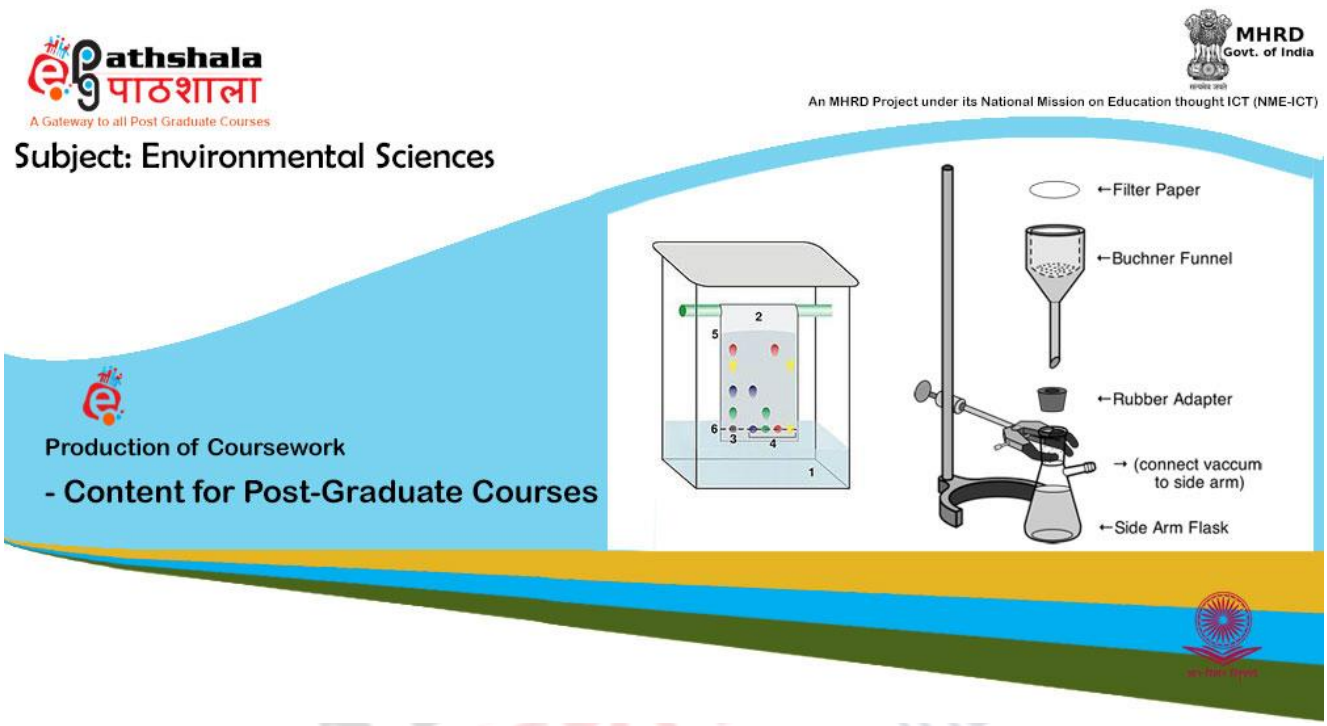

Paper No: 2 **Analytical Chemistry**

Module: 33 **Chromatographic methods: Paper, TLC and Column chromatography**

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Development Team

Principal Investigator & Co- Principal Investigator	**Prof. R.K. Kohli** **Prof. V.K. Garg & Prof. Ashok Dhawan** **Central University of Punjab, Bathinda**
Paper Coordinator	**Dr. J. N. Babu,** **Central University of Punjab, Bathinda**
Content Writer	**Dr. Varinder Kaur** **Department of Chemistry,** **Panjab University, Chandigarh**
Content Reviewer	**Prof. Ashok Kumar** **Punjabi University, Patiala**
Anchor Institute	**Central University of Punjab**

Description of Module	
Subject Name	**Environmental Sciences**
Paper Name	Analytical Chemistry
Module Name/Title	Chromatographic methods: Paper, TLC and Column chromatography
Module Id	EVS/AC-II/33
Pre-requisites	
Objectives	
Keywords	

Module 33: **Chromatographic methods: Paper, TLC and Column chromatography**

Objectives:

1. **To study the basic chromatographic methods**
2. **To study principle, working and applications of paper chromatography**
3. **To study principle, working and applications of column chromatography**
4. **To study principle, working and applications of thin layer chromatography**
5. **To compare paper, column and thin layer chromatography**

PAPER, COLUMN AND THIN LAYER CHROMATOGRAPHY

1. Description

Paper chromatography was first introduced by German scientist Christian Friedrich Schonbein in 1865. It is the technique in which the isolation, identification and quantitation of an unknown substance is carried out on specially designed filter paper. It can be used for the analysis of organic and inorganic compounds.

Principle

This is a type of partition chromatography in which the substances are distributed between two liquids (i.e. stationary liquid held in the fibres of the paper and moving liquid). The stationary liquid is called as stationary phase and moving liquid is named as mobile phase or developing solvent. The components of the mixture migrate with different rates, which is the basis for their separation. The separation can be observed as spots at different positions on the TLC paper.

2. Types of paper chromatography

The paper chromatography is of two types; 1) paper adsorption chromatography, 2) paper partition chromatography. In paper adsorption chromatography, paper immobilized with silica or alumina acts as adsorbent (stationary phase) and solvent as mobile phase. However, in paper partition chromatography, the liquid (mainly moisture) present in the pores of the cellulose fibers of the filter paper act as stationary phase and solvent as mobile phase. Generally, later is referred as paper chromatography.

3. Components of paper chromatography

The various components of paper chromatography are; 1) Stationary phase or paper, 2) Mobile phase, 3) Chromatographic chamber

3.1 Stationary phase or paper used

In paper chromatography, paper is used as stationary phase. The paper used in this techniques contains 98-99% α-cellulose, 0.3-1% β-cellulose. The choice of the filter paper depends upon the nature of solvent and sample to be separated, type of the analysis i.e quantitative and qualitative, and thickness of the paper. Mostly, Whatman filter papers of various grades (No. 1, 2, 3, 4, 20, 40, 42 etc) are used. Other filter papers include the following types.

Modified paper	Hydrophilic paper	Hydrophobic paper
(Acid or base washed filter paper, glass fiber etc)	(Papers modified with methanol, formamide, glycol, glycerol etc)	(Papers having acetylated OH groups

3.2 Mobile phase or developing solvent

The solvents employed for the separation of the components are selected on the basis of the components to be separated. Generally, the polar solvents and buffer solutions are used in paper chromatography. Sometimes, to improve the separation of the components, binary mixtures of the solvents are used. For example; to use hydrophilic mobile phase, mixtures of propanol:ammonia:water, methanol:water and methanol:glacial acetic acid:water can be used as developing solvents. Similarly for hydrophobic systems; mixture of diethyl ether:cuyclohexane and kerosene:isopropanol can be used.

3.3 Chromatographic chamber

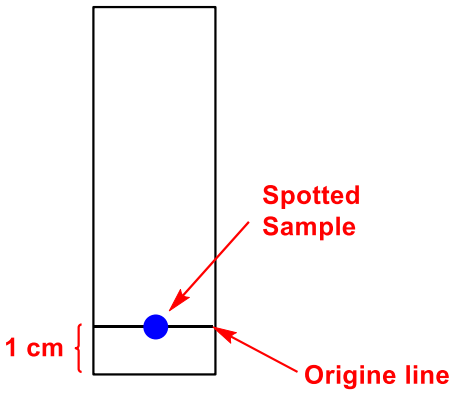
The chromatographic chamber is commonly made up of glass, and less commonly of plastic or stainless steel. The size of the chamber is variable and can be selected on the basis of length of filter paper used. The chamber is saturated with the solvent vapours.

3.4 Mechanism of paper chromatography

The operation of paper chromatography involves following steps.

3.4.1. Preparation of paper

In the first step, paper is cut into desired shape and size depending upon the type of analysis. A horizontal line is drawn on the paper with the help of an ordinary pencil at a distance of 0.5-1 cm from the bottom. The line is marked with points at a distance of 2 cm from each other.

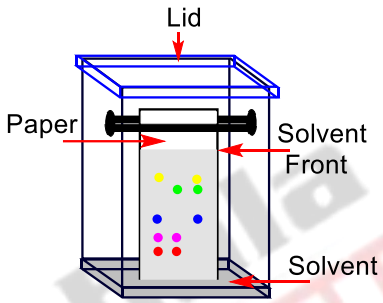


3.4.2. Preparation of solution

The sample is prepared by dissolving a solid sample in a pure solvent. The liquid samples can be applied directly, and biological samples are applied as their extracts.

3.4.3 Application of sample

The sample can be applied by dropping the solution as a small spot on origin line drawn on the chromatographic paper. The spot can be made with the help of a capillary tube or micropipette so that a low concentration can be applied to avoid the larger zone. The spot is dried and is placed in the developing chamber.



3.4.4. Development of chromatogram

The paper with sample spot is placed in the chromatographic chamber saturated with the solvent vapor of selected developing solvent. The edge of the paper is dipped in the solvent in such a way that spot does not dip in the solvent. Allow the solvent to rise up to 2/3rd of the paper height. The filter paper absorbs the developing solvent through its capillary action which reaches the spot of the test solution. The variable mobility of different components in the solvent system separates the components. When solvent travelled to a suitable length the paper is removed from the camber and dried.

Due to the flexibility of the paper, different development techniques can be used to get the efficient and easy separation of components.

1). Ascending development

The ascending development is done by allowing the solvent to move up against the gravity through its capillary action. In this case, solvent is placed at the bottom and lower edge of the paper with spot is dipped in the solvent. Both the sample and developing solvent move in the upward direction.

2). Descending development

This method involves downward movement of solvent as well as spot. In this case, the solvent is kept at the top. The paper is marked with the sample spot at the upper edge. The developing solvent is delivered from the top and it flows down. Therefore, it is called as descending chromatography. This

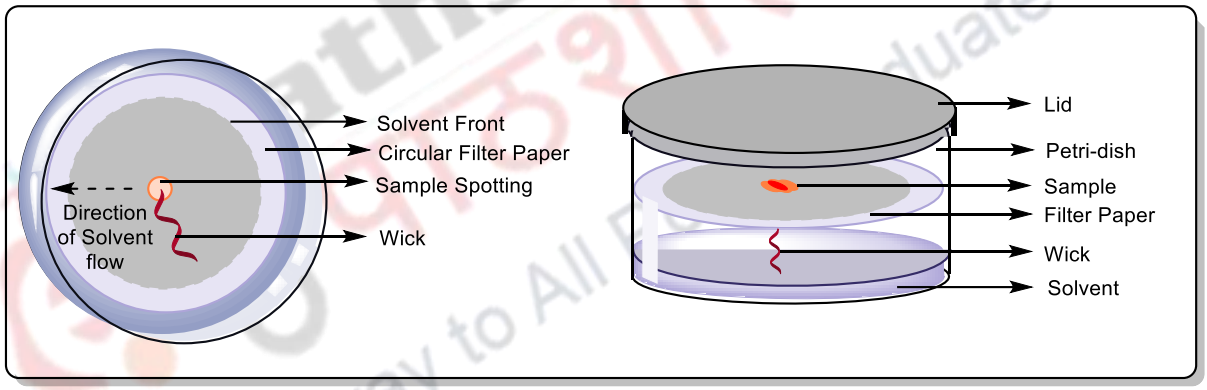
technique involves the movement of solvent under gravity so it is fast as compared to the ascending chromatography.

3). Ascending-descending development

It is the hybrid of ascending and descending chromatography. In this case, 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. Therefore, ascending development is followed by descending development, which increases the length of the run.

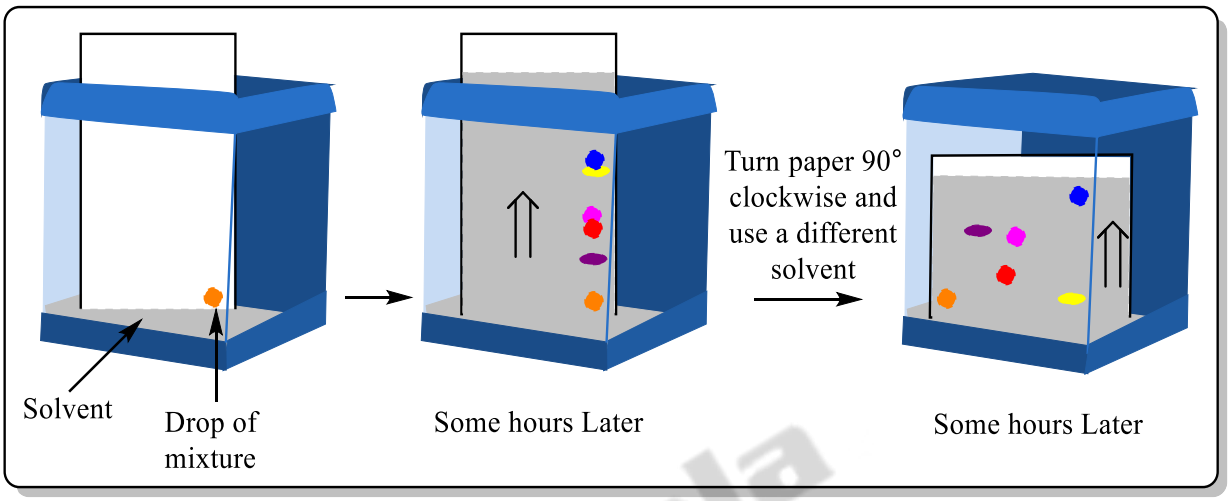
4). Circular/radial development

This method employs a circular filter paper. The sample is placed at the centre of the filter paper and the spot is dried. The paper is placed horizontally on a petry dish containing developing solvent. A wick of the paper is dipped in the solvent and the solvent moves upward resulting in the separation of components in the form of concentric circular spots.



5). Two dimensional development

In this method, chromatogram is developed in two directions using a rectangular paper. The sample is applied on one of the corners of the paper and development is carried out in the upward direction. After the separation of components in the form of various spots, the chromatogram is developed at right angle to the direction of first development.



3.4.5 Drying of chromatogram

The chromatogram is removed from the chamber and position of the solvent front is marked with the pencil. The chromatogram is dried by cold or hot air treatment depending upon the volatility of the developing solvent.

3.4.6 Detection of the chromatogram

The paper chromatograms are detected visually, If the components are colored, the spot can be visualized very easily. However the colourless components are detected by using physical of chemical methods. The detection methods can be categorized into two categories.

1) Non-specific methods (Physical methods)

For the physical detection of colourless components, iodine chamber or UV chamber is used. In iodine chamber, the developed paper is suspended in a closed jar containing crystals of iodine for about one minute. The iodine makes the organic compound spots brown and can be visualized. In UV chamber, the separated components are viewed under UV light. Most commonly, long wavelength (366 nm) and short wavelength (254 nm) light sources are used for this purpose. The spots appear under UV light with a different background.

2) Specific methods

These methods are used specifically for certain compounds or class of compounds. Some of the important examples are given below.

Type of component	Visualizing agent	Visualization
Amino acids, primary and secondary amines	Spraying 0.2% ninhydrin solution in water saturated with butanol on gently heated paper chromatogram	Deep blue or purple spots
Alkaloids	Spraying Dragendroff's agent	Orange or orange-red spots
Phenols	Spraying ferric chloride solution	Blue, green or purple spots
Aldehydes and ketones	2,4-Dinitrophenylhydrazine in a mixture of methanol and sulphuric acid (Brady's reagent)	Bright orange or yellow

4. Measurement methods

These are categorized into two main categories; direct and indirect methods.

1) Direct methods

The direct methods are summarized below.

Method	Features
Comparison of visible spots	It can be used to quantify the components by comparing the intensity and size of the spot with respect to the standard substance
Fluorimetry	Fluorescent compounds or fluorescent derivatives of non-fluorescent compounds can be measured by fluorimetry
Radiotracer method	The compound with radioactive element is labeled with a reagent and measured using Geiger Muller counter
Polarographic and conductometric methods	It is used to quantify the amount of substance in the spot

2) Indirect measurement

In this method the spots are cut and dissolved in an appropriate solvent. The solutions are analyzed by spectrophotometry and electrochemical methods.

5. Measurements in paper chromatography

The movement of a component relative to the solvent is expressed in terms of retardation factor (RF values). It is the movement or migration of a component relative to the solvent front..

Rf = Distance travelled by component /distance travelled by the solvent

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.

The Rf value is affected by the temperature, purity of the solvents, quality of paper, chamber saturation, method of drying and development, pH of the solution etc.

If the solvent front runs off the end of the filter paper, then, movement is expressed in terms of Rx. It is the distance travelled by the substance from the origin line relative to the distance travelled by the standard substance from the origin line.

Rx = Distance travelled by component /distance travelled by the standard substance

Applications of paper chromatography

It can be used for the following applications.

1) Separation of mixture of drugs

2) Separation of mixture of carbohydrates, vitamins, antibiotics, proteins, etc.

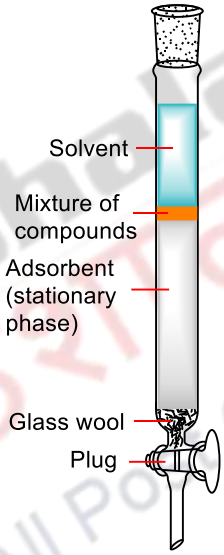
3) Identification of drugs

4) Identification of impurities

COLUMN CHROMATOGRAPHY

1. Description

As discussed earlier, column chromatography was developed by American Chemist D.T. Day in 1900 and M.S. Tswett the Polish botanist in 1906. It is the most useful and common method for the separation and purification of solids as well as liquids. It involves use of solid stationary phase and liquid mobile phase. The separation is based upon the distribution of the components of a mixture between stationary phase and mobile phase.



Principle

The principle of column chromatography is purely adsorption based, in which components are separated on differential adsorption basis. When sample with various components is introduced in the column, the rate at the components move downward is different. The components with lower affinity for the adsorbent move faster and eluted rapidly while the components with higher affinity for the adsorbent move slowly and eluted in the end. This results in the separation of the components.

2. Components of column chromatography

The main components of the column chromatography are column, mobile phase and stationary phase.

2.1 Stationary phase

The stationary phase in the column chromatography is also termed as adsorbent because it works of the principle of adsorption. A stationary phase must fulfill the following requirements.

1) Particles should have uniform size with spherical shape.

2) The adsorbent should be stable chemically as well as mechanically.

3) It should be inexpensive and easily available.

4) It should be used with wide range of compounds.

The stationary phase is selected on the basis of various factors like; nature of the components, length of the column, adsorption affinity of the various components, and quantity of adsorbent required for separation. The main adsorbents used in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch etc. The polar components can be separated on silica and non-polar components are separated on alumina more efficiently.

2.2 Mobile phase

The mobile phase in column chromatography acts performs various functions i.e to introduce sample into the column, to develop the bands in the column (as developing solvent) and to elute the components from the column. Therefore, it should be selected carefully. The main points to be remembered during the selection of mobile phase are;

1) The components of a sample must be soluble in the solvent system chosen.

2) It should be of low boiling point so that components can be recovered at the end.

3) The polarity should be appropriate. Some of polar solvents used in column chromatography are petroleum ether, cyclohexane, acetone, carbon tetrachloride, toluene, esters, benzene, water etc.

2.3 Column

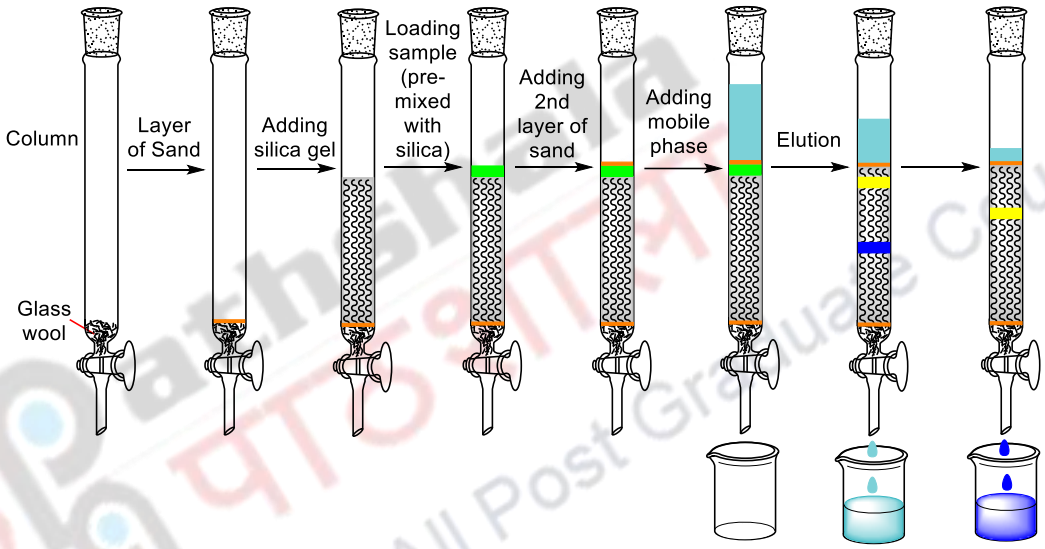
A column is a cylindrical tube made up of glass. It is required for filling the stationary phase. The material used for the fabrication of column should be inert towards various solvents and the components to be separated. The columns may be attached with reservoir at the top and collector at the bottom to make the process easier. A column can have dimensions of 10:1, 30:1 and 100:1, length: diameter. The length of the column is chosen number of components in the sample, quantity of the sample, and type of the adsorbent. Usually long columns are used for better separation.

3. Steps involved in column chromatography

The following steps are followed in column chromatography.

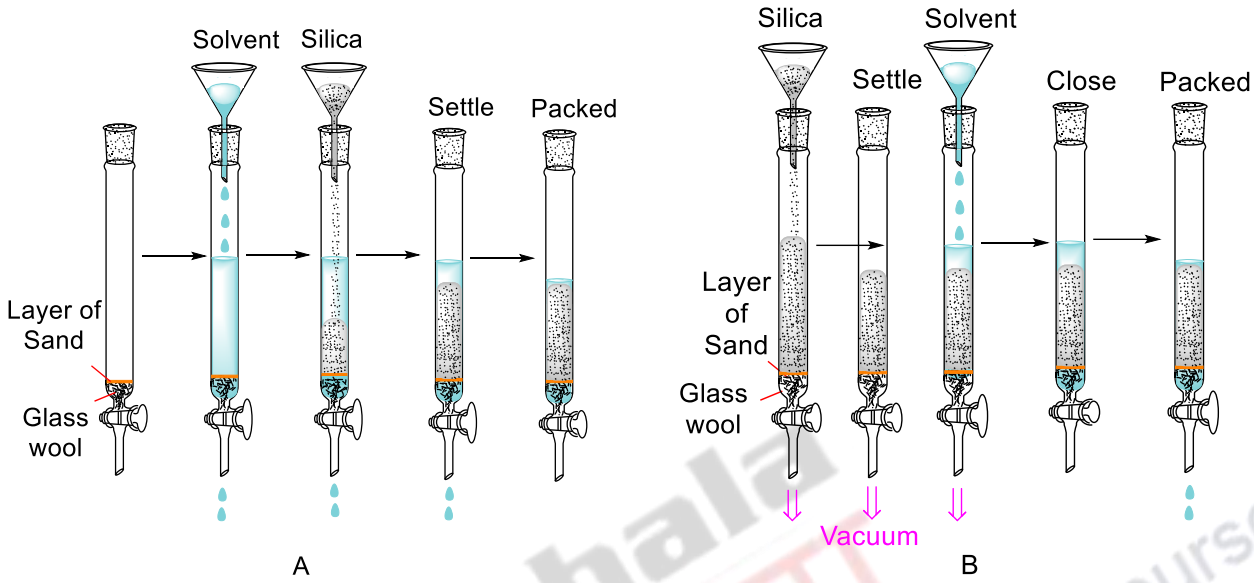
3.1 Preparation of column

Before, the preparation of a column, adsorbent requires pre-treatment. It is activated to increase the adsorption capacity. The activation is done by heating, which removes the adsorbed water. Generally alumina is treated at 400°C and silica at 100°C for activation. To a clean glass column, glass wool/cotton or asbestos pad is inserted at the bottom. Then, adsorbent is introduced in the column to get a uniform packing. At the top of the packing, a paper disc is placed to avoid the disturbance of the packed adsorbent during the application of sample. The packing of a column with adsorbent can be achieved dry packing or wet packing.



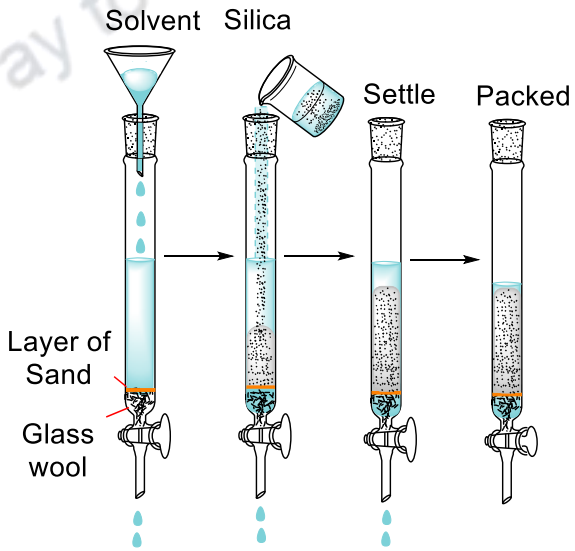
3.1.1 Dry packing (dry filling)

In this method, dry adsorbent is filled in the dry column from the top. After filling the column with adsorbent, the mobile phase is added up to appropriate mark (shown below A). This mixes the adsorbent with the solvent. Sometimes, air bubbles are trapped in the column in dry filling so the column should be tapped after filling to remove the air bubbles. Sometimes solvent is added to the column followed by dry adsorbent as shown below (B).



3.1.2 Wet packing (wet filling)

It is better over dry filling and is most commonly used. In this method, slurry of adsorbent is formed in the mobile phase by stirring. The slurry is added to the column from the top in small portions. A careful addition should be done to avoid the cracks and to get uniform setting of the material in the column (shown below). After setting of material, the solid adsorbent settles at the bottom while solvent covers it at the top. The solvent is removed by knob at the bottom and material is covered with a cotton plug.



3.2 Sample introduction

The sample is prepared by dissolving an appropriate amount in a suitable solvent. The sample solution is added from the top of the column at once to make a zone at the top of the adsorbent. It is allowed to stand for some time so that the components come down and get adsorbed on the stationary phase.

3.3 Elution

The separated components are eluted from the column by the eluting solvent. There are two elution techniques; isocratic and gradient. In isocratic elution, same composition of solvent is used throughout the separation. However, in gradient elution, the composition of the eluting solvent can be varied gradually in a run as per the nature of the components to be separated. For example; when a separation is achieved by using chloroform throughout the separation, elution is isocratic and if the polarity of the solvent is varied from benzene, chloroform and then ethyl acetate, then elution is gradient.

3.4 Detection

The separation of components can be visualized if the separation is in the form of colored bands. In case of colorless components, the elution of component can be detected by thin layer chromatography (to be discussed in next section). For this, the fractions can be collected in separated vials and monitored by TLC. Sometimes, developers are used to visualize the separation as bands like hydrogen sulphide and potassium ferrocyanide.

4. Factors affecting column chromatography

The efficiency of column chromatography can be improved.

1) By increasing the ratio of length to width of the column

2) By decreasing the size of the particles of stationary phase

3) By increasing the temperature during elution

4) By selecting solvent with low viscosity

5) By improving the packing of the column

5. Applications of column chromatography

Column chromatography can be used for the following applications.

1) Separation:

It can be used for the separation of inorganic ions (like metal cations and anions), organic molecules (like dyes, diastereomers, tautomers, racemic mixtures, cis-trans isomers), plant extracts, drugs and pharmaceuticals.

2) Purification

It is used for the purification of a component.

3) Determination of drugs

It can be used for the estimation of drugs; for example, primary and secondary glycosides, phytomeandione in injections and tablets etc.

6. Advantages and disadvantages

The column chromatography can be used for the separation of almost all the compounds. It can be used for wide range of amounts of the components and mobile phase. However, the technique is time consuming, involves loss of components and requires lot of solvents.

THIN LAYER CHROMATOGRAPHY (TLC)

1. Description

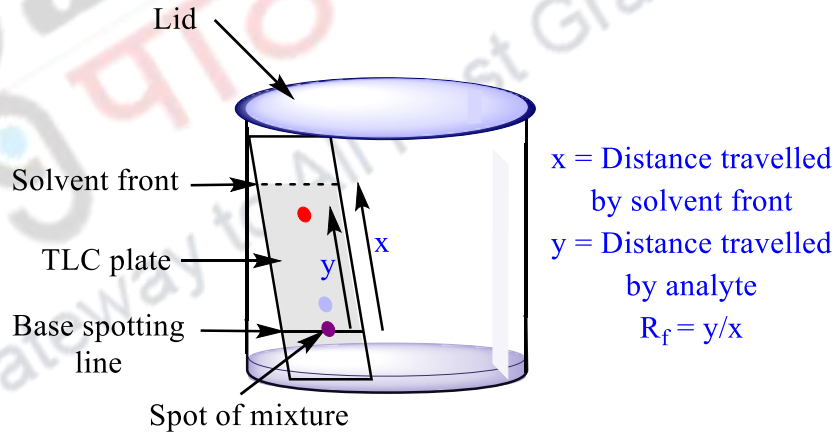
It is the simplest and most commonly used chromatographic technique. It involves the separation, purification and qualitative/quantitative analysis of organic compounds. It utilizes a mobile phase and a stationary phase in the form of a plate or strip. It is used for non-volatile or less volatile substances. The substances can be polar, less polar or non-polar. At the same time, a large number of samples can be analyzed cost-effectively and efficiently.

Principle

The thin layer chromatography works on the principle of adsorption or partition or combination of both. The components with lower affinity for the stationary phase travel downward slowly and the components with higher affinity for adsorbent move slowly, which results in the separation of components.

2. Components of TLC

The main components of TLC are stationary phase i.e. TLC plate and mobile phase.



2.1 Stationary phase or TLC plate

In TLC, the adsorbent is coated as a thin layer on a solid support (either thin sheet of glass or plastic or aluminium). The stationary phase is selected on the basis of the nature of components of the sample. For example; hydrophilic and hydrophobic nature, acidic, basic or amphoteric nature of the components is considered. Secondly, the particle size should be appropriate and adsorbent should not

adhere to the glass plate. Various stationary phases used in TLC include silica, alumina, cellulose, sephadex or their modified forms.

2.2 Mobile phase (solvent system)

The main characteristics of mobile phase are same as discussed previously. It performs the function of a solvent for dissolving substances, as mobile phase to move the components across stationary phase and to separate the components as per their mobility in the solvent. Therefore, the solvents used must be pure, stable, less viscous, less volatile and of low toxicity. The Most commonly, the mobile should have ability to dissolve or desorb the substances effectively. The adsorption of solvent on the adsorbent should be high so that the component can be desorbed efficiently. For example, a mixture of water and n-butanol can be used for the separation of polar or ionic solutes. For hydrophobic stationary phases, mixtures of benzene, cyclohexane and chloroform can be best as mobile phase.

3. Steps involved in TLC

The steps followed in the TLC are given below.

3.1 Preparation of chromatoplates

The glass plates or aluminium plates are used for TLC. The size of the plates is chosen depending upon the size of the sample. The standard sizes available commercially are 20 × 5 cm, 20 × 20 cm, and 20 × 10 cm. The surface of the plates should be smooth and uniform. The plates are coated with the stationary phase to get desired thickness of film, which is usually 250 μm. The plates can be prepared by pouring, dipping, spreading or spraying. In 'pouring', the finely divided adsorbent is mixed with solvent to form homogenous slurry. The slurry is poured on the plate and is kept undisturbed to cover the whole plate with slurry uniformly. In 'dipping', the plates are prepared by dipping in slurry of adsorbent. The volatile solvent is evaporated and the stationary phase is coated on the surface. In 'spraying', the slurry is diluted further in order to spray the mixture on the surface of plate. This technique is not very common these days. In 'spreading', commercially available spreaders are used to spread the adsorbent uniformly on the surface of the plate. Two types of spreaders i.e. moving spreader and moving plate spreader are used to get thin and uniform films of thickness from 0.2 to 2.0 mm. The plates are dried and activated by heating at about 100°C for 30 min.

3.2 Preparation of developing container

The developing container is a specially designed chamber (a jar with a lid or beaker with a watch glass). The solvent is poured into the jar to cover the bottom up to 0.5 cm. The chamber is covered with beaker and allowed to stand.

3.3 Sample application

The sample is disoolved in an appropriate solvent to make 1% of the solution. The spot should be small to get a better and clear resolution. Firstly, two lines are drawn horizonatlly at the bottom and top (1 cm away from the edge) of plate as discussed in paper chromatography. The sample is spotted using a capillary tube or micropipette.

3.4 Separation of components

The TLC plate is placed in the developing chamber and covered. It is left undisturbed. The solvent moves slowly upward by capillary action. When it reached the line marked at the top, the plate is removed from the chamber.

3.5 Visualization of spots

The colored spots can be visualized and marked to calculate the Rf value. For colourless sample, UV lamp is used to visualize the spots. Sometimes, visualizing reagents are used for this purpose like iodine vapour, 2,4-dinitrophenylhydrazine, ninhydrin etc.

4. Application of TLC

1. It is used for the separation of natural products and is used in pharmaceutics.
2. It is used to check the purification of samples.
3. It is used to monitor the reactions.
4. It is used to identify the organic compounds.
5. It is used for the separation of inorganic ions i.e cations and anions.
6. It is used for the separation of vitamin E, vitamin D3 and vitamin A.
7. It is used for the separation of amino acids.

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