Department of Chemistry

Paper:C-14-Analysis of Organic compounds Unit 4 and 5 Separation

Methods-Chromatographic Techniques

Introduction:

Chromatography is a non-destructive procedure for resolving a multi-component mixture of trace, minor or major constituents into its individual fractions. Its variations may be applied to solids, liquids and gases both qualitatively and quantitatively.

Chromatography is a new technique invented by ***M. Tswett***, a botanist in 1906 in Warsaw. He successfully separated chlorophyll, xanthophylls and several other pigments 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 which produced coloured bands at different positions on the column. Tswett termed this system of coloured bands as the ***chromatogram*** and the method as ***Chromatography*** [Greek; *chroma* = colour, *graphos* = writing]. However, in the majority of chromatographic procedures no coloured products are formed and the term is a misnomer.

The column of calcium carbonate in Tswett's method remains stationary and is therefore called as stationary phase. The solution of vegetable extracts moves or flows down the column and is therefore termed as mobile phase.

Chromatography is a method of separation in which the separation of solutes occurs in stationary phase and mobile phase. Chromatography is probably the most important single analytical technique used of today and tomorrow.

Applications:

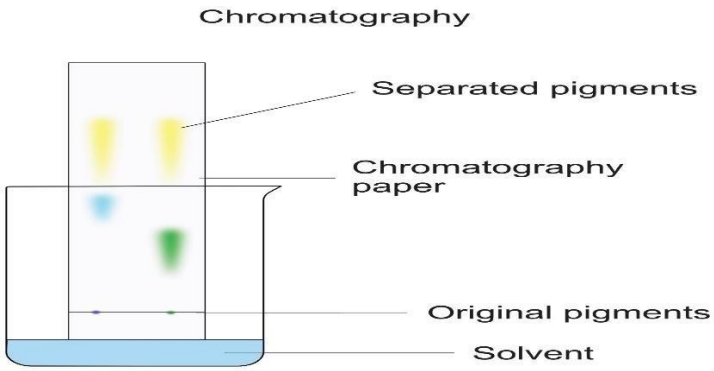
Chromatographic techniques are used

- To establish the purity and authenticity of starting materials and reagents
- To monitor the progress of a reaction
- To check the isolation and purification procedures
- To achieve the separation of product mixtures which were not possible by means of distillation, recrystallisation or sublimation
- To provide a further check on the authenticity of the final product in addition to physical constants and spectroscopic data
- For elemental analysis when coupled with atomic absorption spectroscopy

Definition of Chromatography

In Greek chromatography means colour writing.

Chromatography is a separation technique used for resolving a mixture into individual components through distribution equilibrium between stationary and mobile phases



It is based on the differences in the rate at which the components of a mixture move through a porous medium, stationary phase under the influence of some solvent or gas, mobile phase.

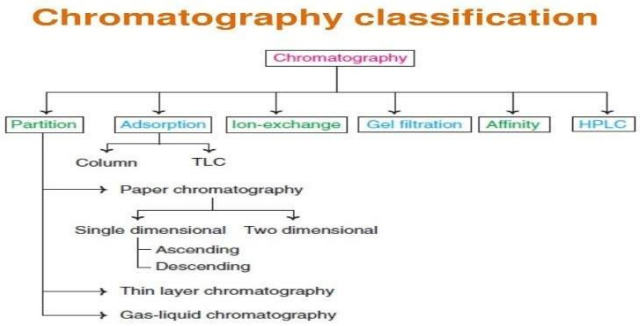
Types of Chromatography - Classification Methods

Classification methods of chromatography are based on

(1) Nature of the mobile phase or two phases

(2) Basis of stationary phase alone

(3) Based on differences in physical properties of compound in stationary phase (or) difference in the interaction of components with stationary phase.



(1) Based on the nature of mobile phase or two phases.

In Chromatography, the stationary phase may be either solid or liquid and the mobile phase may be either liquid or gas. Based on the physical state of the stationary and mobile phases Chromatography can be of six types.

Stationary phase	***Mobile phase***	***Technique***
1. Solid	*Liquid*	*1. Paper Chromatography*
		2. Thin layer Chromatography
		3. Adsorption or Column Chromatography
		4. High performance liquid Chromatography
2. Solid (ion exchange resin)	*Liquid*	*5. Ion exchange Chromatography*
3. Solid	*Gas*	*6. Gas-solid Chromatography*
4. Solid matrix (porous gel)	*Liquid*	*7. Gel permeation Chromatography or Exclusion Chromatography*
5. Liquid	*Gas*	*8. Gas-liquid Chromatography*
6. Liquid	*Liquid*	*9. Liquid-liquid Chromatography*
		10. Countercurrent Chromatography

(2) Based on the nature of stationary phase alone: 4 types

(a) Column chromatography: In this type, powdered solid which is stationary phase is packed in a long narrow glass or metal tube and the mobile phase percolates through the powdered solid.

(b) Paper Chromatography: Filter paper is used as a stationary phase and the solvent is the mobile phase.

(c) Thin Layer Chromatography: Finely divided solid coated as thin layer acts as stationary phase and solvent as mobile phase.

(d) Exclusion Chromatography: Molecular sieves are used as stationary phase. Components are separated on the basis of differences in their sizes by passing through a column containing molecular sieves. This is also called as Gel filtration.

(3) Based on the difference in the physical properties of the components in stationary phase or difference in the type of interaction of components with stationary phase.

The component to be separated must be soluble in mobile phase, when mobile phase carrying dissolved components percolate through or along the stationary phase or the components interact with stationary phase in different ways. On the basis of interactions, chromatographic

methods are classified as follows:

(a) Partition chromatography: When the component gets dissolved in stationary phase, they are partitioned between two phases then it is partition chromatography.

(b) Adsorption chromatography: When the component is adsorbed on a stationary phase it is called adsorption chromatography.

(c) Ion exchange Chromatography: When the ionic component in the mobile phase reacts chemically with ionic component in stationary phase, then it is ion-exchange chromatography.

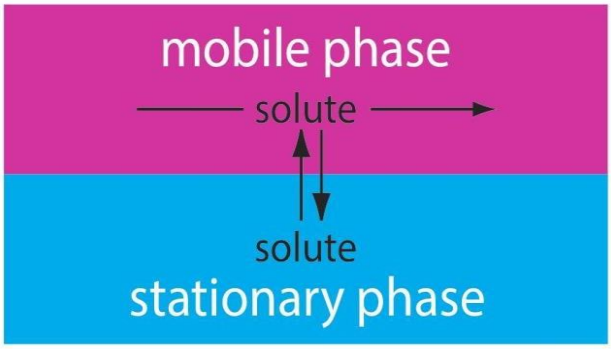
Principles of differential migration

In chromatography the components to be separated from a sample are distributed between two phases.

(1) Stationary Phase: Either solid or liquid held by the solid.

(2) Mobile Phase: Either liquid or gas.

The sample for separation originally present in mobile phase flows through the stationary phase. Separation of individual components from sample is based on the differences in the rates of migration. Two factors are responsible for the different rates of migration of components.



(a) Equilibrium and *(b) Time Duration*

(a) Equilibrium: Removal of component from mobile phase by a stationary phase is an equilibrium process. "The rate of travel of an individual component is directly proportional to partition or distribution of that component between mobile and stationary phase".

Partition or distribution coefficient K_d can be defined as $K_d = \frac{C_S}{C_M}$

where C_S *is conc. of component in stationary phase*

C_M *is conc. of component in mobile phase*

Components which interact strongly with stationary phase will pass on to stationary phase and components which do not interact with stationary phase will pass rapidly through stationary phase. This causes differences in migration rates.

(b) Time Duration: Retardation or retention means the overall time spent by the component in stationary phase. No two components will have the same retention time. Thus the components get separated and affect the migration rates.

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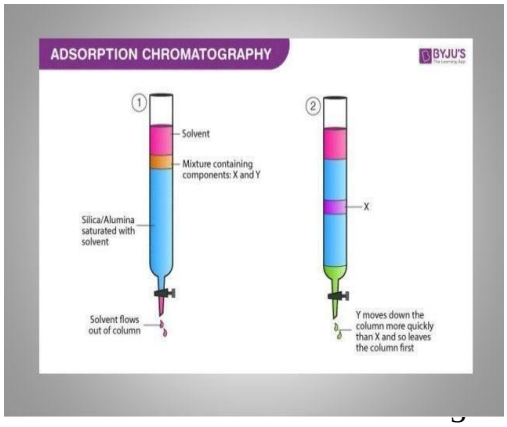
Adsorption

Molecules of a liquid or solid present on the surface behave differently from molecules which are present in the interior on all sides. Hence the intermolecular forces of attraction are exactly equal in all the directions. On the other hand, the molecules at the surface of liquid are surrounded by large number of molecules in liquid phase and few molecules in vapour phase. As a result, net inward force of attraction exerted by the molecule at the surface of the solid. This force



causes liquid or solid to attract and retain the molecules of solute, when comes in contact with them. *This phenomenon of retaining the molecules of a substance on the surface of a liquid or solid is called* ***adsorption****.*

Adsorbents: *Compounds such as silica gel (silicic acid), aluminium oxide, calcium carbonate, magnesium carbonate, magnesium oxide and cellulose may be used as adsorbents.*

Example 1. *Hydroxapatite (calcium phosphate) is widely used for the separation of proteins, nucleic acids and viruses. Care is taken in the choice of adsorption, since some adsorbents may degrade certain compounds during separation, some have a tendency to take up water from the atmosphere and this may affect their adsorption properties.*

Adsorbents for thin layer chromatography *are often impregnated with various ions during plate preparation to enhance the separation.*

For example, the incorporation of silver nitrate with the adsorbent enhances the separation of compounds differing in the number and position of double bonds. This is referred to as argentation TLC. On the basis of relative polarity, adsorbents are classified as (a) Strong (b) Intermediate (c) Weak.

(a) Strong. More polarity, for example Alumina, Silica

(b) Intermediate. $CaCO_3$, $MgC1_2$

(c) Weak adsorbents. Non polar e.g., starch, charcoal.

(d)

Classification of adsorbents: *The stronger adsorbents naturally have more polar structure. Their capacity to take part in hydrogen bonding also plays an important role.*

The strength of adsorption of polar groups on polar compounds increases in the order

—CH=CH—<—OCH_3<—COOR<=C=O<—CHO <—SH <—NH— <— OH <—COOH

In order to minimize chemisorption, acidic adsorbents are preferred for adsorbents of acids and bases.

Classification of adsorbents based on acidity

(1) Acidic, Basic and Neutral Adsorbents used for different separations

Material or adsorbent	***Nature of adsorbent***	***Separation applications***
1. Alumina	Acidic	Sterols, basic esters, alkaloids, inorganic compounds
2. Silica gel	Acidic	Sterols, amino acids
3. Aluminium silicate	Acidic	Sterols
4. Carbon or activated charcoal	Neutral and acidic	Peptides, amino acids and carbohydrates
5. Calcium carbonate	Neutral	Carotenoids, Xanthophylls
6. Starch	Neutral	Enzymes
7. Sugar	Neutral	Chlorophyll, Xanthophylls
8. Magnesia	Basic	Esters, alkaloids etc.

Characteristics of an adsorbent

1. Surface area. Smaller the particle of adsorbent, greater will be the surface area of the adsorbent.

2. Nature of adsorbent. Different adsorbents adsorb the compound with varying attractive forces. This will depend upon the chemical nature of the adsorbent and the different groups in its surface. Binding power of some adsorbents is in the order of -

Activated Charcoal > Alumina > Silica gel > Sugar > Starch > Cellulose > Paper

Activated charcoal has more binding power than others.

A polar compound weakly adsorbed on paper and strongly adsorbed over charcoal.

3. Particle size. Best particle size is 100-120 inch for 2mm column.

(2)

Solvents used in Chromatographic Technique (Eluents)

In chromatographic method, the solution of a mixture is poured on the solid adsorbent or the mixture in gaseous state is introduced over to the closed column. Using a liquid or gaseous solvent the column is eluted.

n-Hexane
Cyclohexane
CCl_4
Benzene
Toluene
$Cl_2C = CHCl$
$H_5C_2 - O - C_2H_5$
$CHCl_3$
$CH_3COOC_2H_5$
n-Butanol
n-Propanol
Acetone
Ethanol
Methanol
Water

↓

Any organic solvent, if it is pure, can be used as the mobile phase. The choice of solvent depends upon the polarity of the compounds to be resolved and upon their distribution coefficient.

$$K_d = \frac{\text{Concentration of compound in stationary phase}}{\text{Concentration of compound in mobile phase}}$$

If the concentration of compound is more in mobile phase, that compound gets eluted first i.e. compounds with low distribution coefficient values will get eluted first.

Low polarity solvents: n-hexane, n-heptane, toluene, acetonitrate, diethyl ether and chloroform.

Intermediate polarity solvents: ethanoic acid, dichloromethane and pyridine.

Highly polar solvents: propan-1-ol, butan-l-ol, acetone, ethanol, methanol and water.

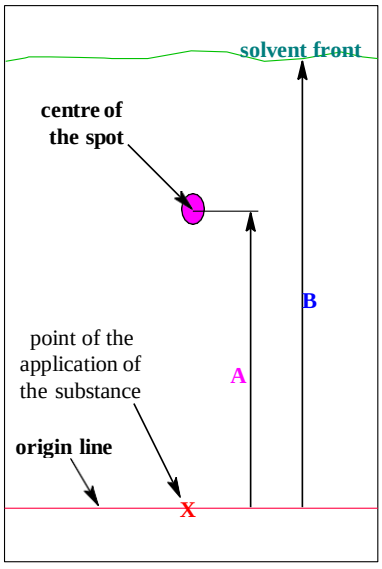
The arrangement of all the solvents in increasing order of polarity is called ***elutotropic series****.*

Migration Parameters (Rf)

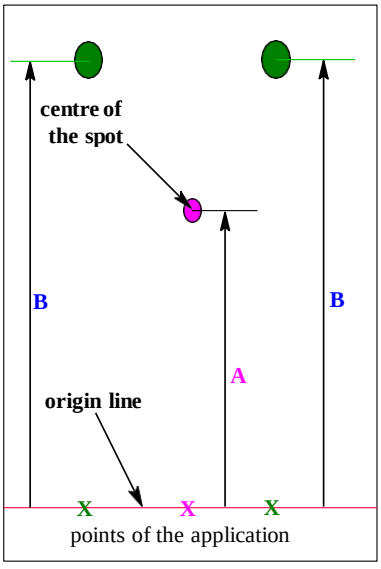
The positions of migrated spots on the chromatogram are indicated by different terms such as R_F, R_X, R_M, and R_C. These are qualitative and quantitative parameters.

(a) R_F (Retention Factor) Values: *The R is related to the migration of the solute front relative to the solvent front as*

$$R_F = \frac{\text{Distance travelled by thesolute front from the origin line}}{\text{Distance travelled by the solvent from the origin line}}$$



Diagramatic representation of R_F



Diagramatic representation of R_x

R is a function of the partition coefficient. It is a constant for a given substance. The R_F defines the movement of a substance relative to the solvent front in a given chromatographic system.

The factors affecting R_F value:

(i) The solvent employed
(ii) The medium used for separation
(iii) The nature of the mixture
(iv) The temperature
(v) Size of the vessel or the developing chamber

(b) R_X Values: *When the solvent front runs off the end of filter paper the movement of a substance is expressed as R_X but not R_F.*

$$R_X = \frac{\text{Distance travelled by thesubstancefrom the origin line}}{\text{Distance travelled by the standard substance (X) from the origin line}}$$

(c) R_M Values: *The R_F values of chemically related compounds are very close. The influence of individual functional groups was presumed to be added to a rough approximation. According to Bate-Smith and Westall, R_M is defined as follows:*

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

R_M is additive and is composed of the partial R_M value of the individual functional groups or other grouping of atoms in the molecules.

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THIN LAYER CHROMATOGRAPHY (TLC or tlc)

Thin-layer chromatography is a smart technique which utilizes a thin (0.25 mm) layer of an inert material such as alumina, magnesia or silica and a mobile phase. It is also called as ***drop, strip, spread layer, surface or open column chromatography.***

Thin-layer chromatography was first introduced by Izmailov and Shraiber in 1938 for separating plant extracts. Consden, Gordon and Martin used filter papers for amino acid analysis. Kirchner separated terpenes using impregnated filter paper and glass – fibre paper coated with silicic acid or alumina. Stahl introduced it as an analytical adsorption chromatography.

Types of TLC: *Adsorption TLC, High Performance TLC, Ion exchange TLC, Partition TLC, Reversed phase TLC and Reversed phase partition TLC techniques are also in use.*

Advantages:

Thin-layer chromatography [TLC] has the following advantages over Paper and Column Chromatography.

- ✓ It requires simple equipment.
- ✓ Short development time [about 1 hour].
- ✓ It has wide choice of stationary phase.
- ✓ It allows early recovery of separated components.
- ✓ Conspicuous separation effects.
- ✓ Easy visualization of separated compounds.
- ✓ Sensitivity is 10 to 100 times that of Paper chromatography.
- ✓ It utilizes variable thickness of thin layers.
- ✓ It utilizes chemically inert stationary phase.

Principle

The basis is the ***partition*** *or* ***distribution coefficient*** which describes the way in which *a compound distributes itself between two immiscible phases. For a compound distributing itself between equal volumes of two immiscible solvents A and B, the value of distribution coefficient is a constant at a given temperature.*

$$K_d = \frac{\text{Concentration in solvent A}}{\text{Concentration in solvent B}}$$

The distribution of a compound can also be described by its distribution between any two phases such as solid / liquid or gas / liquid phases.

Experimental Details or Process of TLC technique

It includes the following sub titles.

(i) The Coating materials
(ii) Preparation of thin layers on plates
(iii) Activation of adsorbent and purification of silica gel ‘g’ layers
(iv) Choice of Solvent systems
(v) Sample application
(vi) Development tank
(vii) Plate development
(viii) Detection of components
(ix) Evaluation of the chromatogram

1. **The Coating materials:** the coating material contains **1. The adsorbent 2. A binder and 3. A visualizing reagent.**

1. The Adsorbent: The choice of adsorbent depends on

- the nature of the components to be separated,
- chemical reactivity of the adsorbent with the solvent or binder.

The following adsorbents are used.

S. No.	*Adsorbent*	*Nature*	*Separatory mechanism*	*Components to be separated*
1.	Silica gel	Acidic	Adsorption partition	Acidic and neutral substances
2.	Alumina	Basic	Adsorption partition	Basic and neutral
3.	Kieselguhr	Neutral	Partition	Hydrophilic substances
4.	Cellulose powder	Neutral	Partition	Water soluble compounds

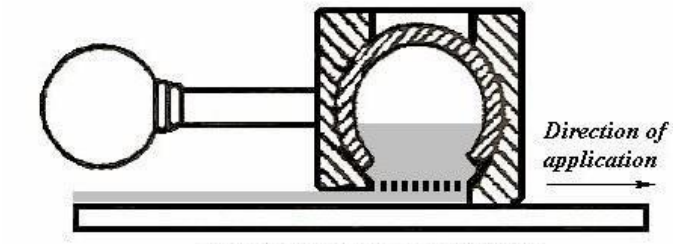
2. A Binder: To bind the adsorbent to the glass plate some binders like gypsum, starch, hydrated silicon oxide, etc are added to adsorbents. Gypsum is probably the most widely used. Trade materials, Silica gel G, Alumina G, etc contain Gypsum as the binder.

3. Visualizing Reagent: The usual practice is to add an inert fluorescent indicator such as zinc silicate to coating materials to facilitate visualization on exposure to UV light after the development of the chromatogram.

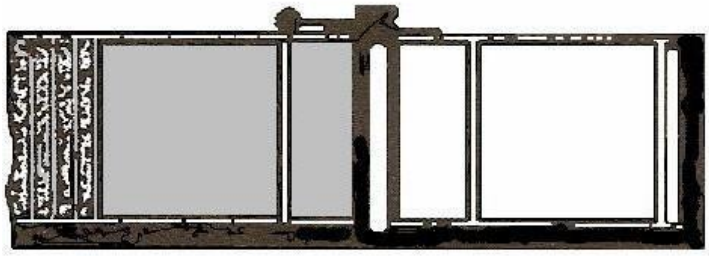
2. **Preparation of thin layers on plates:**

Thin layer should be consistent through out. A large number of applicators are commercially available which are used for coating the glass plates with different adsorbent layers of uniform thickness. The coating material is used in the form of a suspension or slurry in some liquid.

1. **Pouring:** In this method amount of the slurry is put on the given size plate which is kept on level surface. The plate is then tipped back and forth to spread the slurry uniformly over the surface.
2. **Dipping:** In this method plates are prepared by dipping them at a time, back to back, in chloroform-methanol slurries of the adsorbent.
3. **Spraying:** He employs a small point sprayer for distribution of the slurry on the glass plate.
4. **Spreading:** the slurry is placed in an applicator. This is either moved over the stationary plate or it is hold stationary and the plate is pushed or pulled through.



Operation of Thin-layer Spreader


Aligning tray with glass slide partially coated (grey = adsorbent)

- In another apparatus the spreader takes up the spreading mixture and applies it uniformly as a thin layer on the glass slide in an aligning tray.
- An improved TLC spreader can give a layer with thickness of 0.0 to 2.0 mm.

Precoated plates: Ready to use thin layers [0.1 to 0.2 mm] of common adsorbents precoated on glass or plastic sheets are available but expensive.

3. Activation of adsorbent and purification of silica gel 'G' layers:

To remove the liquid associated with the thin layer as completely as possible, the plates are dried for 30 minutes in air and then in oven at 110 ^{o}C for another 30 minutes. This drying makes the adsorbent layers active. In order to obtain very active layers silica gel and alumina plates can be heated to 150^{o}C for about 4 hours.

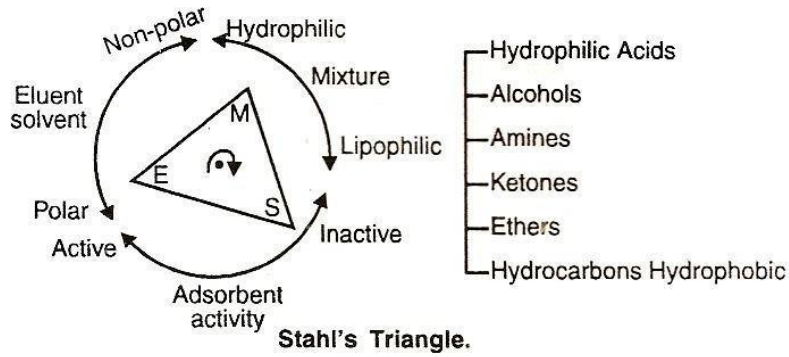
Silica gel G contains iron as an impurity which causes considerable distortion of the chromatographs. The coated and air-dried plates on preliminary development with methanol- Con. HCl [9:1, v/v] the iron gets migrated with the solvent front to the upper edge of the plate.

The plates are again dried and activated at 110 ^{o}C.

4. Choice of Solvent systems:

The choice of the best developing solvent is one of the most important decisions in practical TLC. To know a suitable solvent ***Stahl's triangle*** which inter relates *adsorbent activity, nature of the solute and solvent* is used.

If the triangle is rotated the *corner M* points to the *type of mixture to be separated*, S *corner points to the activity of the adsorbent* and corner *E points to the optimum polarity of the eluent.*



Stahl's Triangle.

Along the Stahl's diagram, there is listed the relative order of some solutes.

With mixtures of solvents it is possible to obtain intermediate elution behaviour.

High Boiling Paraffins
Cyclohexane
Benzene
Benzene, Ethyl Acetate (95:5)
Chloroform
Chloroform, Acetone (9:1)
Diethyl Ether
Ethyl Acetate
Ethyl Acetate, Methanol (99:1)
Benzene, Acetone (1:1)
Acetone
Methanol
Dimethyl Formamide
Dimethyl Sulfoxide
Water
Increasing polarity

5. Sample application:

Agla micro syringe is used for transferring the sample solution to thin layers for quantitative work. However, capillary tubes may be used for qualitative work. Solvent used for sample solution should be volatile and as non-polar as possible.

6. Development tank:

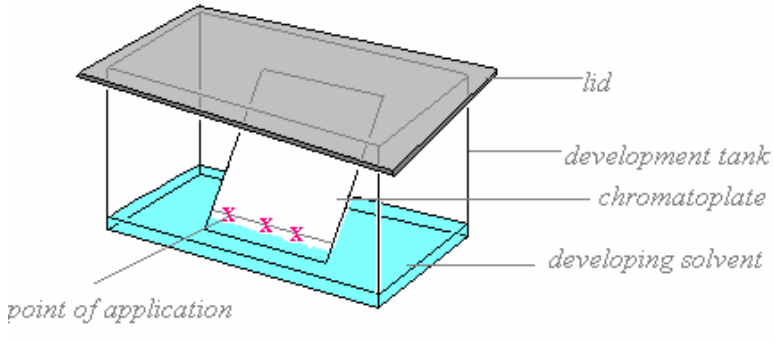
The development tank used in paper chromatography is also used in the TLC.

a. Ascending chromatography is the most common technique in TLC.

In TLC the plate is placed in development chamber at an angle of 45^{o}. The bottom of the chamber is covered up to 1 mm by the solvent. Three sides of the tank are lined with solvent impregnates paper while top is covered tightly with the lid.

b. Descending TLC with more advantages over ascending technique is also possible.

c. Horizontal TLC [= Radial Chromatography] is also possible.

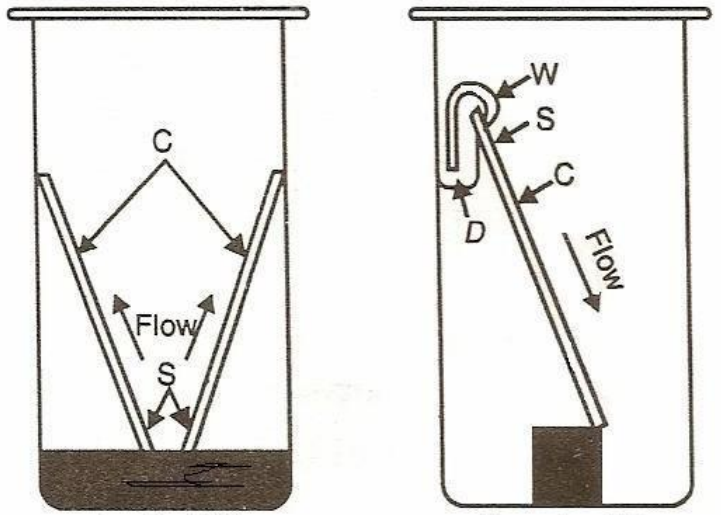


Apparatus for TLC

7. Plate development:

A drop of the sample is

placed near one edge of the plate and its position is marked with a pencil. After the sample solvent has evaporated, the plate is placed in closed container saturated with vapours of the developing solvent. One end of the plate is wetted with the developer by means of special arrangement.



Ascending TLC *Descending TLC*

S = initial position of sample D = developer C = chromatographic surface W = cotton wick

8. Detection of components:

One can see coloured substances usually.

Uncoloured components are seen under UV light or by treating the plate with a visualizing reagent.

The corrosive reagents like chromic acid / Sulphuric acid can be used in TLC.

9. Evaluation of the chromatogram:

After detecting the separated solutes on the plate and making their positions, their evaluation may be either qualitative or quantitative.

i. **Qualitative:** This is the same as with paper chromatograms except to say that RF values for TLC show inferior reproducibility.

ii. **Quantitative:** The quantitative evaluation chromatographically separated substances can be carried out in two ways.

i. **Direct Methods:** The quantitative determination is undertaken directly on the layer.
   a. Visual assessment of chromatograms
   b. Determination by measurement of spot areas
   c. Quantitative TLC incorporating densitometry
   d. Direct Spectrophotometry on thin layer chromatograms

ii. **Indirect Methods:** The substances removed from the adsorbent and then determined after elution.
   a. Gravimetric
   b. UV Spectrophotometry
   c. Colorimetry
   d. Fluorimetry
   e. Polarography
   f. Coulometry
   g. Radiometry
   h. Flame photometry

<u>Applications of TLC</u>

1. As a Check on Processes:- TLC has been used for checking of the other separation procedure and purification processes.

2. In Organic Chemistry:- The main use of TLC lies in the isolation and separation of individual Components of a mixture.

a) For checking the purity of samples:- TLC is mainly used as a check on samples obtained by other methods.

b) As a purification process:- A small amount of pure organic compound is required for its characterization by elemental analysis and other physical methods.

c) Examination of reaction:-TLC has enabled the chemist to assess that a reaction is complete and it has followed the expected course. This also gives information about the nature of the by products.

d) For identifying organic compounds:-TLC is being increasingly employed as a rapid method for isolation, purification and identification of organic substances.

i) Acids:- The normal aliphatic acids with even-numbered carbon atoms from decanol to docosanoic acid have been separated on layers of Kieselguhr "G" using Cyclohexane as the developing agent.

ii) Alcohols:- even-numbered alcohols from decanol through hexacosanol can be separated by chromatography on Kieselguhr "G" with Cyclohexane as a developing solvent.

iii) Glycols:- some glycols have been separated on loose layers of alumina with an activity grade of III by using a hexane-acetone(4:1) solvent or a mixture of ether-ethanol(99:1) separated with water.

iv) Alkaloids:- TLC has been used for the isolation and determination of alkaloids in toxicology.

v) Amines:- Several alkyl amines have been chromatographed on silica gel, buffered silica gel or as cellulose layers by using solvents like 95% ethanol-25% ammonia (4:1).

vi) Amino acids, Proteins and Peptides:- A mixture of amino acids can be separated by two-dimensional chromatography. **Rokkons** was successful in isolating 34 ninhydrin positive substances isolated from using and separated on silica gel plates.

vii) Antibiotics:- tetracyclines, penicillins, neomycin, erythromycins and other antibiotics can be separated by TLC using various solvents.

viii) Besides these, compounds like carbohydrates, aldehydes, ketones, dyes, pharmaceutical products, natural pigments, phenols steroids, terpenes, essential oils, vitamins, adhesives, explosives, plasticizers, etc, have been separated and characterized by

Limitations of TLC:

1. **It is used only for small scale preparative work.**
2. **It demands sacrificing resolving power.**

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PAPER CHROMATOGRAPHY

Cellulose filter paper usually covered with a thin film of water is often used as the stationary phase and a moving liquid as the mobile phase in paper chromatography. The procedure is often regarded as liquid – liquid chromatography.

The credit for the present status of paper chromatography in the realm of separation techniques goes to *A.J.P. Martin* and his co-workers, R. Consden, A.H. Gordon and R.L.M. Syngle.

Principle:

This is a partition chromatography in which the *substances are distributed between a stationary liquid (water) which is held in the fibers of the paper and a mobile liquid or developing solvent.* The components of the mixture to be separated migrate at different rates and appear as spots at different points on the paper.

Types of Paper Chromatography

(i) ***Paper Partition Chromatography:*** *This is a standard method of analysis in which a special paper containing adsorbed water is used as stationary phase another solvent as mobile phase.* Other liquids can replace the water to provide a different type of stationary phase.

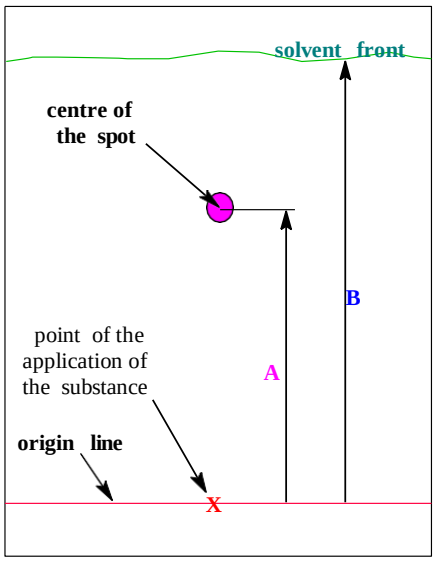
(ii) ***Paper Adsorption Chromatography:*** Commercially available special papers containing an *adsorbent* permit *adsorption paper chromatography*.

(iii) ***Reverse Phase Paper Chromatography:*** Paper treated with *silicone* or *paraffin oil* permits *reverse phase- paper chromatography*, in which the mobile phase is a *polar solvent*.

(iv) ***Ion-exchange Paper Chromatography*** Commercially available special papers that contain an ion-*exchange resin* permit *ion-exchange paper chromatography.*

Procedure:

In this technique, a drop of the test solution is applied on the filter paper and the spot is dried. The paper is kept in a closed chamber and the edge of the filter paper is dipped into a solvent called developing solvent. As the developing liquid percolates through the filter paper it reaches the spot of test solution, the various substances are moved by the solvent system at various speeds. When the solvent has moved these compounds to various suitable heights the paper is dried and the various spots are visualized by suitable visualizing reagents. The movement of substances relative to the solvent is expressed in terms of migration parameters.



Diagramatic representation of R_F

Migration parameters:

The positions of migrated spots on the chromatogram are indicated by different terms such as R_F, R_X and R_M. These are qualitative and quantitative parameters.

(a) R_F (Retention Factor) Values: *The R is related to the migration of the solute front relative to the solvent front as*

$$R_F = \frac{\text{Distance travelled by the solute front from the origin line}}{\text{Distance travelled by the solvent from the origin line}}$$

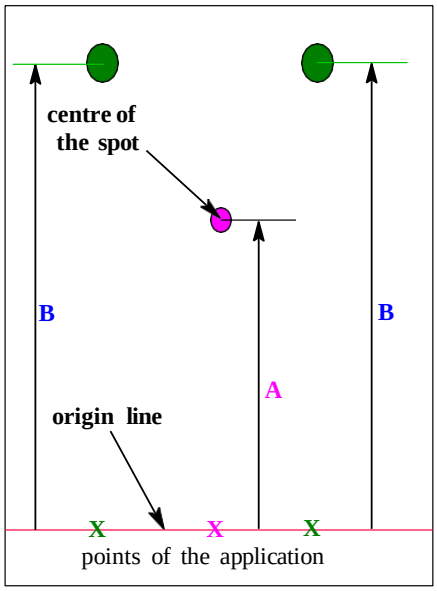
R is a function of the partition coefficient. It is a constant for a given substance. The R_F defines the movement of a substance relative to the solvent front in a given chromatographic system.

The factors affecting R_F value:

(i) The solvent employed

(ii) The medium used for separation
(iii) The nature of the mixture
(iv) The temperature
(v) Size of the vessel or the developing chamber

(b) R_X Values: *When the solvent front runs off the end of filter paper the movement of a substance is expressed as R_X but not R_F.*



Diagramatic representation of R_x

$$RX = \frac{\text{Distance travelled by the substance from the origin line}}{\text{Distance travelled by the standard substance (X) from the origin line}}$$

(c) R_M Values: *The R_F values of chemically related compounds are very close. The influence of individual functional groups was presumed to be added to a rough approximation. According to Bate-Smith and Westall, R_M is defined as follows:*

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

R_M is additive and is composed of the partial R_M value of the individual functional groups or other grouping of atoms in the molecules.

Experimental details for qualitative analysis or Process of Chromatography

The following operations are involved in the Paper chromatographic technique.

1] Choice of the Chromatographic technique,
2] Choice of the Filter paper,
3] Choice of the developing solvent,
4] Preparation of samples,
5] Application of Samples or Spotting,
6] Development of the Chromatogram
7] Drying of the chromatogram,
8] Visualization and
9] Calculation of R_F values.

1] Choice of the Chromatographic technique:

The choice of type of technique depends upon the nature of the substances to be separated, i.e., partition, adsorption, reverse-phase, ion-exchange or ascending, descending, ascending-descending, radial, two-dimensional, etc.

2] Choice of the Filter paper:

The prime factors that govern the choice:

i] whether the paper is used for qualitative or quantitative analysis;
ii] whether it is used for analytical or preparative chromatography; or
iii] whether the substances used are hydrophilic, lipophilic, neutral or charged species

- Various types of Whatmann Chromatography papers are available to suit the type of separation. Chemical Composition of which is

α—Cellulose 98—99%
β—Cellulose 0.3—1%
Pentosans 0.4—0.8%
Ash 0.07—0.01%
Other Soluble matter 0.015—0.1%

- Generally coarser and *faster paper*, Whatmann – 31 ET, is used when substances to be separated have sufficiently wide – apart R_F values.
- Slow papers (Whatmann – 20) are rarely used.
- Heavy papers like Whatmann – 3 MM are generally used for *preparative purposes.*
- Cellulose paper is impregnated with alumina, silica, zirconium oxide, etc. to get *adsorbent papers.*
- Papers have been impregnated with powdered or liquid ion exchangers to produce *ion exchange papers.*
- **Preparation of Paper:** After selecting the quality of paper, the shape and size is taken into consideration. The most commonly used paper is rectangular. After taking the paper, it is washed with water to remove any reducing agent if present.
- To *increase the capillarity of the paper*, it is soaked in 7% HCl for 24 hours followed by washing with water and ethanol.

3] Choice of the developing solvent:

n-Hexane
Cyclohexane
CCl_4
Benzene
Toluene
$Cl_2C = CHCl$
$H_5C_2 – O – C_2H_5$
$CHCl_3$
$CH_3COOC_2H_5$
n-Butanol
n-Propanol
Acetone
Ethanol
Methanol
Water

Elutotropic series

Selection of proper developing solvent depends upon the fact that RF values should be different for different constituents present in a mixture.

Criteria for a good solvent system:

- The R_F values of the sample should lie between 0.05 and 0.85.
- The solvent should not undergo any chemical reaction with any of the components of the sample mixture.
- The solvent should not interfere with detection of the spots.
- The composition of the solvent should not alter with time.

A suitable solvent with desired polarity can be selected from the *elutotropic series* to meet the separation needs.

If a pure solvent is not satisfactory, solubility of suitable polarity may be obtained by trying out mixtures in various proportions of solvents provided they are not completely miscible with each other.

Stationary phase	**Mobile phase**
Water	iso-propanol-ammonia – water [9:1:2]
	n-Butanol-Acetic acid- water [4:1:5]
	Phenol saturated with water
Formamide	Benzene-Cyclohexane [from 1:9 to 9:1]
Kerosene	70 % iso-prapanol
Liquid paraffin	Dimethylformamide-methanol-water [10:10:1]

Improvement in the spot resolution can be attained by ***multiple (two-dimensional) development*** *technique.*

4] Preparation of samples:

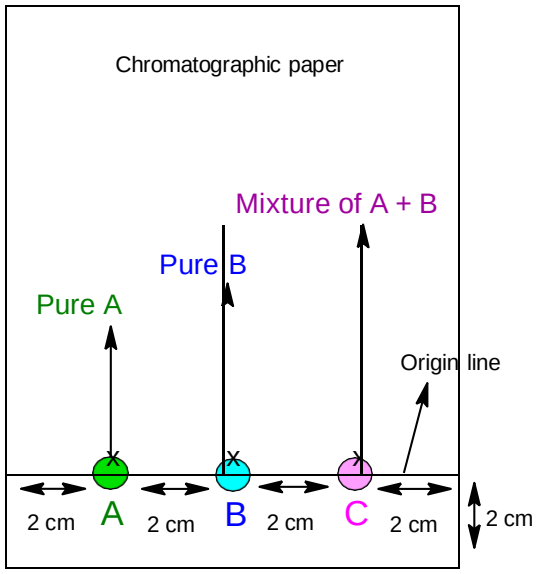
For solid substance, a weighed amount is dissolved in a volatile solvent and carefully a minimum volume of the concentrated solution is applied on the paper. Generally, a sample volume of 10 – 20 µg having as many µg of the substance is ideal to be spotted.

5] Application of Sample or Spotting:

The sample may be applied either as a spot or band.

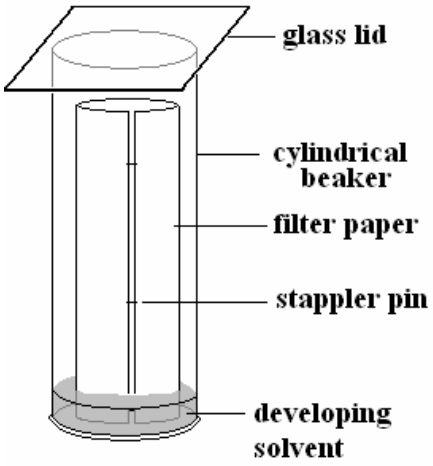
For ascending technique, a strip of Whatmann filter paper of suitable size, 25 x 7 cm is generally used. A horizontal line, called ***origin line*** is drawn on the filter paper by a lead pencil. On the origin line *'x' marks* are made with a pencil in such a way each cross is 2 cm away from each other. By the help of graduated micropipette, the test solutions are applied on 'x' marks and the spots are dried cautiously by a stream of hot or cold air. A, B and C are three 'x' marks.

On the first two marks, solutions of pure substances are applied while at C a mixture of A and B is applied.



6] Development of the chromatogram:

Roll the paper sheet in a cylindrical form (the spots should lay at the bottom) and put it into a beaker containing a suitable developing solvent to a depth of about 0.25" and cover the beaker with a watch glass. It should be done in such a way that the paper does not touch the sides of the beaker.

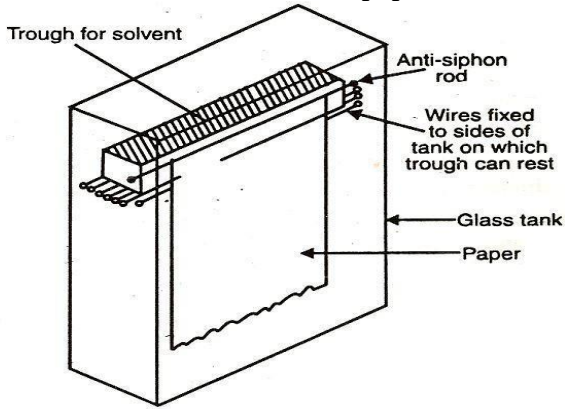


Paper chromatography [Ascending technique]

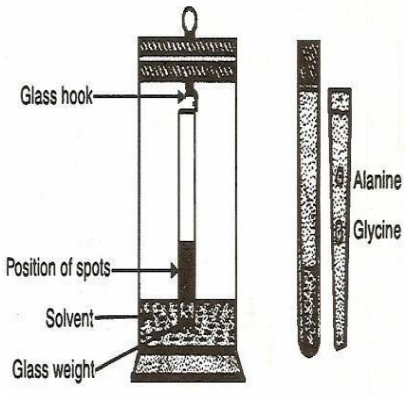
The top and the bottom of the paper are stapled. Allow the eluting agent to rise about 6". This process may take about 3-4 hours.

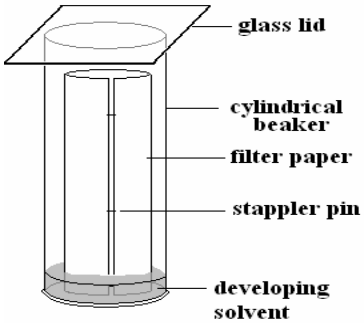
Types of Development

1. ***Descending Development*:** When the development of the paper is done by allowing the solvent to travel down the paper, it is known as descending technique.



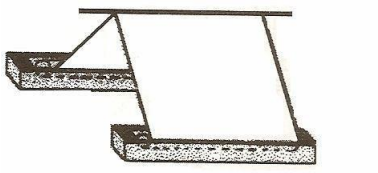
2. ***Ascending Development:*** When the development of the paper is done by allowing the solvent to travel up the paper, it is known as ascending technique.





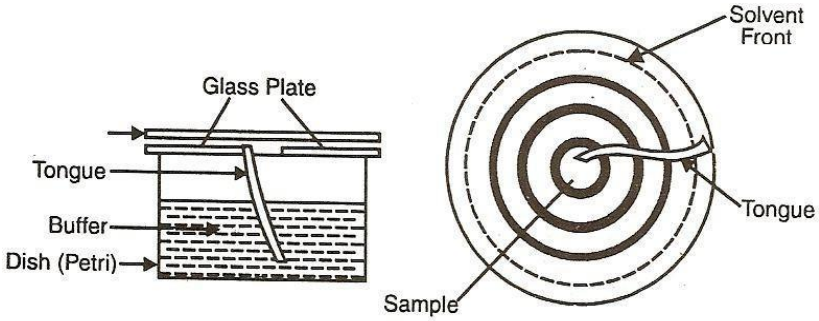
Paper chromatography [Ascending technique]

3. ***Ascending-Descending Development*:** It is a hybrid of the above two techniques. In this technique, the upper part of the ascending chromatography can be folded over a glass rod allowing the ascending development to change over in to the descending after crossing the glass rod.



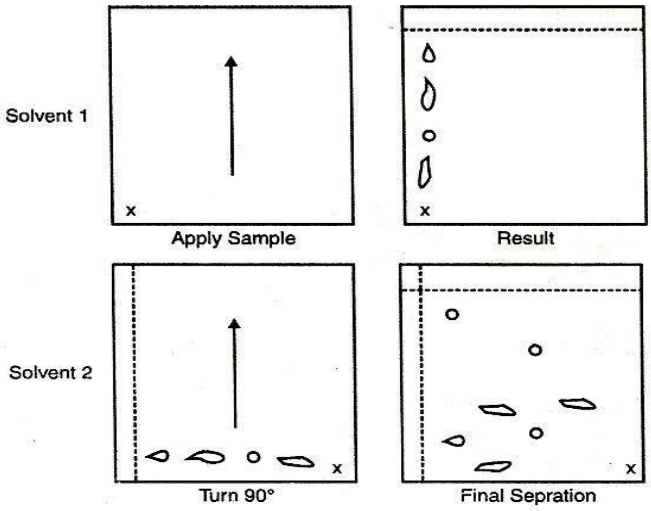
Ascending-Decending Chromatography

4. ***Radial or Circular Development:*** In this technique, a circular filter paper is taken upon which the sample is placed at the centre. After drying, the paper is fixed horizontally on the Petri-dish possessing the solvent so that the tongue or the wick of the paper dips into the solvent. Cover the paper with Petri-dish cover. The solvent rises through the tongue or the wick. The solvent front has moved through a sufficient large distance, the components get separated in the form of concentric circular zones.



Radial Paper Chromatography.

5. ***Two-dimensional Development:*** In this technique, a square or rectangular paper is used. The sample is applied to one of the corners. The second development is performed at right angle to the direction of the first run. It can be carried out with identical solvent systems in both the directions or by two solvent systems.



Separation of a Mixture by Two–dimensional Paper Chromatography

7] Drying the chromatogram:

The wet chromatogram after development is dried in special drying cabinets which are being heated electrically with temperature controls.

8] Visualization:

Visualization of the spots can be done in two ways:

- ***Chemical detection:*** Chemical treatment can develop the colour of colourless spots on the paper. The reagents used for visualizing the spots are known as ***chromatogenic or visualizing reagents.***

The reagents are applied either by the pressure spraying or by dipping the chromatogram. Reagents in organic solvents are more suitable for spraying than aqueous solutions. Then the spots are dried.

- ***Physical detection:*** Some colourless spots when held under a UV lamp fluoresce and reveal their existence.

When the substance is coloured, the spots can be observed either by reflected light or transmitted light.

9] Calculation of R_F values:

The distances of chromatographed species are noted from its centre to the origin line. The distance of solvent front from the origin line gives the R_F values.

Applications

Paper chromatography has been applied to the separation of many organic and biochemical products. Eg:

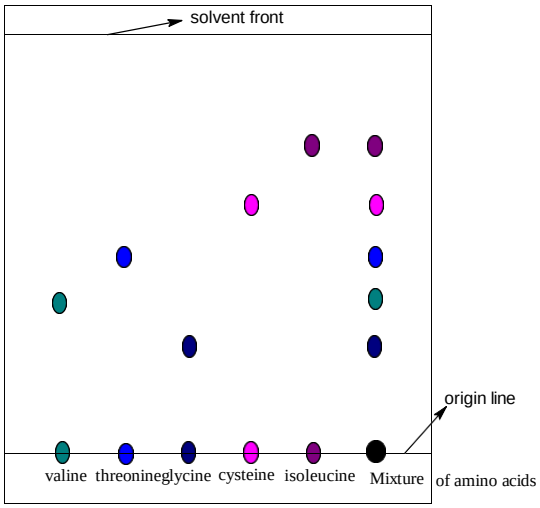
- in the determination of indoles in the whole urine
- In the study of barbiturates, antibiotics, carbamyl phosphates, hormones and amino acids
- In the study of inorganic metal salts and complex ions.
- In the analysis of mixture of sugars

Procedure for the separation of amino acids

Amino acids can be separated by paper chromatography.

The sample is spotted on the paper, eluted followed by visualization using ninhydrin which gives purple or brown colour. R_F values give the qualitative identification. Quantitative identification is carried out by comparing the intensity of the colour with the standards.

Prepare 0.1 g / 100 mL each of valine, threonine, glycine, cysteine and isoleucine; as well as a mixture of all these. Take 8" x 10" sheet of Whatmann Chromatography paper No. 1 and make six equally spaced small circle with a pencil 0.5" from the bottom. Label the paper at the top with the name of each amino acid and label the *sixth* as unknown.



Place four small drops of an amino acid on one circle and dry it carefully over between each drop of application.

Similarly repeat the process for other amino acids.

Roll the paper sheet in a cylindrical form (the spots should lie at the bottom) and put it into a beaker containing 95% alcohol to a depth of about 0.25" and cover the beaker with a watch glass. It should be done in such a way that the paper does not touch the sides of the beaker. The

top and the bottom of the paper are stapled. Allow the eluting agent to rise about 6". This process may take about 3-4 hours.

Now remove the paper from the beaker and mark the solvent front position immediately with the help of a pencil. The chromatogram is dried and then sprayed with 0.2% of ninhydrin solution in water saturated with butyl alcohol. The chromatogram is then dried in an oven at about 100 – 105 ^{o}C until coloured spots appear.

Find the R_F values of each amino acid and the spots for the unknown and thus find out quantitatively the composition of the unknown.

The most important precaution is to handle the paper by forceps or surgical gloves.

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COLUMN CHROMATOGRAPHY

* *

Introduction

It was developed by the American petroleum chemist *D. T Day* in 1900, *MS. Tswett*, the Polish botanist, in 1906 used adsorption columns in his investigations of plant pigments. It was only after 1930 the method was used extensively by chemists. Column chromatography is also known as **adsorption chromatography**.

Principle

"*Rate of adsorption varies with a given adsorbent for different materials". This principle of selective adsorption is used in column chromatography.*

In this method, the mixture to be separated is dissolved in a suitable solvent and allowed to pass through a tube containing the adsorbent. The component which has greater adsorbing power is adsorbed in the upper part of the column and the component which has lesser adsorbing power is adsorbed in the lower part of the column. This process is continued and as a result the materials are partially separated and adsorbed in the various parts of the column.

The portion of a column which is occupied by a particular substance is called its ***zone.***
The banded column of adsorbent is termed a ***chromatogram****, and the operation is spoken of as the* ***development of chromatogram****.*

After development, the column may be washed with more solvent, now termed the **eluent**, and each component is collected separately as it reaches the end of the column and is released.
The process of recovery of constituents from the chromatogram is known as ***elution****.*

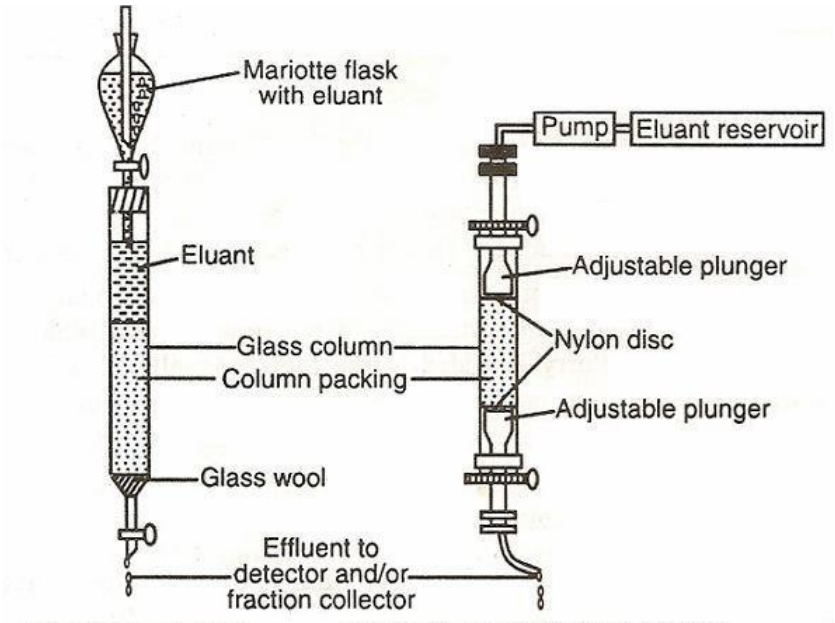
Procedure or Experimental Details

It includes the following subtitles:

(a) Apparatus
(b) Adsorbents
(c) Preparation of Adsorption Columns
(d) Solvents Used
(e) Method of introducing the solution
(f) Elution
(i) Successive Elution
(ii) Gradient Elution
(g) alysis

(a) Apparatus.

A simple straight glass tube tapered at the bottom and fitted with a tip is commonly used. This tube is about 20-30 cm long and 2-3 cm in diameter. The adsorbent is supported on a cotton plug or glass wool.



Equipment for column chromatography

(b) Adsorbents

To obtain efficient

columns, the adsorbents should have following characteristics

(i) Particles should be spherical in shape and uniform in size.

(ii) They should not react chemically, either with the eluting solvents or with the sample components.

(iii) They should contain as small amount of soluble components as possible.

(iv) They should be categorically inactive and have neutral surface except for ion exchangers.

Example:

(1) Alumina activated by heating to about 200-300°C in a current of air or carbon dioxide.

(2) Silica gels, activated charcoal, magnesium oxide, magnesium carbonate, calcium carbonate, calcium sulphate, barium carbonate, etc.

(3) Various polyamides such as Nylon, Perlon, and powdered polyacryl amide *are* suitable for the separation of lower fatty acids, phenols, quinones and synthetic dyes.

(4) Starch is suitable for the separation of racemates.

(5) Powdered sugar is used for the separation of colouring pigments of leaves and phenols.

(c) Preparation of Adsorption Columns

The cotton plug or glass wool is kept in position in the tube which is used as a support for the column. The tube is then clamped vertically and adsorbent is added in small amounts so that the tube is packed uniformly. This is continued till nearly two-thirds of it is filled.

An alternative procedure involves the preparation of slurry of the adsorbent in petroleum ether or eluting solvent. Now the slurry is added to the tube gradually, a little at time; Suction is applied after each addition. This process is continued till a column of desired height is obtained.

(d) Solvents Used

The choice of solvent depends upon the solubility of the compound and possesses boiling point between 40°C and 85°C.

The most widely used is *light petroleum*; others are *cyclohexane, carbon disulphide, benzene, chloroform, carbon tetrachloride, ethyl acetate, ethyl alcohol, acetone, ether and acetic acid.*

The solvents used have three functions to perform.

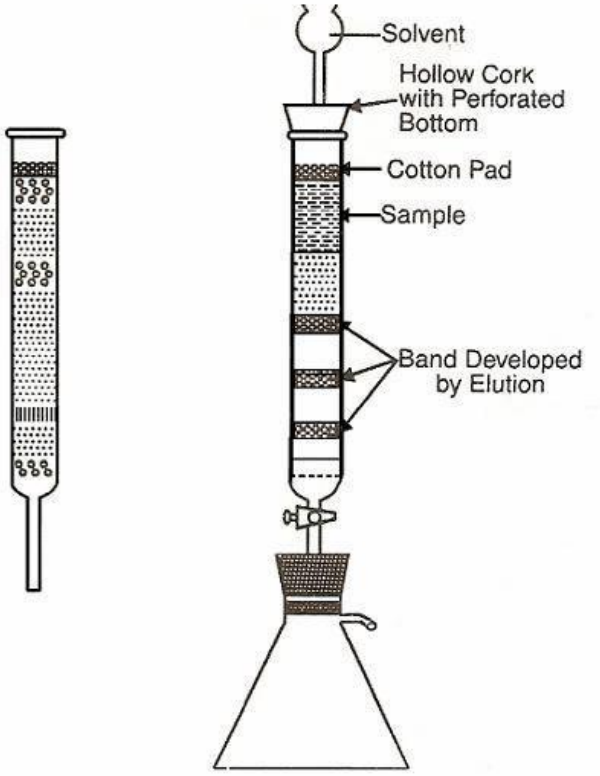
(1) They serve to introduce the mixture into the column.

(2) They affect the process of development by which the zone of chromatogram is separated to their fullest extent. In such case, the solvents are termed as ***developers.***

(3) They are also used to remove the required content of each zone. The solvents used for this purpose are called ***eluents***. The eluting power of the solvents is practical parallel to their dielectric constants.

(e) Method of introducing the solution

The top of the column is covered by a cotton plug. Some solvent is put over it. The rate of flow of solvent is controlled by suction



A Complete Set-up for Column Chromatography.

pump. The solvent should percolate the rate of 4-10 cm per minute. Now the solution is added. After the entire portion of the solution has passed through the developing liquid, is led into the column slowly.

(f) Elution

After all the liquid has passed through and various zones become well defined, the system is allowed *to dry* in air or oxygen or *to elution.*

Elution can be achieved by two techniques: a. Successive elution b. Gradient elution

(i) Successive Elution

The complex samples are eluted with *a series of solvents successively* for complete separation of various compounds containing different functional groups.

For example, a particular solvent may dissolve and elute only one group of compounds, such as hydrocarbons, leaving the rest of the compounds containing other functional groups undissolved at the top of the column. After the group of compounds that are soluble in this first solvent has been eluted from the column, a new solvent may be used to dissolve and separate a second group of compounds on the column and so on.

Each change in solvent brings about the elution from the sample of a different set of organic functional groups. Each set of fractions can be studied separately by further chromatography with a different column or by IR, UV absorption spectroscopy, or NMR.

(ii) Gradient Elution

It's problematic to collect fractions near the interface between the two succeeding mobile phases in successive elution which can be overcome by gradient elution.

In this system, *two miscible solvents with different dielectric constants* may be used.

After the sample is loaded on the top of the column, the first solvent, which has a lower solvent strength, is used as the mobile phase. After a suitable period of time a small amount of the second solvent is introduced. With time, its concentration in the first solvent progressively increases until finally the mobile phase consists entirely of the second solvent.

Throughout the operation there is a gradient in the concentrations of the two solvents. This leads to a progressive change in solvent strength and in the solubility of some of the components of the sample. Popular pairs of solvents include water-methanol, water-acetonitrile, and dioxane - tetrahydrofuran.

(g) Analysis

In case of air-dried columns the adsorbent may be pushed out completely with the wooden pestle or plunger and the zones may be separated as they leave the tube. Each zone is then dropped immediately into the eluent and the suspension is filtered on a sintered glass funnel to get rid of the adsorbent.

In case of eluted columns the developed chromatogram is treated either with a single eluent or with a succession of solvents having increasingly powerful eluent actions (successive elution) or with two solvents (gradient elution). The various portions of the column are thus washed out one by one and collected in different receivers.

The fraction is treated with suitable drying agent and the solvent or eluent is evaporated to get the compound.

Theory of development

If a solution of *concentration 'C'* is passed through an adsorbent column in such a manner that *n* gram get adsorbed per gram of the adsorbent, then *'n'* may be given as follows $n = f(C)$. When only one gram column is considered, then only pure solvent will be flowing until *n* gram of the solution get adsorbed on the column. This volume of solvent is known as '*retention volume'* is given as $R = \frac{f(C)}{C}$

The retention volume 'R' is found to be independent of initial concentration of the solution.

Applications of Column Chromatography

For analytical purposes, column chromatography finds limited applications.

(a) Analytical Uses: The internal surface of the narrow tubing (0.05-2 mm internal diameter) serves as adsorbent or support for the liquid phase.

- Glass capillaries whose internal surface is treated with conc. ammonia at 300°C can separate amino acids using butanone + pyridine + dilute acetic acid (5 : 5 : 1) or xylose + glucose + maltose using butanone + acetic acid + water (3 : 1 : 6).
- *Vestergaard* and Sayegh could separate seven urinary steroids within 5 hours with narrow Teflon tubing packed with aluminium oxide or silica by gradient elution using acetone in chloroform.

(b) Separation of Geometrical Isomers: The separation of cis-trans isomers is based on the steric factors. Isomers whose functional groups can approach the surface of the adsorbent more easily are more strongly adsorbed.

- Winterstein reported the first chromatographic separations of cis / trans isomers of bixin and crocetin dimethyl ether.
- Zechmeister separated cis / trans isomers of carotenoids on calcium carbonate, aluminium· oxide and other adsorbents.
- Biphenyl octatetraene have been separated into all- Trans, trans-cis-trans and trans-cis-cis-trans isomers.
- Cis / trans isomers of carboxylic acids have been separated on charcoal and silica gel.

(c) Separation of Diastereomers: To separate optically active counterparts CC can be used.

- For example, the separation of diastereomeric *7-Chloro-azibicyclo-(4:1:O)-heptane* has been done on silica gel using pentane / diethyl ether as solvent.

(d) Separation of Tautomeric Mixtures:

- The keto and enol-forms of p-hydroxyl-phenyl pyruvic acid and indolyl pyruvic acid could be separated in the liquid phase. The separation is done in weakly acidic medium. The enol form appears in the eluate before the keto form.

(e) Separation of racemates.

- The first successful separations of racemates using organic solvents were achieved on lactose, e.g., the famous separations of enantiomers of Troger's base.