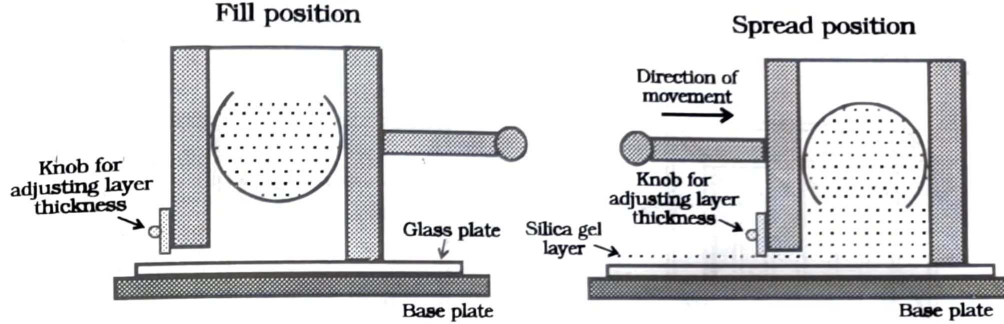


Fig 14.1. TLC Spreader

Activation of TLC plates is nothing but removing water / moisture and other adsorbed substances from the surface of any adsorbent, by heating at high temperature so that adsorbent activity is retained. The activated plates can be stored in thermostatically controlled oven or in desiccator and can be used whenever required.

4. APPLICATION OF SAMPLE

Usually to get good spots, the concentration of the sample or standard solution has to be minimum. 2 - 5μl of a 1% solution of either standard or test sample is spotted using a capillary tube or micropipette. The spots can be placed at random or equidistant from each other by using a template, with markings. The spots should be kept atleast 2cm above the base of the plate and the spotting area should not be immersed in the mobile phase in the development tank. Atleast 4 spots can be spotted conveniently on a quarter plate (20cm x 5cm).

5. DEVELOPMENT TANK

For the purpose of development, a developing tank (Fig 14.2) or chamber of different sizes to hold TLC plates of standard dimensions are used. These require more solvents for developing the chromatogram. When a new method is developed, it is better to develop in glass beakers,

specimen jars, etc, to avoid more wastage of solvents. When developed method or standard method is used, it is better to use development tank. New type of development tanks (Fig 14.3) have hump in the middle, which require less solvent. The development chamber or tank should be lined inside with filter paper moistened with the mobile phase so as to saturate the atmosphere. If this kind of saturation of the atmosphere is not done, **"edge effect"** occurs where the solvent front in the middle of TLC plate moves faster than that of the edge. Therefore the spots are distorted and not regular (Fig 14.4).

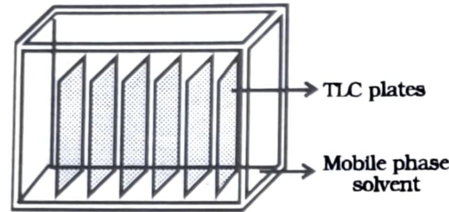


Fig 14.2. Developing tank

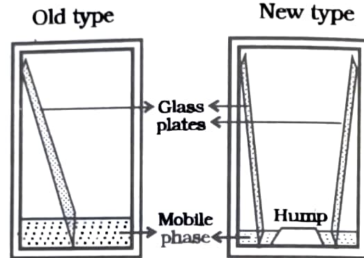


Fig 14.3. Lateral view of developing tank

6. Mobile Phase

The solvent or the mobile phase used depends upon various factors as mentioned in column chromatography. Some of the factors are:

i. Nature of the substances to be separated

ii. Nature of the stationary phase used

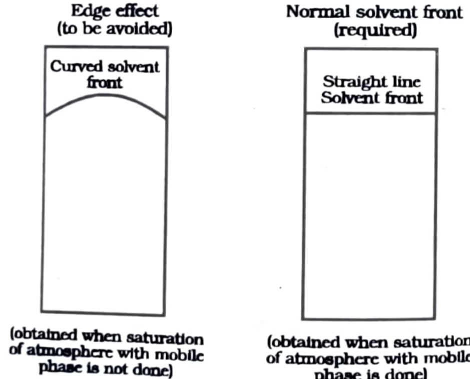


Fig 14.4. Edge effect and Normal TLC

iii. Mode of chromatography (Normal phase or reverse phase)

iv. Separation to be achieved - Analytical or preparative

Pure solvents or mixture of solvents are used. The following gives a list of solvents (of increasing polarity)

Petroleum ether, Carbon tetrachloride, Cyclohexane, Carbondisulfide, Ether, Acetone, Benzene, Toluene, Ethyl acetate, Chloroform, Alcohols

(Methanol, Ethanol), Water, Pyridine, Organic acids, mixture of acids or bases with Pyridine or Alcohols, etc. The solvent composition is done by trial and error method only but with a review of literature and other logical considerations like solubility of the substance, polar or non polar character of the samples, etc. Some examples of solvent compositions are given in the applications of TLC.

7. DEVELOPMENT TECHNIQUE

Different development techniques are used for efficient separations. They are

i. One dimensional development (Vertical)

ii. Two dimensional development

iii. Horizontal development

iv. Multiple development

i. **One dimensional development (Vertical):** In this technique, the plates are kept vertical and the solvent flows against gravity, because of capillary action. Most separations done practically are of this type only.

ii. **Two dimensional technique:** Although one dimensional technique is sufficient for most samples, for complex mixtures two dimensional technique is used. First, the plates are developed in one axis and the plates after drying are developed in the other axis. When large number of compounds cannot be separated by using one dimensional technique, this technique is followed. Fig 14.5. explains the two dimensional development of separation of mixture of several amino acids.

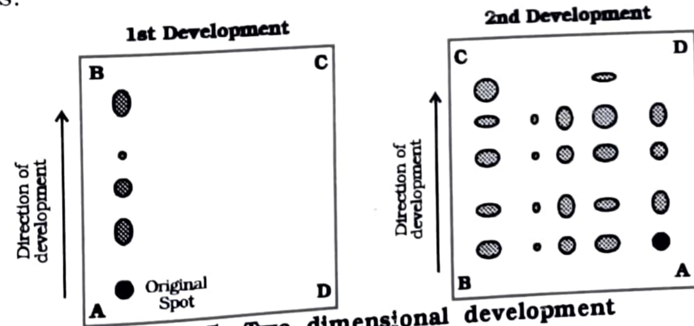


Fig 14.5. Two dimensional development

8. DETECTING OR VISUALISING AGENTS

After the development of TLC plates, the spots should be visualised. Detecting coloured spots can be done visually. But for detecting colourless spots, any one of the following techniques can be used.

a. **Non specific methods:** Where the number of spots can be detected, but not the exact nature or type of compound.

Examples

i. **Iodine chamber method:** where brown or amber spots are observed when the TLC plates are kept in a tank with few iodine crystals at the bottom.

ii. **Sulphuric acid spray reagent:** 70 - 80% v/v of sulphuric acid with few mg of either potassium dichromate or potassium permanganate or few ml of nitric acid as oxidising agent is used. This reagent after spraying on TLC plates is heated in an oven. Black spots are seen due to charring of compounds.

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

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

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

i. Ferric chloride - for Phenolic compounds and tannins
ii. Ninhydrin in acetone - for amino acids
iii. Dragendroff's reagent - for alkaloids
iv. 3,5 - Dinitro benzoic acid - for cardiac glycosides
v. 2,4 - Dinitrophenyl hydrazine - for aldehydes and ketones

The detecting techniques can also be categorised as

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

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

In densitometric method, ***Densitometer*** is used which measures quantitatively the density of the spots. When the optical density of the spots for the standard and test solution are measured, the quantity of the substance can be calculated. The plates are neither destroyed nor eluted with solvents to get the compounds. This method is also called a ***in-situ method***.

QUALITATIVE ANALYSIS

The ***Rf*** value (Retardation factor) is calculated for identifying the spots i.e. in Qualitative analysis. ***Rf value is the ratio of distance travelled by the solute to the distance travelled by the solvent front***.

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

The R_f value ranges from 0 to 1. But ideal values are from 0.3 to 0.8. R_f value is specific and constant for every compound in a particular combination of stationary and mobile phase. When the R_f value of a sample and reference compound is same, the compound is identified by its standard. When the Rf value differs, the compound may be different from its reference standard.

R_x value

R_x value is nothing but the ratio of distance travelled by the ***sample*** and the distance travelled by the ***standard***. R_x value is always closer to 1.

R_m values

R_m value is used in qualitative analysis to find out whether the compounds belong to a homologous series. If they belong to a homologous

series, the ΔR_m values are constant. The ΔR_m values for a pair of adjacent member of a homologous series is determined by using the formula:

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

QUANTITATIVE ANALYSIS (Direct and Indirect methods)

Direct method: (Using densitometer) The quantity of the individual spots can be determined by using densitometric method. Densitometric technique is called a in-situ method and is described earlier in detecting techniques.

Indirect method: Quantitative analysis can be done after eluting the individual spots with solvent and filtering off the stationary phase. The solution can be concentrated and the exact quantity of the compound determined by conventional methods like colorimetry, UV spectrophotometry, flourescence method, flame photometric method, electrochemical methods of analysis, etc.

APPLICATIONS OF TLC

The applications are wider and there is no limitation to the compounds that can be analysed by TLC. Anyhow different types of applications are listed below.

1. Separation of mixtures of drugs of chemical or biological origin, plant extracts, etc.
2. Separation of carbohydrates, vitamins, antibiotics, proteins, alkaloids, glycosides, etc.
3. **Identification of drugs**

Drug	Stationary phase	Mobile phase	Detecting agent
Aminocaproic acid	Silica Gel G	Alcohol:H_2O:NH_3 (25 : 3 : 4)	Ninhydrin in alcohol and pyridine
Amoxycillin trihydrate	Silica Gel H.F-254	Buffer pH 6 : acetone (4 : 1)	NaOH+Starch+glacial acetic acid + Iodine in potassium iodide
Ampicillin for oral suspension	Cellulose M.N-300	Citric acid : Butyl alcohol (5 : 1)	Starch iodide reagent
Chlorpromazine HCl	Silica Gel G	Ether : Ethyl acetate : NH_3	UV 254nm
Cimetidine	Silica Gel G	Alcohol : Ammonia (10 : 1)	Iodine vapour
Digitoxin	Kiesalguhr G	Xylene : Ethylmethyl ketone : HCHO (50 : 4 : 4)	Trichloro acetic acid + Chloramine T
Levodopa	Micro crystalline cellulose	n-Butanol : Glacial acetic acid : water (50 : 25 : 25)	Potassium ferricyanide
Methyldopa	Micro crystalline cellulose	n-Butanol : Glacial acetic acid : water (50 : 25 : 25)	Potassium ferricyanide

4. **Identification of related compounds in drugs**

Drug	Name of the related compound	Stationary phase	Mobile phase	Detecting agent
Allopurinol	3-amino pyrazole-4-carbonamide hemisulphate	Cellulose powder with flourescent additive	n-butanol : ammonia	UV 254nm
Bethanidine sulphate	Methylamine Benzylamine Trimethyl guanidine sulphate	Silica Gel G	ethyl acetate : glacial acetic acid : water : alcohol (25 : 12 : 8 : 5)	1. Ninhydrin in isopropanol 2. Potassium iodobismuthate

5. **To detect the presence of foreign substances in drugs**

Drug	Name of the foreign substance	Stationary phase	Mobile phase	Detecting agent
Amodiaquin HCl	4(7-chloro-4-quinolylamino) phenol HCl	Silica Gel G	Chloroform : Ethylmethyl ketone : diethyl amine (5 : 4 : 1)	$FeCl_3$ + Potassium ferricyanide
Butylated Hydroxy anisole	2 & 3-t-Butyl-1-4-methoxy phenol	Silica Gel G	Chloroform	Phospho molybdic acid + NH_3 vapour
Carbimazole	Methimazole	Silica Gel	Chloroform : acetone (4 : 1)	Potassium iodobismuthate

6. **To detect decomposition products in drugs**

Drug	Name of the decomposition product	Stationary phase	Mobile phase	Detecting agent
Chlordiazepoxide	7-chloro-1,3-dihydro -5-phenyl-1,4-benzo diazepine-2-one-4-oxide	Silica Gel	Alcohol : Ethyl acetate (1 : 24)	N-1-(NED)
Diazepam	-	Silica Gel G.F-254	Hexane : Ethyl acetate (1 : 1)	UV 254nm

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

HPTLC is a sophisticated and automated form of TLC. The following are features of HPTLC:

1. The use of precoated plates with stationary phase particle size of ***less than 10μ*** in diameter.
2. Wide choice of stationary phases like Silica gel, for Normal phase and C18, C8, etc., for Reverse phase mode.
3. Auto sampler instead of manual spotting and streaking for preparative purposes.
4. New type of development chambers which requires less amount of solvents for developing.
5. More efficiency because smaller and uniform size of adsorbents.
6. The use of UV/Vis/Fluorescence Scanner which scans the entire chromatogram qualitatively and quantitatively. The scanner is an advanced type of densitometer.
7. Improved Data processing capabilities by the use of computers.

Preparative TLC

The apparatus, principle, procedure and other requirements are similar to that of analytical TLC. The thickness of adsorbent layer used is 2mm and a non-destructive detecting technique like UV or iodine chamber method is used. The spots are eluted with solvent after scrapping the distinct spots. The solvent is evaporated leaving behind pure component.

15. PAPER CHROMATOGRAPHY (PC)

- Introduction - Paper Partition Chromatography
  - Paper Adsorption Chromatography
- Principle of Separation
- Practical Requirements
  - ☆ Stationary Phase & Papers used
  - Application of sample
  - Mobile phase
  - Development Technique
    - Ascending development
    - Descending development
    - Ascending-Descending development
    - Circular / Radial development
    - Two dimensional development
  - Detecting or visualising agents
    - Non-specific & specific methods
    - Destructive & Non-destructive technique
- Quantitative analysis
  - ☆ Direct and Indirect technique
- Qualitative analysis - R_f, R_x, R_M
- Applications

INTRODUCTION

Paper chromatography is defined as the technique in which the analysis of unknown substances is carried out mainly by the flow of solvents on specially designed filter paper. There are two types of paper chromatography. They are

Paper adsorption chromatography: in which paper impregnated with silica or alumina acts as adsorbent (stationary phase) and solvent as mobile phase.

Paper partition chromatography: in which moisture/water present in the pores of cellulose fibres present in filter paper acts as stationary phase and another mobile phase is used as solvent.

In general, Paper chromatography refers to paper partition chromatography only since most separations are based on partition type only.

PRINCIPLE OF SEPARATION

The ***principle of separation*** is mainly ***partition*** rather than adsorption. Cellulose layers in filter paper contains moisture which acts as stationary phase. Organic solvents or buffers are used as mobile phases. Instead of water as stationary phase, other organic solvents can be used by suitable modification.

PRACTICAL REQUIREMENTS

1. Stationary phase and Papers used
2. Application of sample
3. Mobile Phase
4. Development technique
5. Detecting or visualising agents

1. STATIONARY PHASE AND PAPERS USED

Paper of chromatographic grade consists of α-cellulose - 98-99%, β-cellulose 0.3-1%, pentosans - 0.4-0.8%, ether soluble matter - 0.015 -

0.02%, ash - 0.01 - 0.07%. Whatman filter papers of different grade like No.1, No.2, No.3, No.3MM, No.4, No.17, No.20 etc are used. These papers differ in sizes, shapes, porosities and thickness.

- ☞ Choice of filter paper depends upon thickness, flow rate, purity, technique, etc.
- ☞ **Modified papers** - Acid or base washed filter paper, glass fibre type paper.
- ☞ **Hydrophilic papers** - Papers modified with methanol, formamide, glycol, glycerol etc.
- ☞ **Hydrophobic papers** - Acetylation of OH groups leads to hydrophobic nature, hence can be used for reverse phase chromatography. Silicone pretreatment and organic non-polar polymers can also can be impregnated to give reverse phase chromatographic mode.
- ☞ Impregnation of silica, alumina or ion exchange resins can also be made.
- ☞ **Size of the paper used:** Paper of any size can be used. Paper should be kept in a chamber of suitable size.

2. APPLICATION OF SAMPLE

The sample to be applied is dissolved in the mobile phase and applied using capillary tube or using micropipette. Very low concentration is used to avoid larger zone.

3. MOBILE PHASE

Pure solvents, buffer solutions, or mixture of solvents are used. Some of the examples of ***Hydrophilic mobile phases***:

Isopropanol : Ammonia : Water	- 9:1:2
n-Butanol : glacial acetic acid : water	- 4:1:5
Methanol : water	- 3:1 or 4:1
t-Butanol : water : Formic acid	- 40:20:5

Examples of Hydrophobic mobile phases

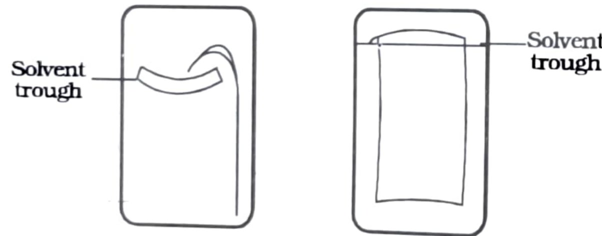
Kerosene : 70% Isopropanol
Dimethyl ether : cyclohexane
Single/two phase or three phase solvent systems are also used.

4. DEVELOPMENT TECHNIQUE

Since paper is flexible when compared to glass plate used in TLC, several types of development are possible which increases the ease and efficiency of operation. They are

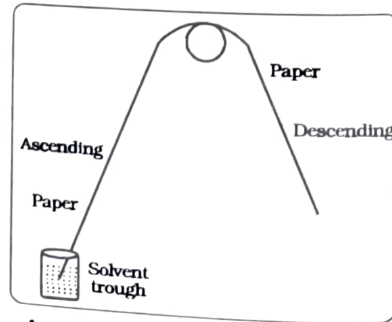
i. **Ascending development:** Like conventional type, the solvent flows against gravity. The spots are kept at the bottom portion of paper and kept in a chamber with mobile phase solvent at the bottom. (same as Fig 14.2 & 14.3 in TLC chapter)

ii. **Descending development:** This is carried out in a special chamber where the solvent holder is at the top. The spot is kept at the top and the solvent flows down the paper. The advantage is that the flow of solvent is assisted by gravity and hence the development is faster.



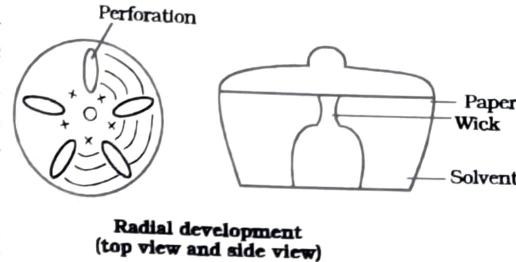
Development chamber (lateral & posterior view)

iii. **Ascending-Descending development:** This is a combination of ascending and descending type. Only the length of separation is increased by using a combination of techniques. First ascending takes place followed by descending development.



Ascending cum descending development

iv. **Circular/ radial development (Horizontal):** Here, the spot is kept at the centre of a circular paper. The solvent flows through a wick at the centre and spreads in all directions uniformly. Hence the individual spots after development look like concentric circles. By making perforations radially, number of quadrants can be created allowing more number of samples to be spotted.



Radial development (top view and side view)

v. **Two dimensional development:** This technique is similar to 2-Dimensional TLC. The paper is developed in one direction and after development, the paper is developed in the second direction allowing more compounds or complex mixtures to be separated into individual spots. In the second direction, either the same solvent system or different solvent system can be used for development. (same as Fig 14.5 in TLC chapter)

5. DETECTING OR VISUALISING AGENTS

After the development of chromatogram, the spots should be visualised. Detecting coloured spots can be done visually. But for detecting colourless spots, any one of the following techniques can be used.

a. **Non specific methods:** Where the number of spots can be detected, but not the exact nature or type of compound.

Examples

i. **Iodine chamber method:** where brown or amber spots are observed when the TLC plates are kept in a tank with few iodine crystals at the bottom.

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

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

Examples

i. Ferric chloride - for Phenolic compounds and tannins

ii. Ninhydrin in acetone - for amino acids

iii. Dragendroff's reagent - for alkaloids

iv. 3,5 - Dinitro benzoic acid - for cardiac glycosides

v. 2,4 - Dinitrophenyl hydrazine - for aldehydes and ketones

The detecting techniques can also be categorised as

i. **Destructive technique:** eg. Specific spray reagents, etc where the samples are destroyed before detection. eg. Ninhydrin reagent.

ii. **Non-Destructive technique:** Like UV chamber method, Iodine chamber method, densitometric method, etc where the sample is not destroyed even after detection.

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

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

QUANTITATIVE ANALYSIS: (Direct and Indirect techniques)

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

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

QUALITATIVE ANALYSIS

R_f VALUE

The R_f value (Retardation factor) is calculated for identifying the spots i.e. in Qualitative analysis. R_f value is the ratio of distance travelled by the solute to the distance travelled by the solvent front.

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

The R_f value ranges from 0 to 1. But ideal values are from 0.3 to 0.8. R_f value is constant for every compound in a particular combination of stationary and mobile phase. When the R_f value of a sample and reference compound is same, the compound is identified by its standard. When the Rf value differs, the compound may be different from its reference standard.

R_x value

R_x value is nothing but the ratio of distance travelled by the sample and the distance travelled by standard. R_x value is always closer to 1.

R_m values

R_m value is used in qualitative analysis to find out whether the compounds belong to a homologous series. If they belong to a homologous series, the ΔR_m values are constant. The ΔR_m values for a pair of adjacent member of a homologous series is determined by using the formula:

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

APPLICATIONS

The applications are wider and there is no limitation to the compounds that can be analysed by paper chromatography. Paper chromatography is more useful for the analysis of polar compounds like amino acids, sugars, natural products, etc. The different types of applications are listed below.

1. Separation of mixtures of drugs of chemical or biological origin, plant extracts, etc

2. Separation of carbohydrates (sugars), vitamins, antibiotics, proteins, alkaloids, glycosides, aminoacids, etc

3. Identification of drugs

Drug	Mobile phase	Detecting agent
Erythromycin estolate	Isobutyl methyl ketone	Nutrient agar containing Bacillus pumilus
Gentamycin	Chloroform : Methanol : Ammonia : Water (10:5:3:2)	Ninhydrin in pyridine - acetone mixture
Vancomycin	t-Amyl alcohol : Acetone : Water (2:1:2)	Nutrient agar containing Bacillus subtilis

4. Identification of impurities

Drug	Mobile phase	Detecting agent
Hydroxocobalamin	s-Butyl alcohol : acetic acid : Potassium cyanide	Elution and measurement of absorbance at 361nm

5. Identification of related compounds

Drug	Mobile phase	Detecting agent
Phenformin HCl	Ethyl acetate : ethanol : water (6:3:1)	Potassium ferricyanide, Sodium nitroprusside & NaOH
Ergotamine injection	Chloroform : methanol	p-dimethyl amino benzaldehyde reagent
Vitamin A	Dioxan : methanol : water with BHA (70:15:5)	UV 366nm

6. Identification of foreign substances in drugs

7. Identification of decomposition products

8. Analysis of metabolites of drugs in blood, urine etc.