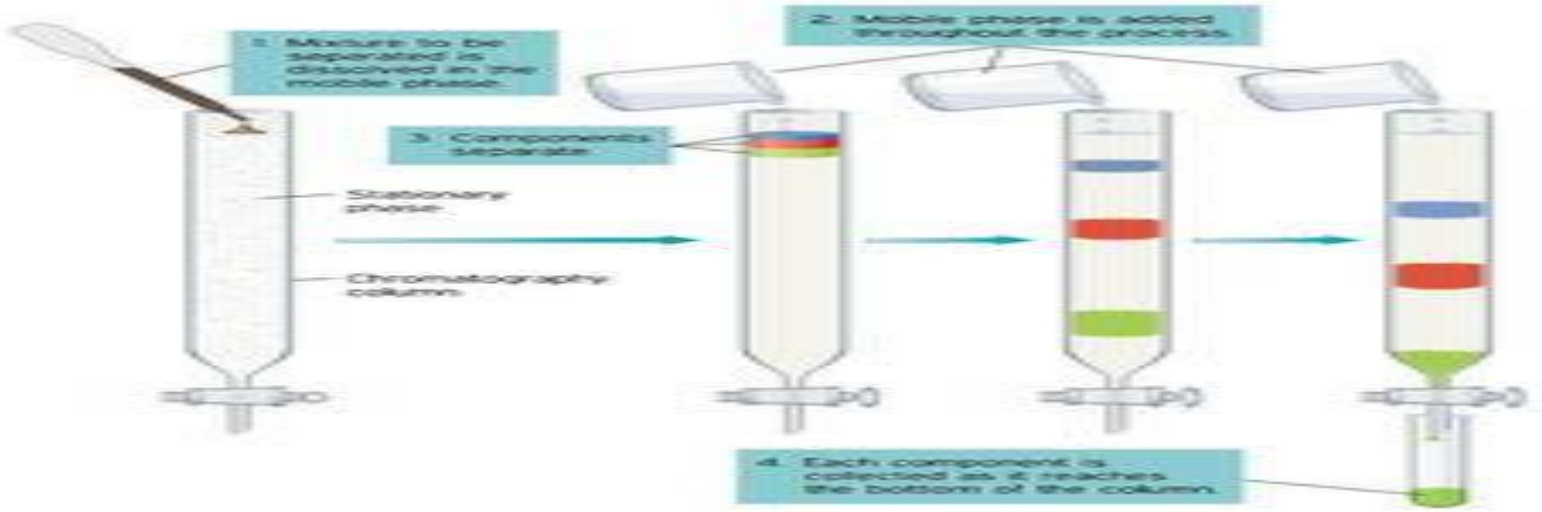
Chromatography



History

- Mikhail Tswett, Russian, 1872-1919

  Botanist
- In 1906 Tswett used to chromatography to separate plant pigments
- He called the new technique chromatography because the result of the analysis was 'written in color' along the length of the adsorbent column
- Chroma means “color” and graphein means to “write”

Importance

Chromatography has application in every branch of the physical and biological sciences

- 12 Nobel prizes were awarded betwe 1972 alone for work in which chromatogr vital role

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases

one of which is stationary (stationary phase) while the other (the mobile phase) moves through it in a definite direction.

The chromatographic process occurs due to differences in the distribution constant of the individual sample components.

Chromatography

Is a technique used to separate and identify the components of a mixture.

Works by allowing the molecules present in the mixture to distribute themselves between a stationary and a mobile medium.

Molecules that spend most of their time in the mobile phase are carried along faster.

Classification of chromatography according to mobile phase:

1- Liquid chromatography: mobile phase is a liquid. (LLC, LSC)

. 2- Gas chromatography : mobile phase is a gas. (GSC, GLC).

Classification according to the packing of the stationary phase:

1- Thin layer chromatography (TLC): the stationary phase is a thin layer supported on glass, plastic or aluminium plates.

2- Paper chromatography (PC): the stationary phase is a thin film of liquid supported on an inert support.

3- Column chromatography (CC): stationary phase is packed in a glass column.

Classification according to the force of separation:

1- Adsorption chromatography.

2- Partition chromatography.

3- Ion exchange chromatography.

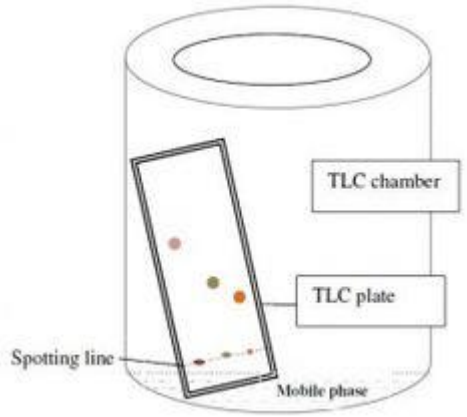
4- Gel filtration chromatography.

5- Affinity chromatography**.**

Thin layer chromatography (TLC)

is a method for identifying substances and testing the purity of compounds.

TLC is a useful technique because it is relatively quick and requires small quantities of material.



Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase.

- The stationary phase:

is a thin layer of adsorbent (usually silica gel or alumina) coated on a plate.

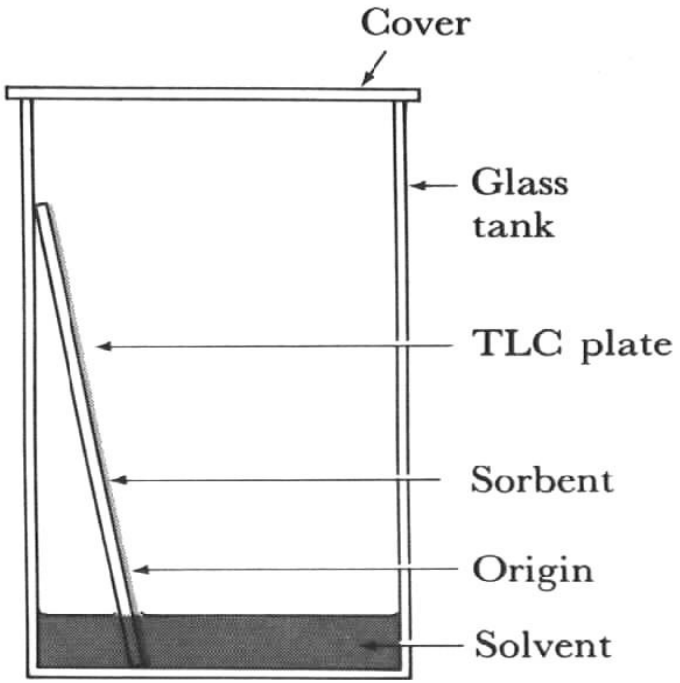
- The mobile phase:

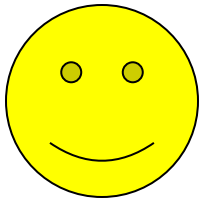
is a developing liquid which travels up the stationary phase, carrying the samples with it.

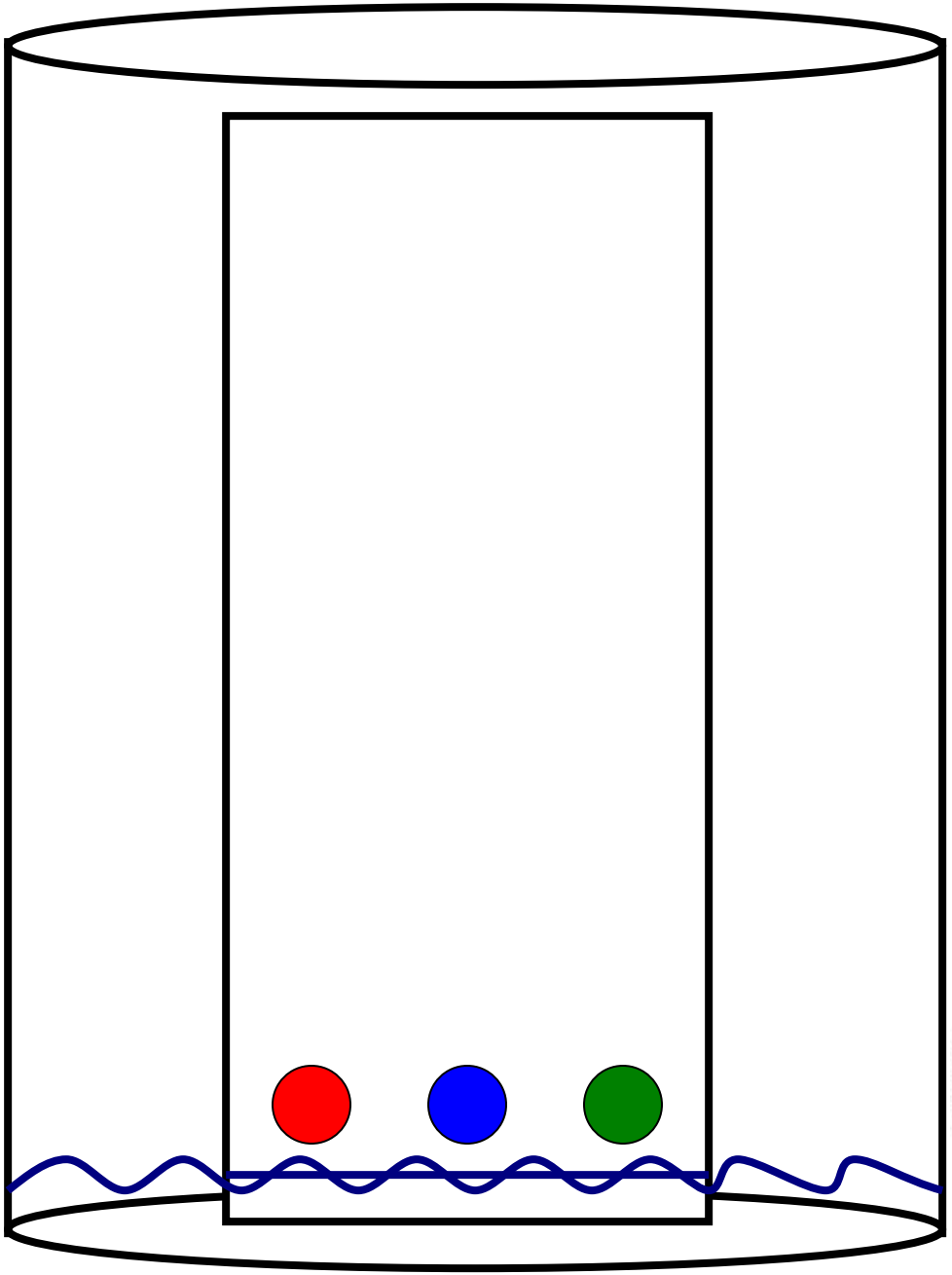
Components of the samples will separate on the stationary phase according to

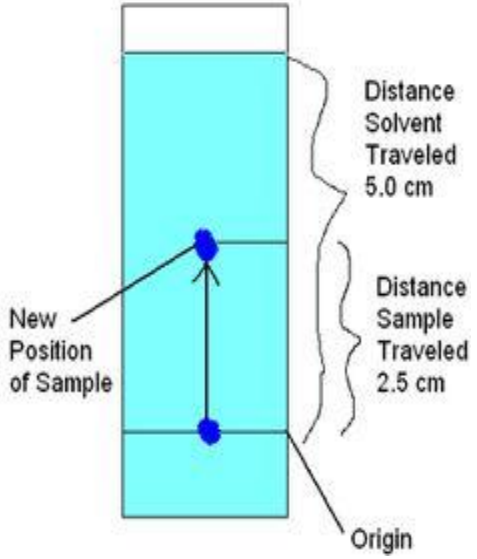
how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase.

Thin Layer Chromatography (TLC)

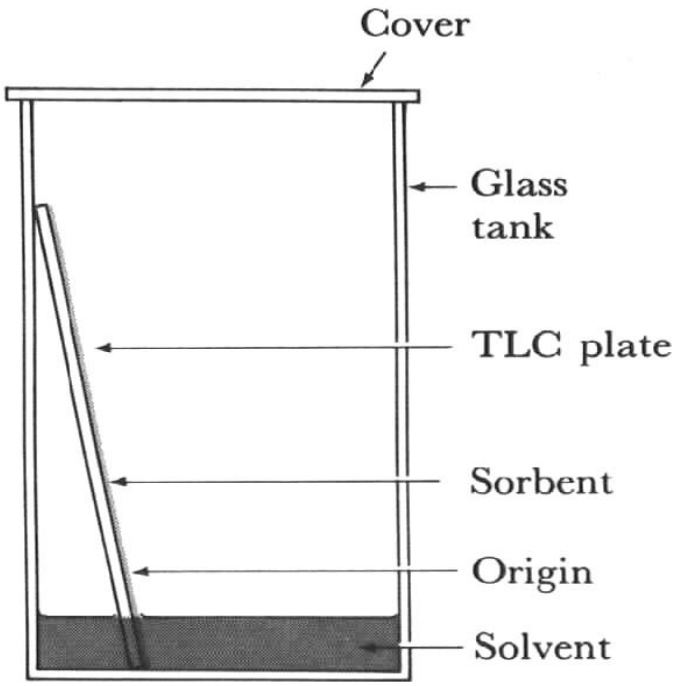


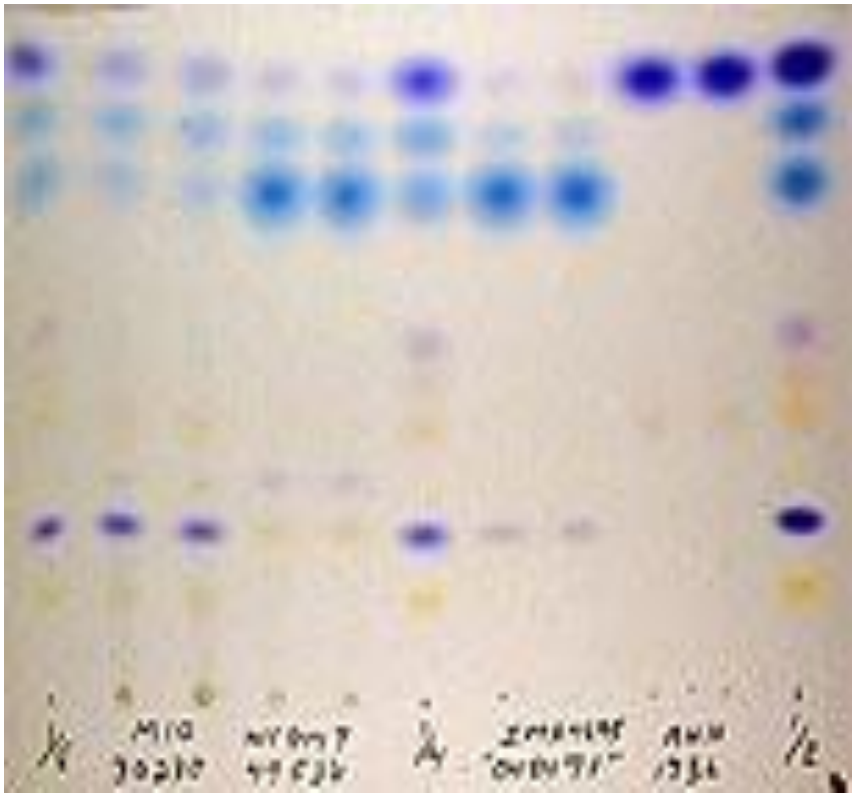





Distance
Solvent
Traveled
5.0 cm
Distance
Sample
Traveled
2.5 cm
New
Position
of Sample
Origin

TLC





Preparing the Chamber

- To a jar with a tight-fitting lid add enough of the appropriate developing liquid so that it is 0.5 to 1 cm deep in the bottom of the jar.
- Close the jar tightly, and let it stand for about 30 minutes so that the atmosphere in the jar becomes saturated with solvent.

Preparing the Plates for Development

With a pencil, etch two small notches into the adsorbent about 2 cm from the bottom of the plate.

The notches should be on the edges of the plate, and each notch should be the same distance up from the bottom of the plate.

The notches must be farther from the bottom of the plate than the depth of the solvent in the jar.

Using a drawn-out capillary tube, spot the samples on the plate so that they line up with the notches you etched.

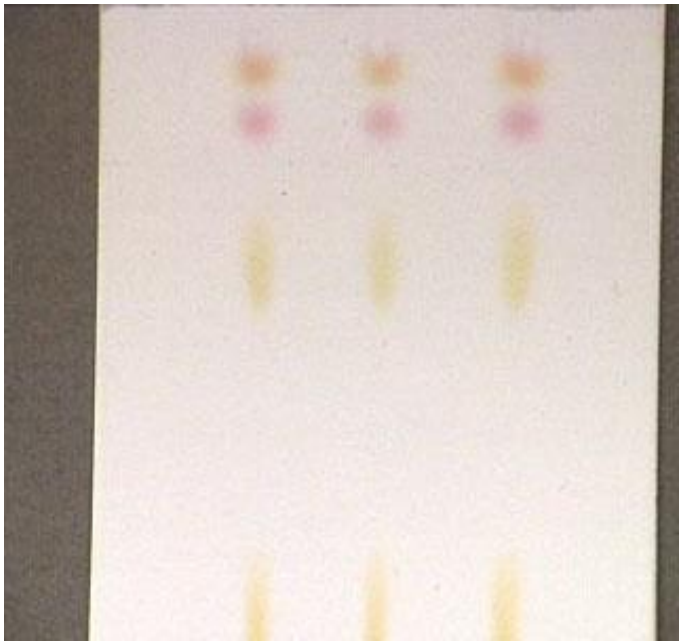
Developing the Plate

After preparing the development chamber and spotting the samples, the plates are ready for development.

Be careful to handle the plates only by their edges, and try to leave the development chamber uncovered for as little time as possible.

When the plates are removed from the chamber, quickly trace the solvent front (the highest solvent level on the plate) with a pencil.

Identifying the Spots (visualization)



If the spots can be seen, outline them with a pencil.

If no spots are obvious, the most common visualization technique is to hold the plate under a UV lamp.

Many organic compounds can be seen using this technique, and many commercially made plates often contain a substance which aids in the visualization of compounds.

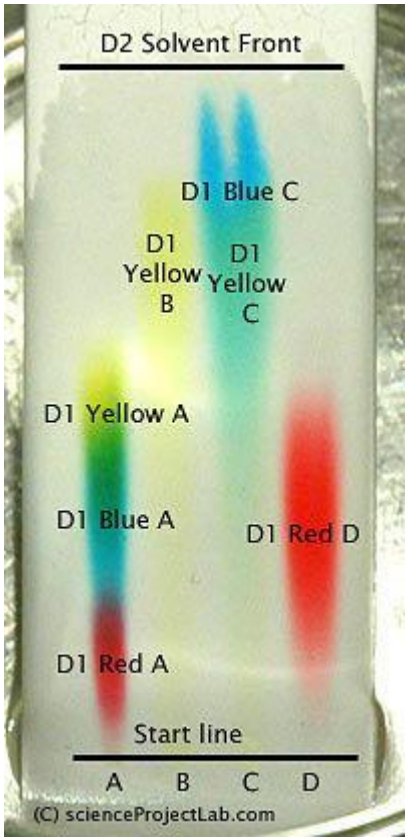
Some visualizing Agents

Alkaloids: Dragendorff's reagent

Cardiac glycosides: Antimony trichloride

Sugar: Aniline phthalate

Amino acids: Ninhydrin



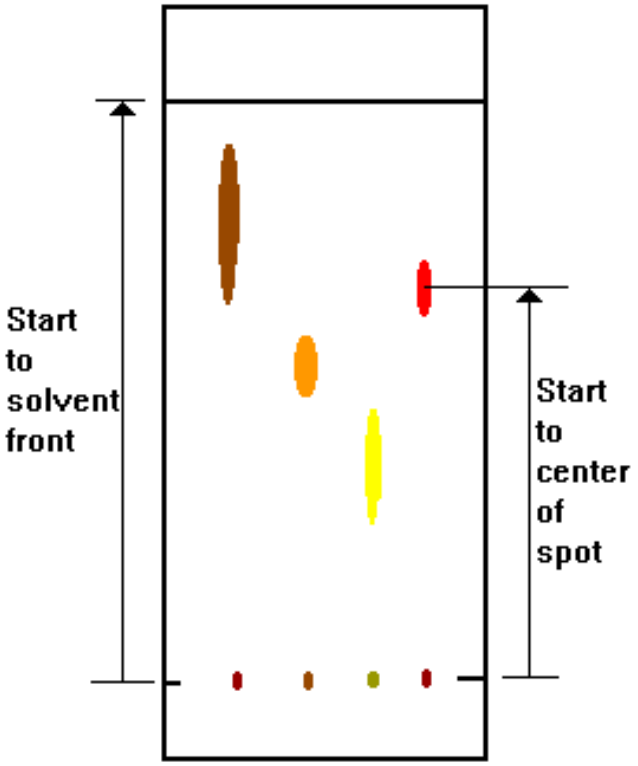
Interpreting the Data

The R_f (retention factor) value for each spot should be calculated.

It is characteristic for any given compound on the same stationary phase using the same mobile phase for development of the plates.

Hence, known R_f values can be compared to those of unknown substances to aid in their identifications.

$$R_f = \frac{\text{Distance from start to center of substance spot}}{\text{Distance from start to solvent front}}$$



(Note: R_f values often depend on the temperature and the solvent used in the TLC experiment.

the most effective way to identify a compound is to spot known substances – authentic - next to unknown substances on the same plate.)

In addition, the purity of a sample may be estimated from the chromatogram.

An impure sample will often develop as two or more spots, while a pure sample will show only one spot

Summary

A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina).

A small amount of the mixture to be analyzed is spotted near the bottom of this plate.

The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid.

This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action.

As the solvent moves past the spot that was applied, an equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution.

In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others.

When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized.

If the compounds are colored, visualization is straightforward. Usually the compounds are not colored, so a UV lamp is used to visualize the plates.

مقطع فيديو عنكروماتوغرافيا الطبقات الرقيقة من يوتيوب

Thin layer chromatography animation from youtube

https://www.youtube.com/watch?v=rMGQavOMAmc&ab_channel=BiologywithAnimations

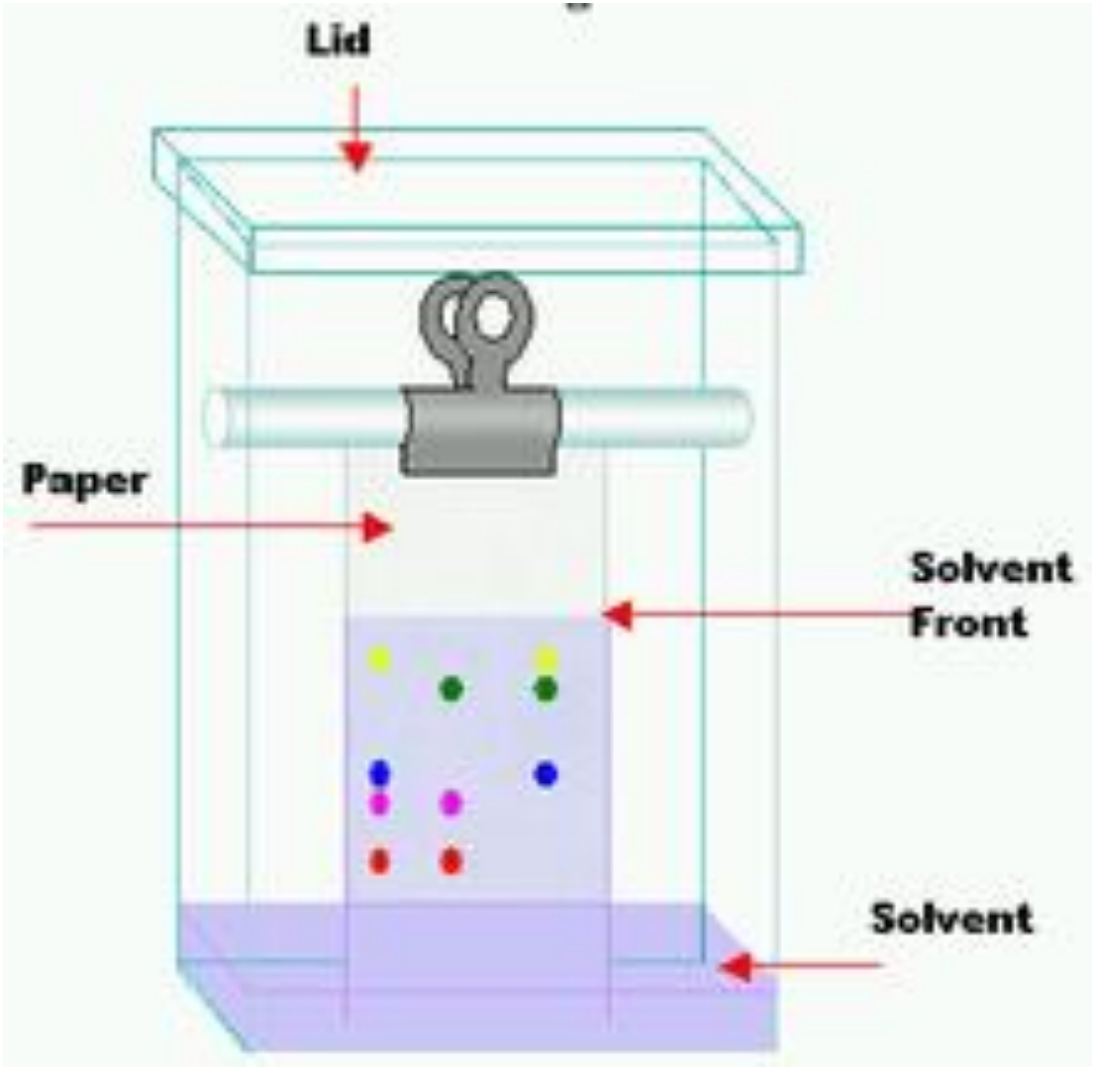
Paper Chromatography

A method of partition chromatography using filter paper strips as carrier or inert support.

The factor governing separation of mixtures of solutes on filter paper is the partition between two immiscible phases.

One is usually water adsorbed on cellulose fibres in the paper (stationary phase).

The second is the organic solvent flows past the sample on the paper (stationary phase).


Lid
Paper
Solvent
Front
Solvent

Partition occurs between the mobile phase and the stationary aqueous phase bound by the cellulose (paper).

The isolation depends on partition coefficient of the solute.

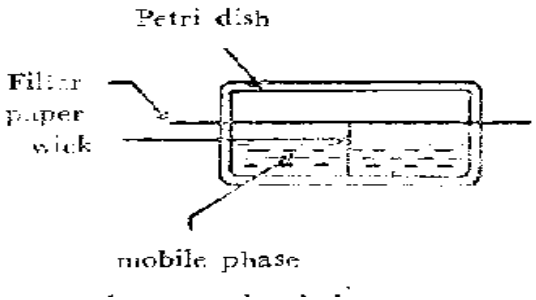
$$K = \frac{c(stationary)}{c(mobile)}$$

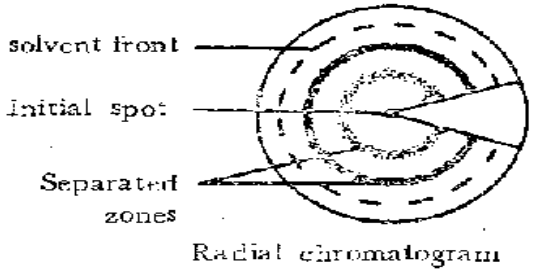
General Procedure

1- Choice of paper and solvent to be used.
2- Desalting of sample.
3- Application of the sample.
4- Equilibration of paper.
5- Development.
6- Detection.
7- Identification of substances.

Techniques of development with various flow

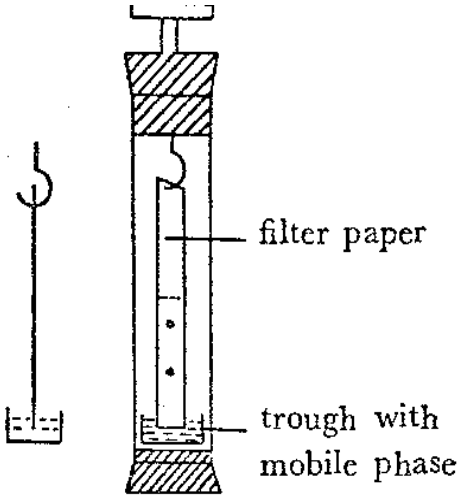
Radial development



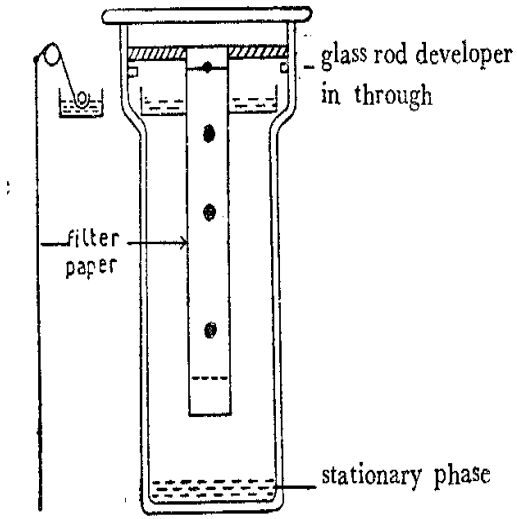


Radial chromatogram

Ascending development



Cylinder for ascending chromatography



Descending development

Descending development

Multiple chromatography

Multiple chromatography includes all procedures in which the development is repeated after one development is completed.

A- multiple development: the chromatogram is repeatedly developed in the same direction and thus the complete resolution of two or more substances which have R_f values close together can be obtained.

As the mobile phase one can use either the same solvent system or different solvent systems.

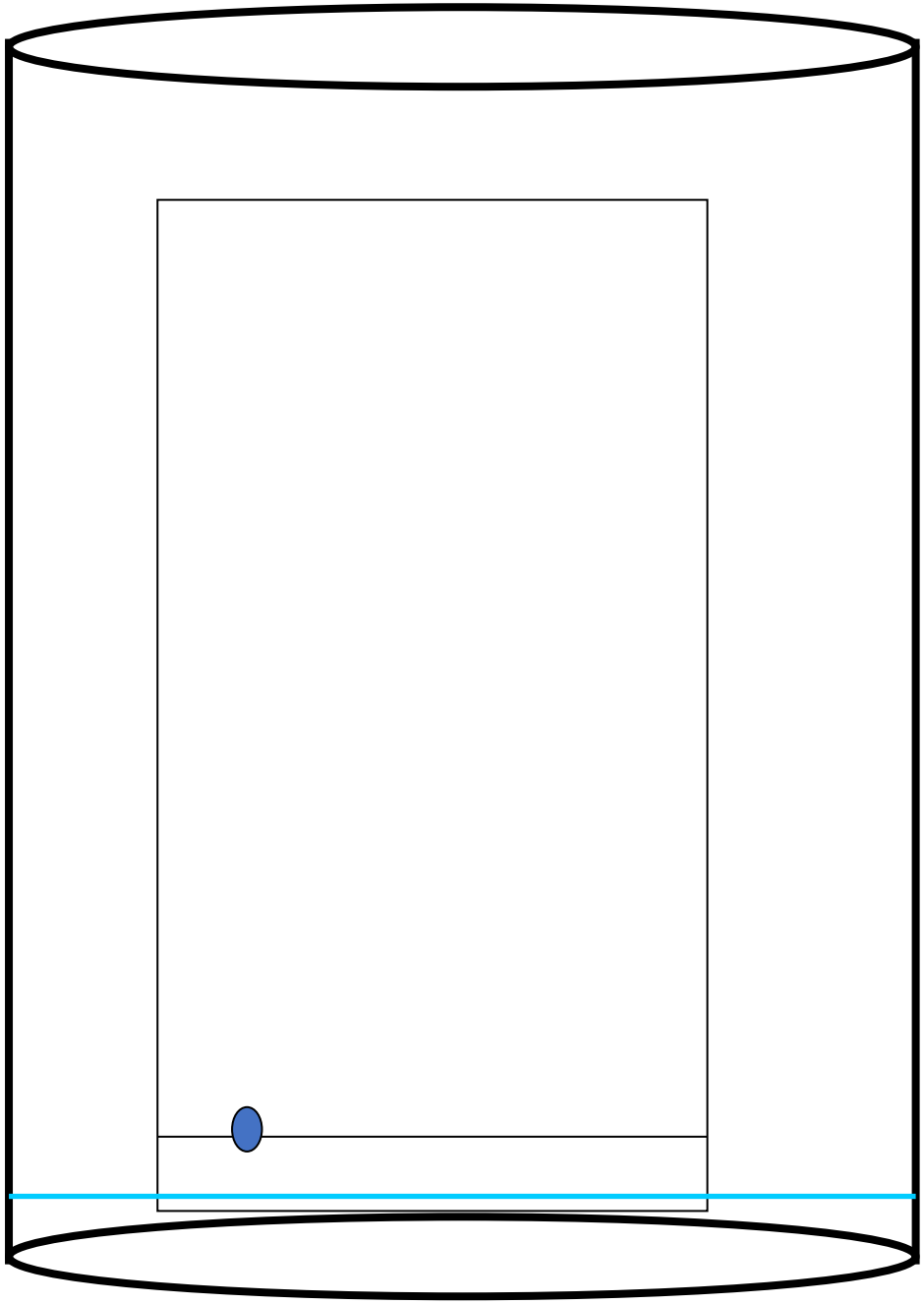
B- two-dimensional chromatography:

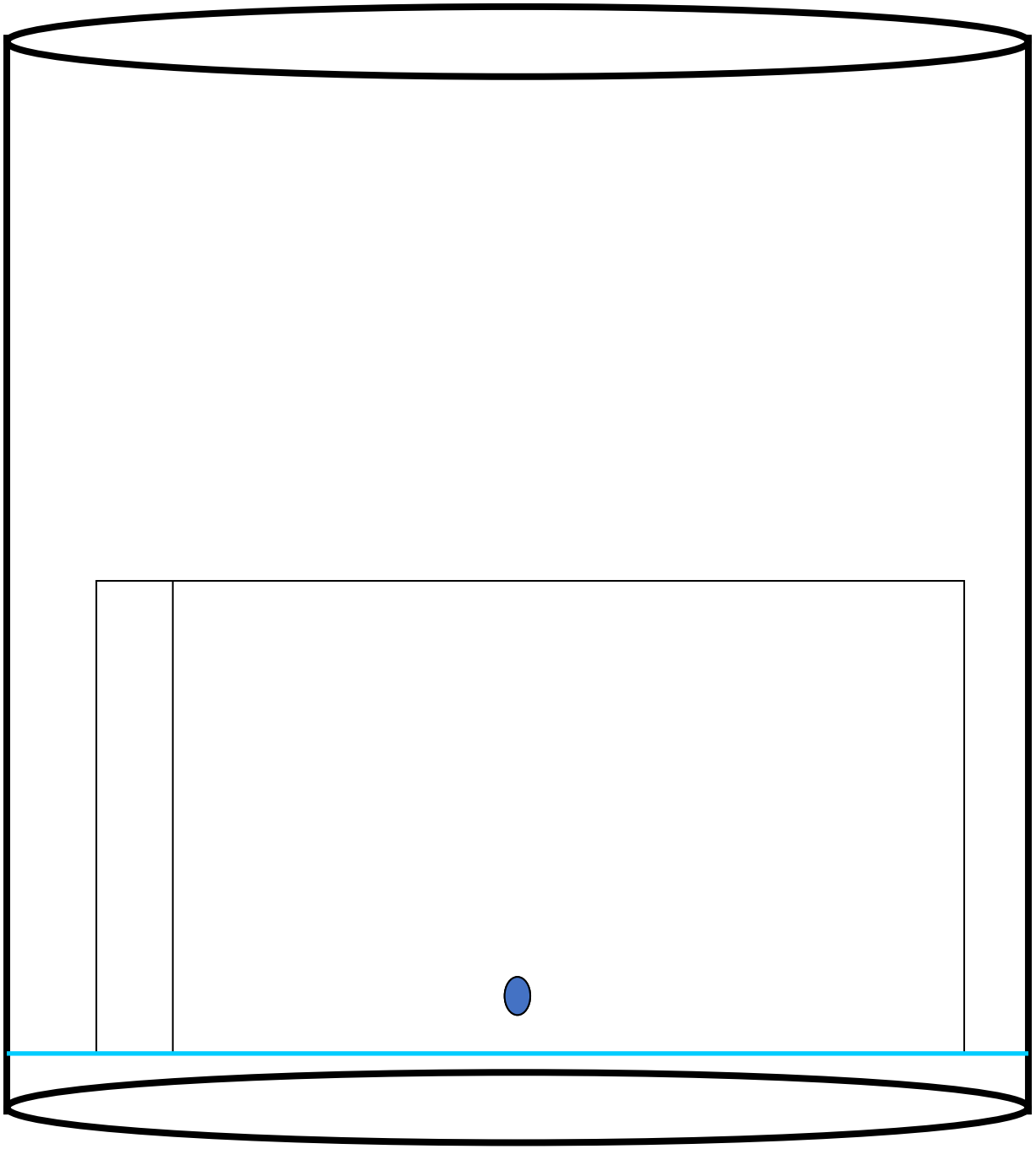
When large numbers of substances are to be separated on a single chromatogram.

Development in a direction perpendicular to the first, and with a solvent system different from that used initially is often necessary.

The sample is applied on one corner of a square piece of paper and after development with the first solvent, the paper is dried , rotated 90^{o} and developed in the second direction.

Usually, different types of solvents systems are used in each direction. It is essential that the first solvent be completely volatile.





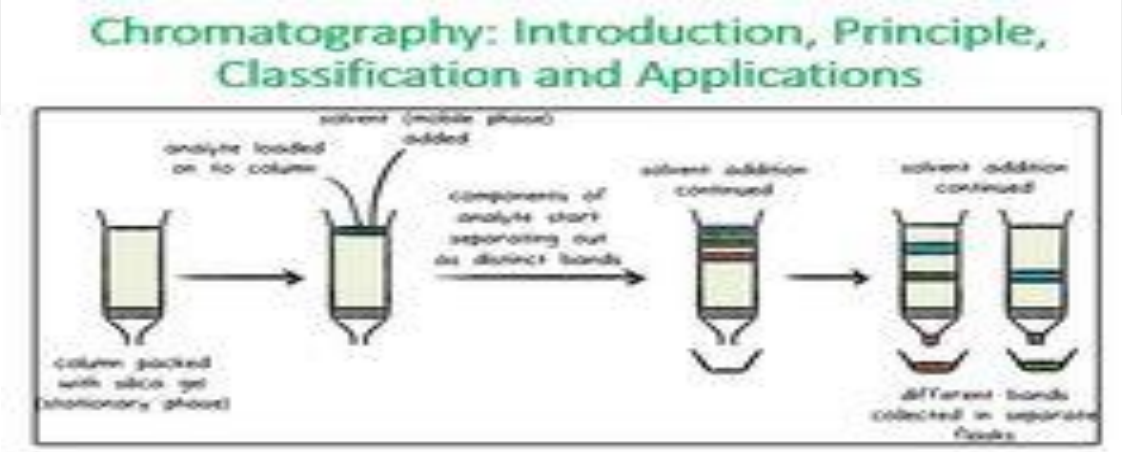
Column Chromatography (CC)

This includes chromatographic methods in which:

The stationary phase is packed into a column.

The mobile phase is a moving liquid or gas.

According to the mechanism of separation of solutes, five major types of CC are distinguished. Usually, one mechanism predominates but does not exclude the others

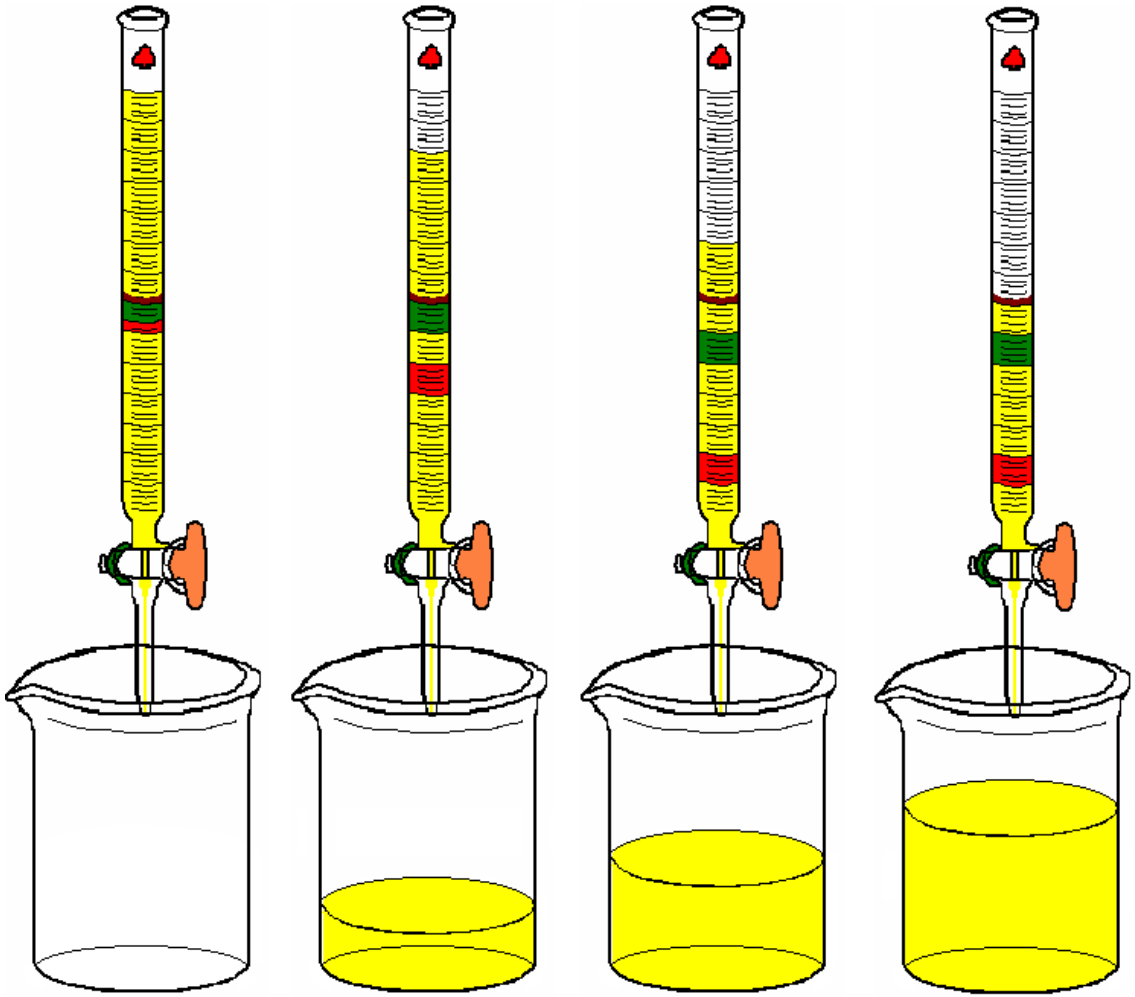


Different Types of chromatography

Mode or type	Stationary phase	Mobile phase	Mechanism
Adsorption Chromatography	Solid that attracts the solutes	Liquid or gas	Solutes move at different rates according to the forces of attraction to the stationary phase.
Partition Chromatography	Thin film of liquid formed on the surface of a solid inert support	Liquid or gas	Solutes equilibrate between the 2 phases according to their partition coefficients
Ion Exchange Chromatography	Solid resin that carries fixed ions & mobile couterions of opposite charge attached by covalent bonds	Liquid containing electrolytes	Solute ions of charge opposite to the fixed ions are attracted to the resin by electrostatic forces & replace the mobile counterions.
Molecular Exclusion Chromatography	Porous gel with no attractive action on solute molecules	Liquid	Molecules separate according to their size: 1.Smaller molecules enter the pores of the gel, and need a larger volume of eluent. 2.Larger molecules pass through the column at a faster rate.
Affinity Chromatography	Solid on which specific molecules	Liquid or gas	Special kind of solute molecules interact with those immobilized on

Column Chromatography

- Column chromatography
  - Stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or under the effect of gravity



Column Chromatography

https://www.youtube.com/watch?v=Zke6xGhbbho&ab_channel=UniversityofLeeds-ChemistryLaboratories

Term	Definition
Solvent	**Mobile liquid phase with no affinity to the stationary phase (i.e. inert towards it) & no effect on solutes.**
Developer	**Any liquid with more affinity to the stationary phase than the solvent but less than solutes and just capable to move them through the column.**
Effluent	**Any liquid that passes out of the column.**
Eluent	**Any liquid that has lesser affinity to the stationary phase than solutes but is capable to move them out of the column.**
Eluate	**Fraction of eluent containing a required specific substance.**
Retention volume (V_R)	**(or retardation volume): Volume of mobile phase that passes out of the column, before elution of a specific substance.**

Open Column Chromatography (Traditional column chromatography)

Traditional column chromatography is characterized by addition of **mobile phase** under **atmospheric pressure** and the **stationary phase** is **packed in a glass column**.

Packing & operating the column
1- Packing

The selection of the method of packing depends mainly on the density of the solid.Techniques used are the wet, dry & slurry methods.

In all cases avoid inclusion of air bubbles

2- Sample Application

- Apply **evenly & in a concentrated solution** to the top of the column which is protected
- from disturbance (e.g. add glass wool or filter paper).

Elution techniques

Technique	Procedure
Isocratic elution	**Addition of solvent mixture of fixed composition during the whole process.**
Gradient elution	**Continuous or linear elution: in which there is continuous change in the composition of the mobile phase over a period of time (e.g. polarity, pH or ionic strength).**
	Step wise or fractional elution: in which the change is not continuous i.e. a sudden change in the composition of the mobile phase is followed by a period where the mobile phase is held constant.

Detection

On-column detection for colored or fluorescent compounds directly after developing the chromatogram.

Monitoring of eluted fractions (PC or TLC).

Using special detectors connected to the column such as refractive index, UV detectors, etc…

Factors affecting solutes separation in CC (Factors affecting column efficiency)

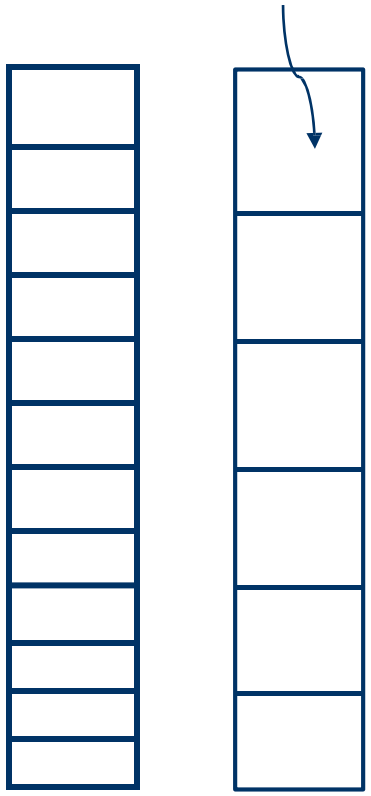
Factor	Effect
Particle size of solid stationary phase (or of support)	**Decrease of size improves separation (but very small particles need high pressure).**
Column dimensions	**Efficiency increases as ratio length / width increases.**
Uniformity of packing	**Non uniform packing results in irregular movement of solutes through column & less uniform zone formation, (i.e. band broadning or tailing).**
Column temperature	**Increase in column temperature results in speed of elution but does not improve separation (tailing).**
Eluting solvent	**Solvents should be of low viscosity (to give efficient resolution) & h igh volatility (to get rapid recovery of the substances).**
Solvent flow rate	**Uniform & low flow rate gives better resolution.**
Continuity of flow	**Discontinuous flow disturbs resolution**
Condition of adsorbent	**Deactivation of adsorbent decreases separation.**
Concentration of solutes	**Substances of high concentration move slowly.**

Number of Theoretical Plates (N)

H = Theoretical Plate Height
L = Length of the Column.

N = L / H

As HETP decreases efficiency of the column increases.



Adsorption Column Chromatography

Adsorbents:

The most common are Alumina & Silica gel in which the interactions with solute molecules is due to OH groups present on their surface.

More polar molecules are adsorbed more strongly & thus, will elute more slowly

Strength of adsorption of polar groups (solutes) on polar support is in the following order:

-C=C- < O-CH3 < -COOR < >C = O < -CHO < -NH2 < -OH < -COOH

Olefins < Ethers < Esters < Lactones < Aldehydes < Amines < Phenols < Acids.

Applications in separation of natural products

Alumina: sterols, dyestuffs, vitamins, esters, alkaloids & inorganic compounds.

Not used for compounds containing phenolic or carboxylic groups

Silica gel: sterols & amino acids.

Carbon: peptides, carbohydrates & amino acids.

Calcium carbonate: carotenoids & xanthophylls.

Selection of the mobile phase

The **stationary & mobile phases** should have a considerable difference between their **polarity**.

The polarity of some common solvents is decreasing in the following order:

pure water > methanol > ethanol > propanol > acetone > ethyl acetate> ether > chloroform > dichloromethane >benzene > toluene > carbon tetrachloride > cyclohexane > hexane > pentane.