

The background of the slide features a repeating pattern of stylized, light blue leaves. The leaves are rendered in a flat, graphic style with visible veins, set against a light blue gradient background. The pattern is centered and covers the entire area.

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 between 1937 and 1972 alone for work in which chromatography played a 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.

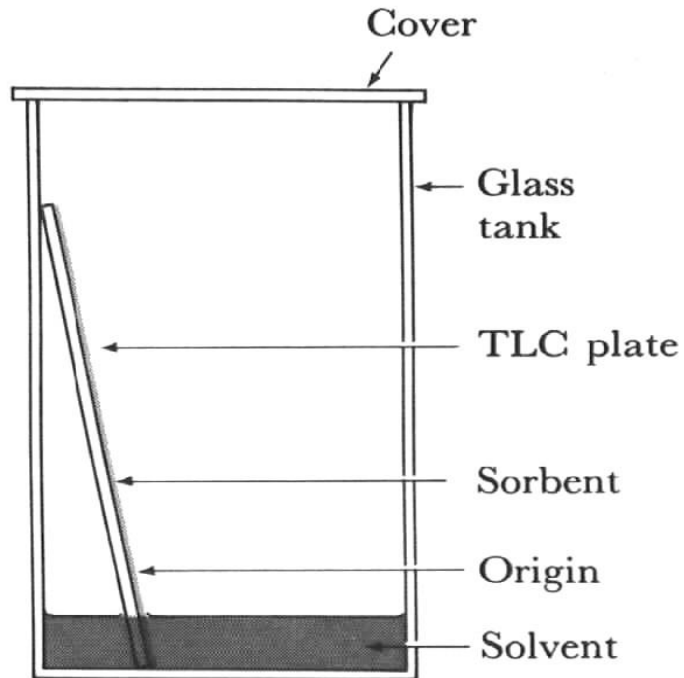
The mobile phase:

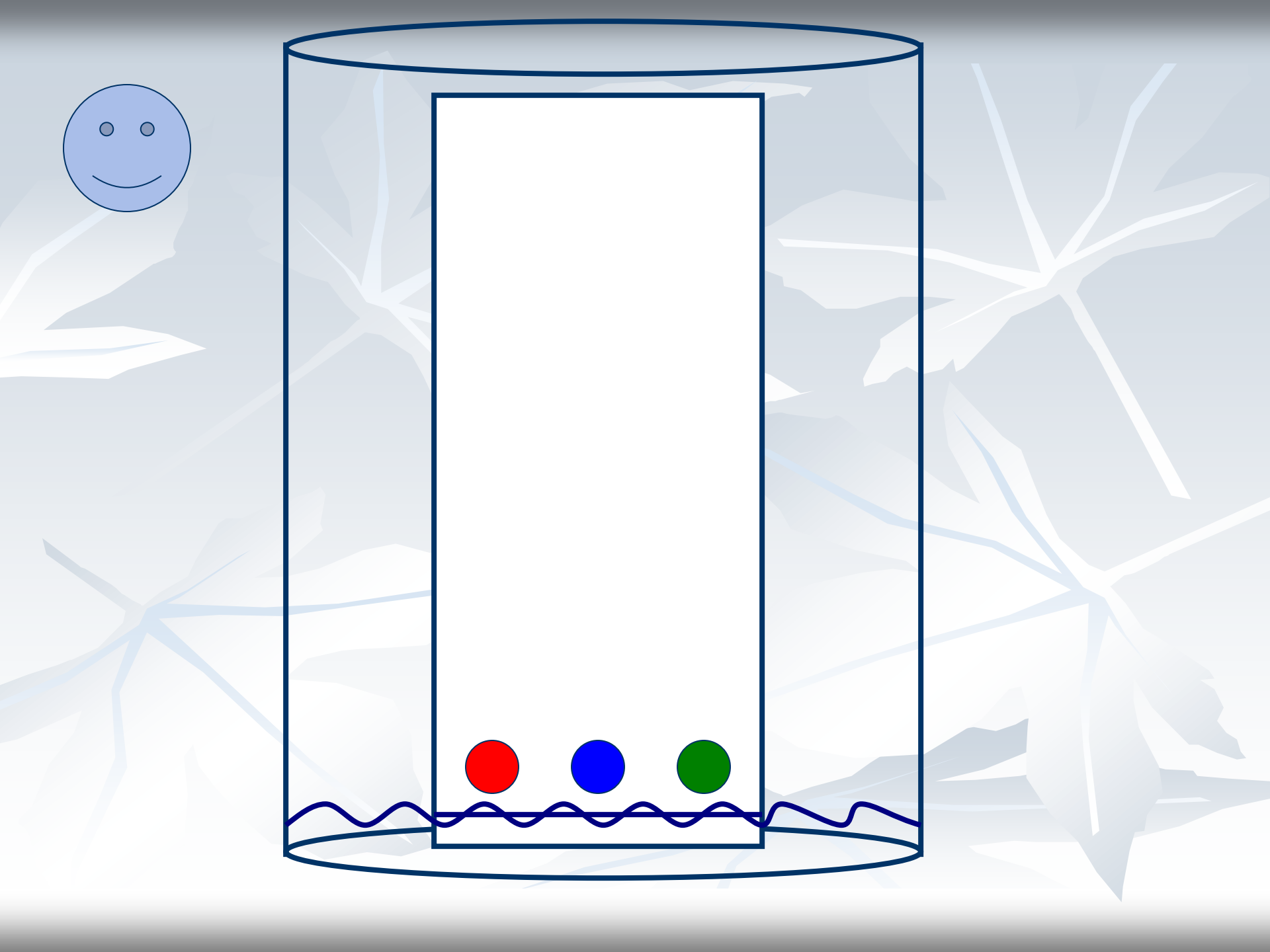
is a developing liquid which travels up the stationary phase, carrying the samples with it.

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

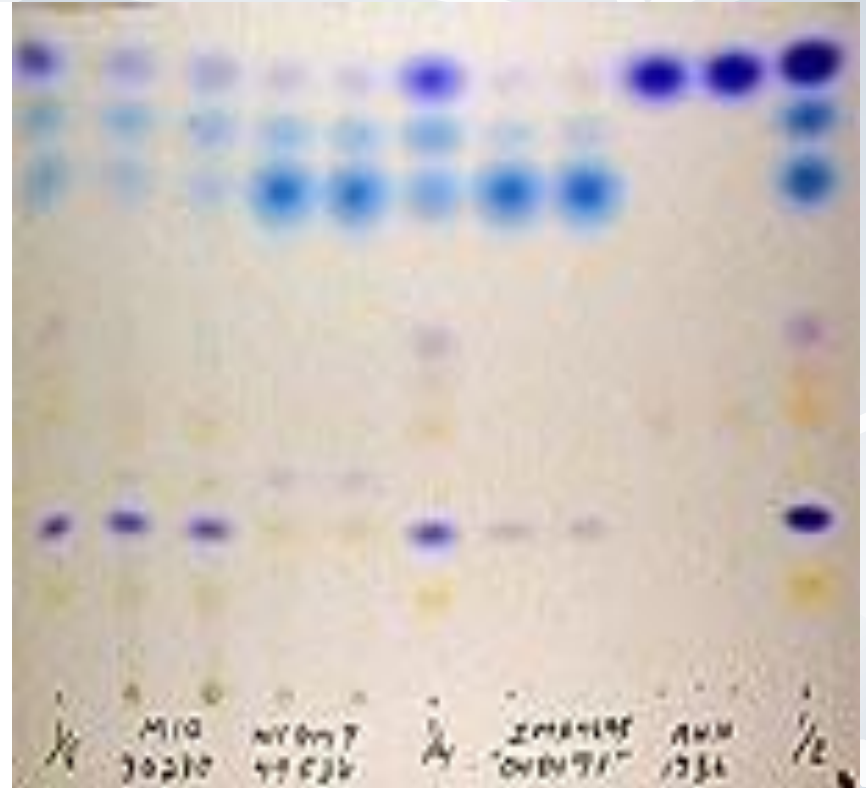
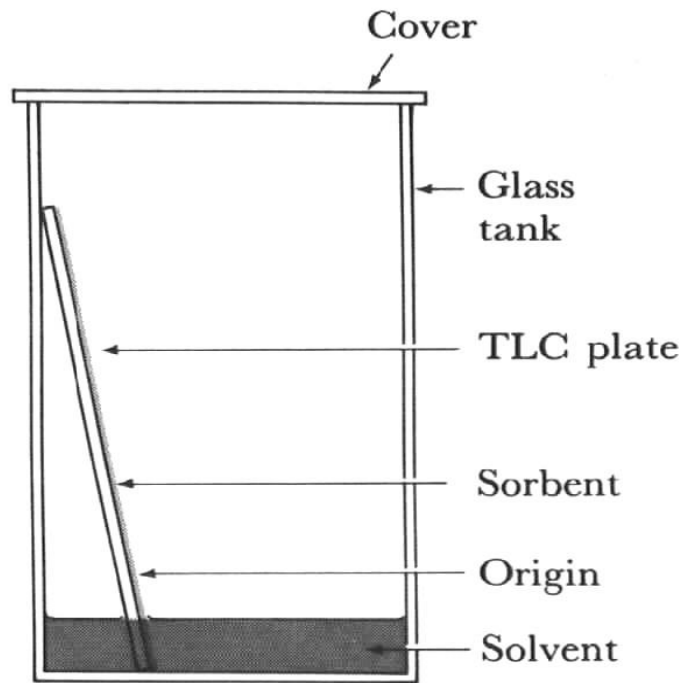
how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase.

Thin Layer Chromatography (TLC)





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

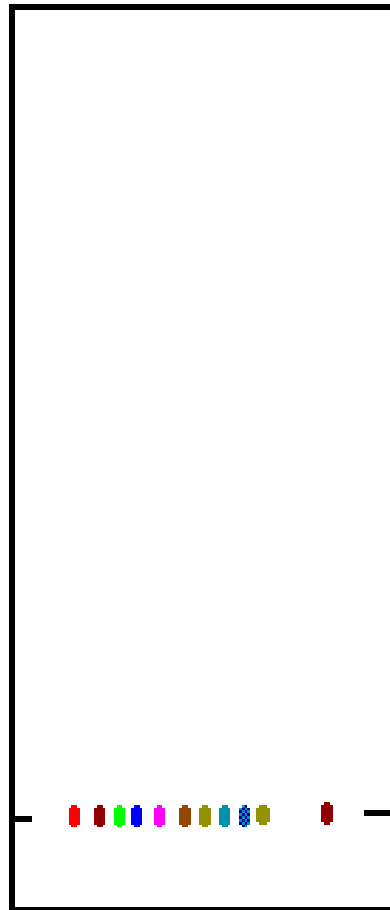
With a pencil, etch two small notches into the adsorbent about 2 cm from the bottom of the plate.

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.

Question: What is wrong with the plate shown below?



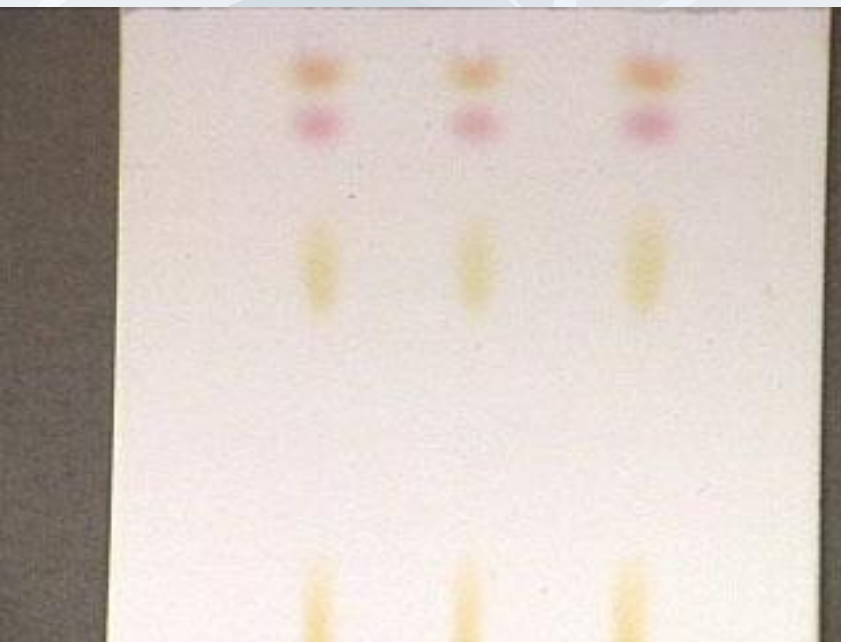
Developing the Plates

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.

Visualizing Agents

Alkaloids: Dragendorff's reagent

Cardiac glycosides: Antimony trichloride

Sugar: Aniline phthalate

Amino acids: Ninhydrin

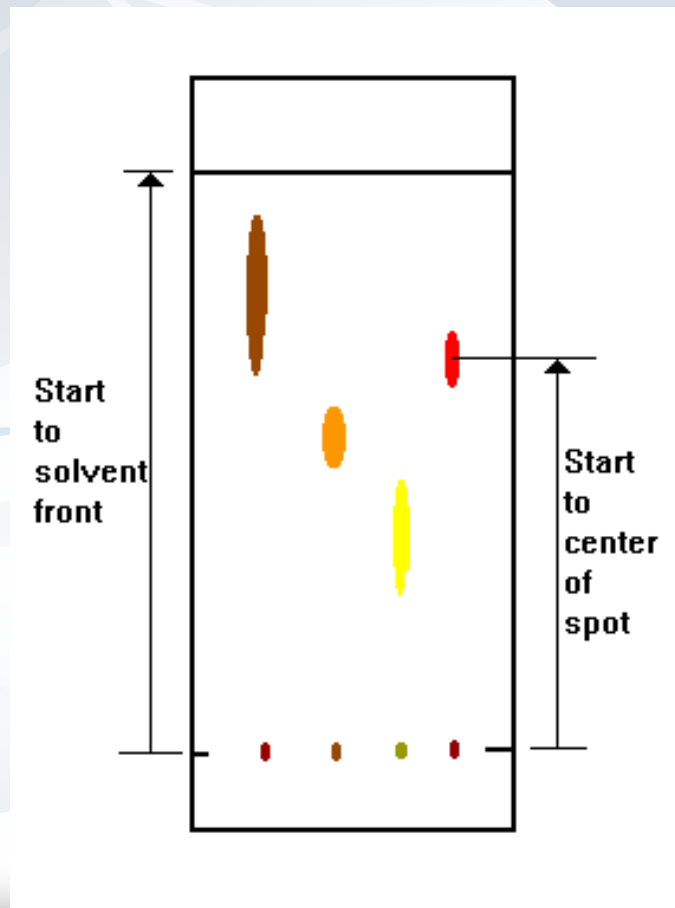
Interpreting the Data

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.

Paper Chromatography

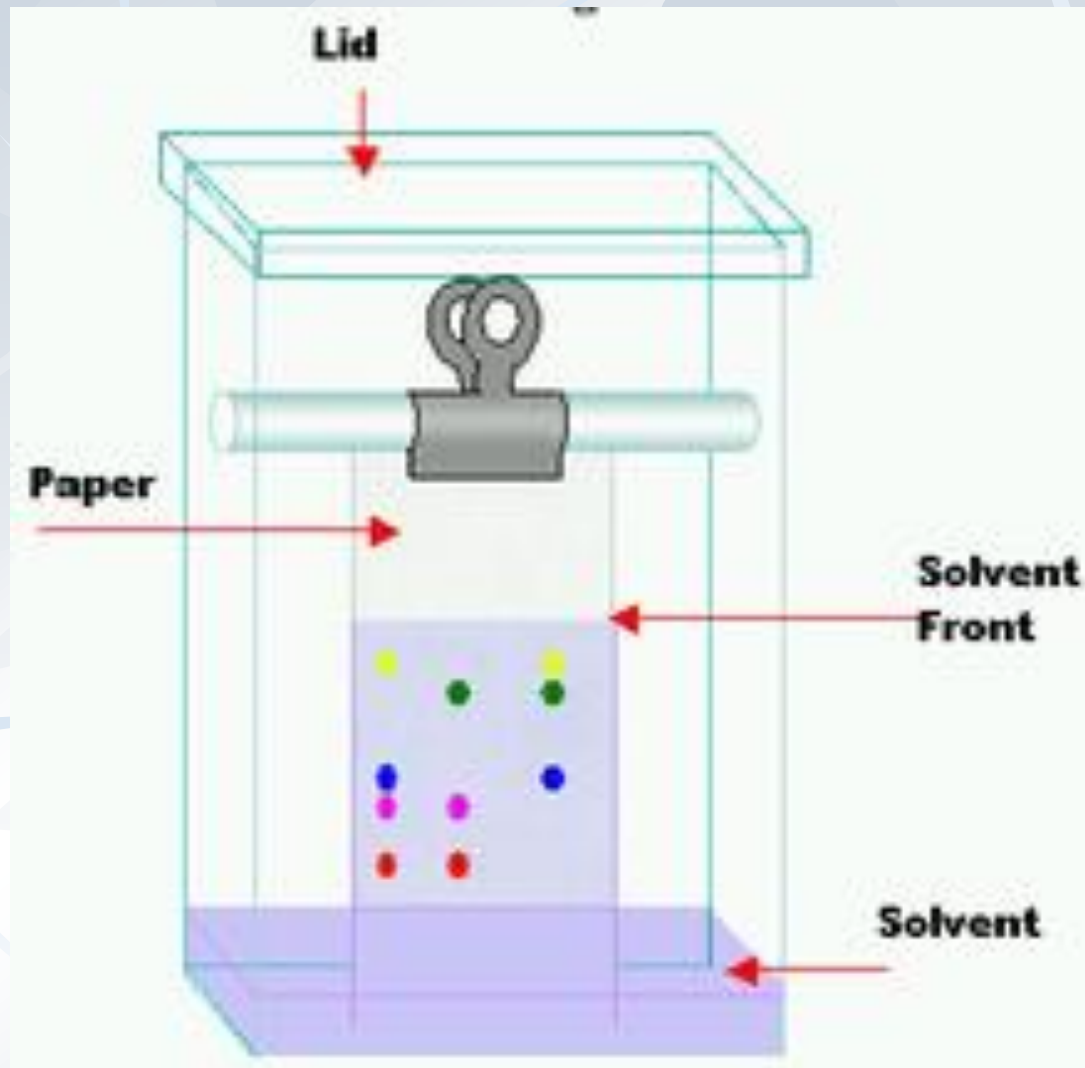
Paper Chromatography

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).



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

The isolation depends on partition coefficient of the solute.

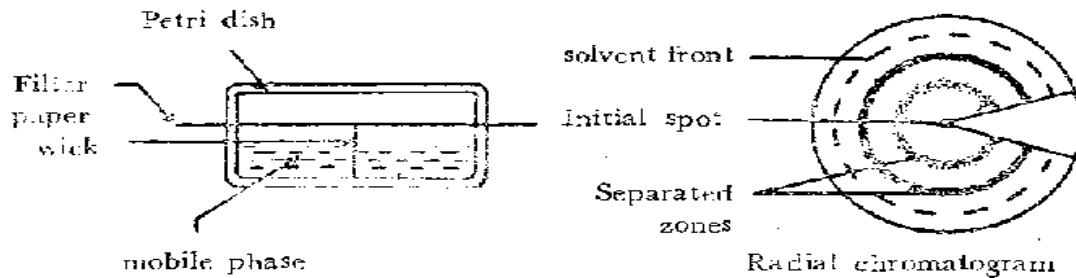
$$K = \frac{c(\textit{stationary})}{c(\textit{mobile})}$$

General Procedure

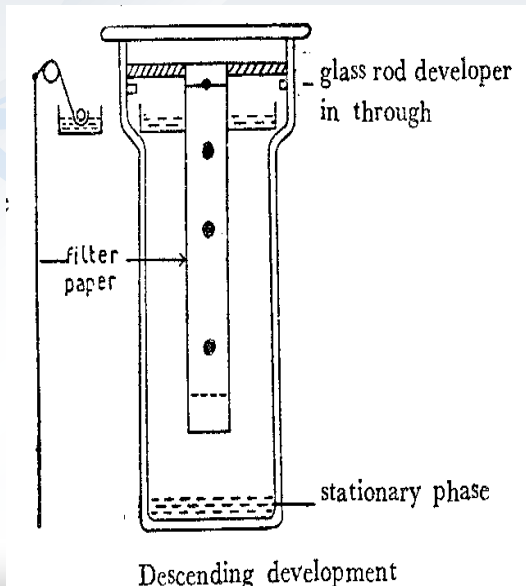
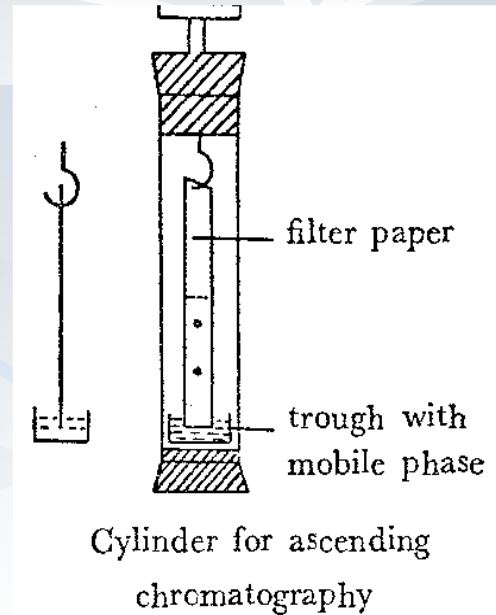
- 1- Choice of paper and solvent to be used.
- 2- Desalting of sample.
- 3- Application of the sample.
- 4- Equilibration of paper.
- 5- Development.
- 6- Detection.
- 7- Identification of substances.

Techniques of development with various flow directions

Radial development



Ascending 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.

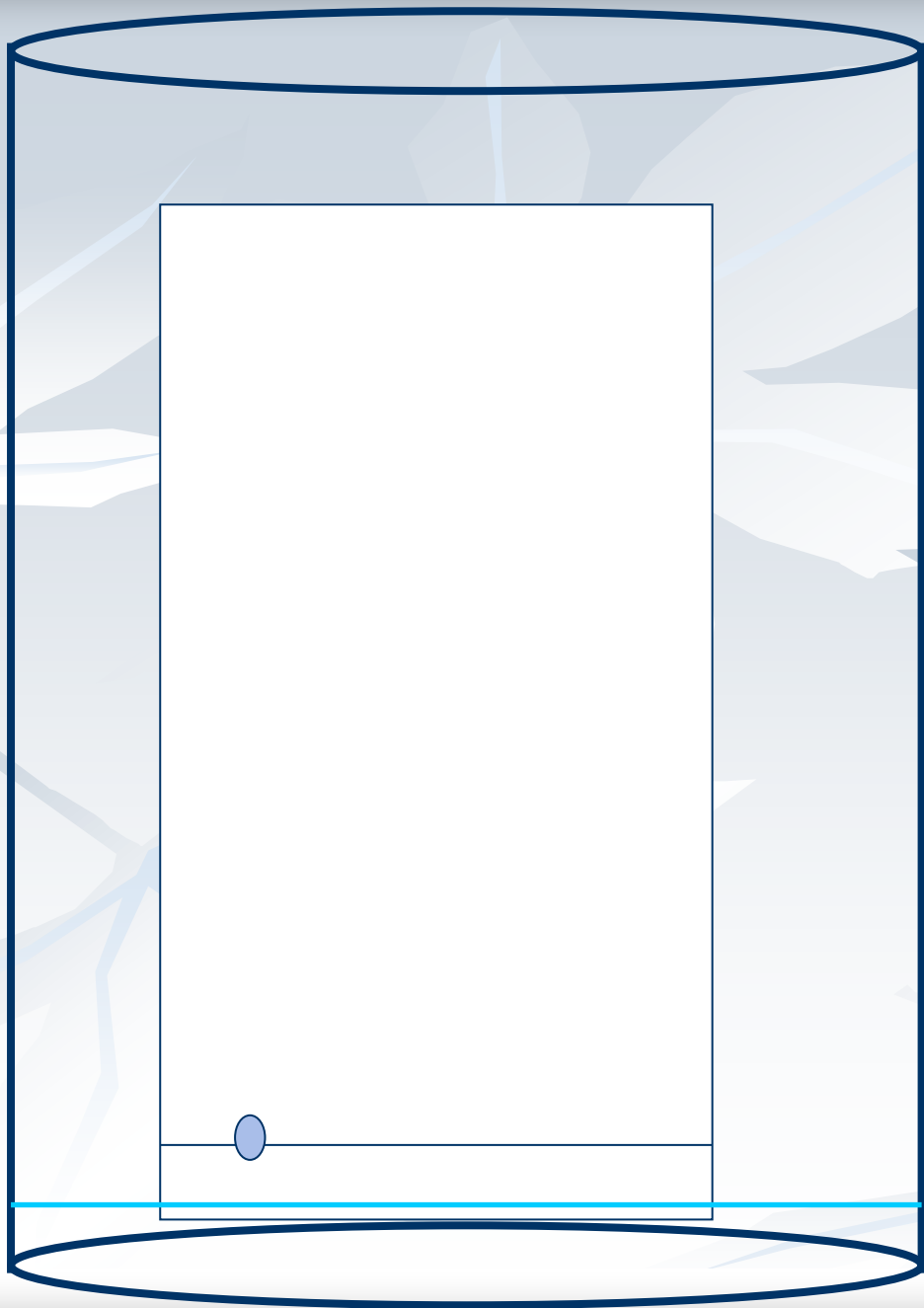
B- two-dimensional chromatography:

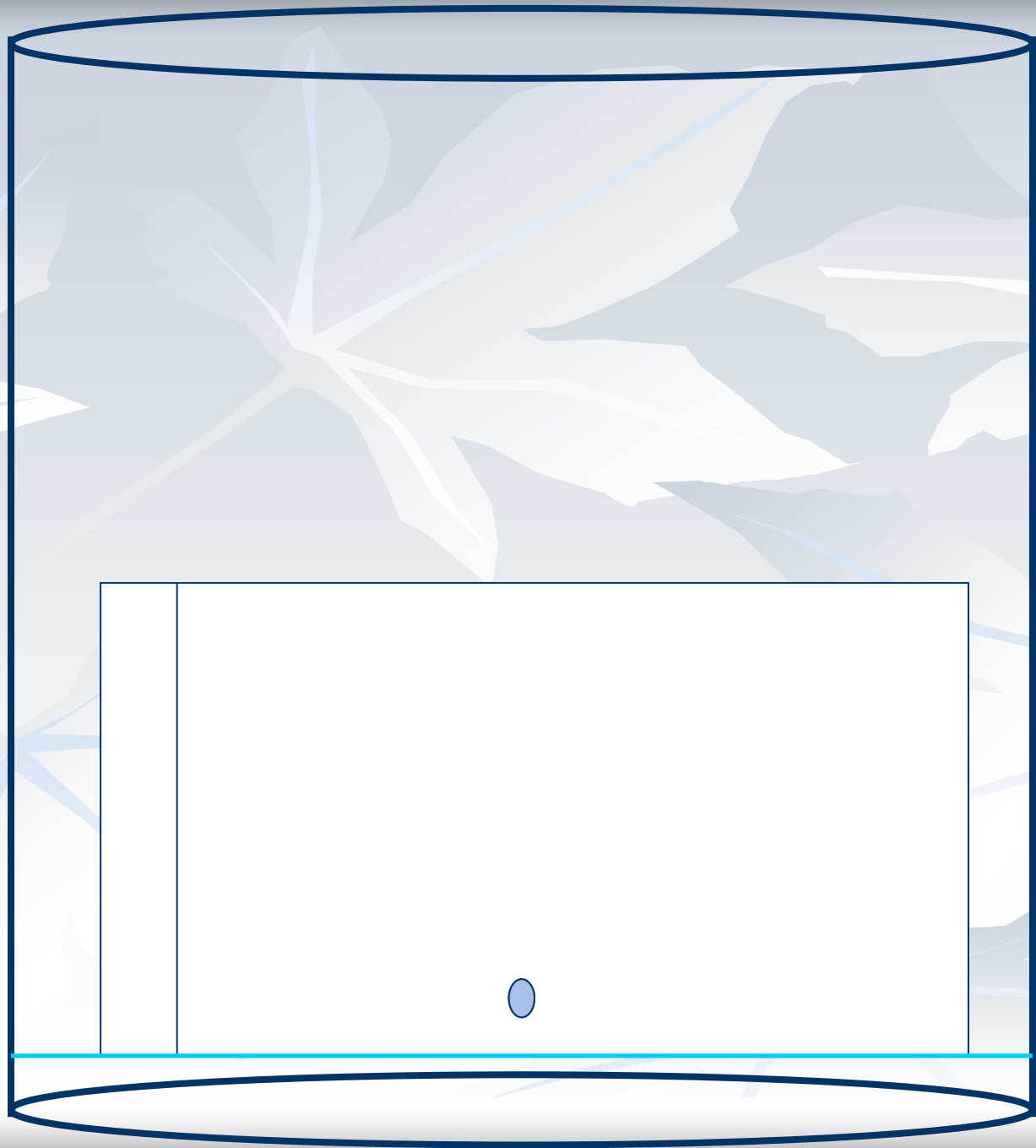
When large numbers of substances are to be separated on a single chromatogram.

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° 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.





Columnar 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

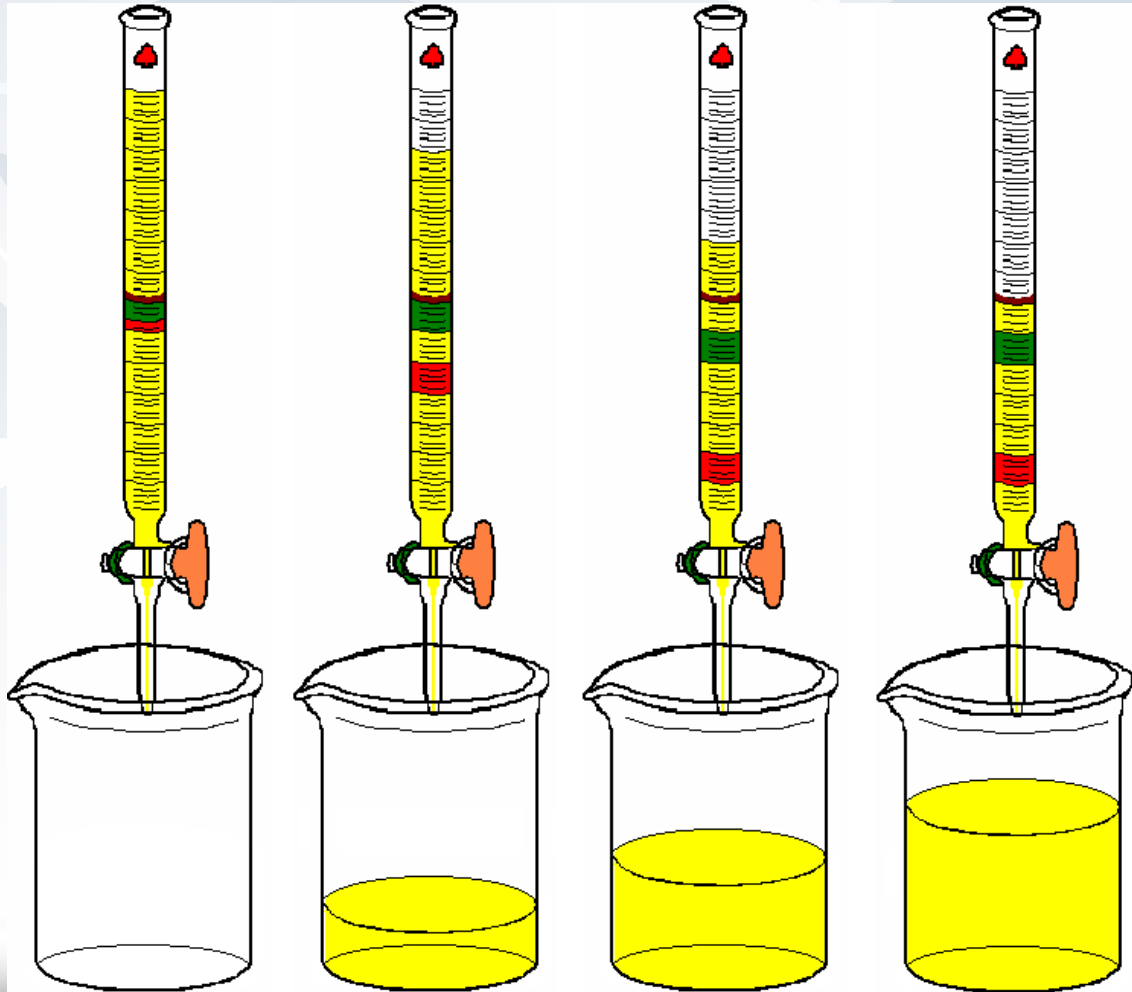
Different Types of chromatography

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 counterions 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 are immobilized	Liquid or gas	Special kind of solute molecules interact with those immobilized on the stationary phase

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



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.1

Technique	Procedure
Isocratic elution	Addition of solvent mixture of fixed composition during the whole process.
Gradient elution	<u>Continuous or linear elution</u> : 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).
	<u>Step wise or fractional elution</u> : 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.

4- 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) & high 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.

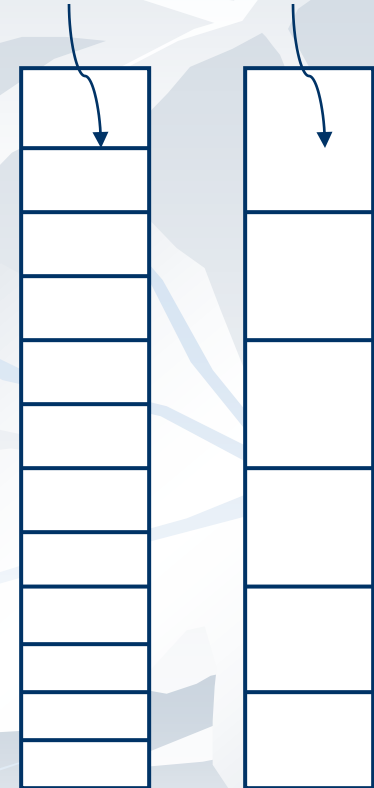
Number of Theoretical Plates (N)

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.

Partition Column Chromatography

In this type, the **packing consists of a theoretically inert support material coated with a film of the liquid stationary phase.**

The division into adsorption & partition is only of theoretical significance as in partition chromatography the adsorption effects of the support can be felt.

Selection of the solid support

The support material should:

- adsorb & retain the mobile stationary phase.
- expose as large surface as possible to the mobile phase
- be mechanically stable.
- be easy to pack.
- not retard the solvent flow

Examples of solid supports:

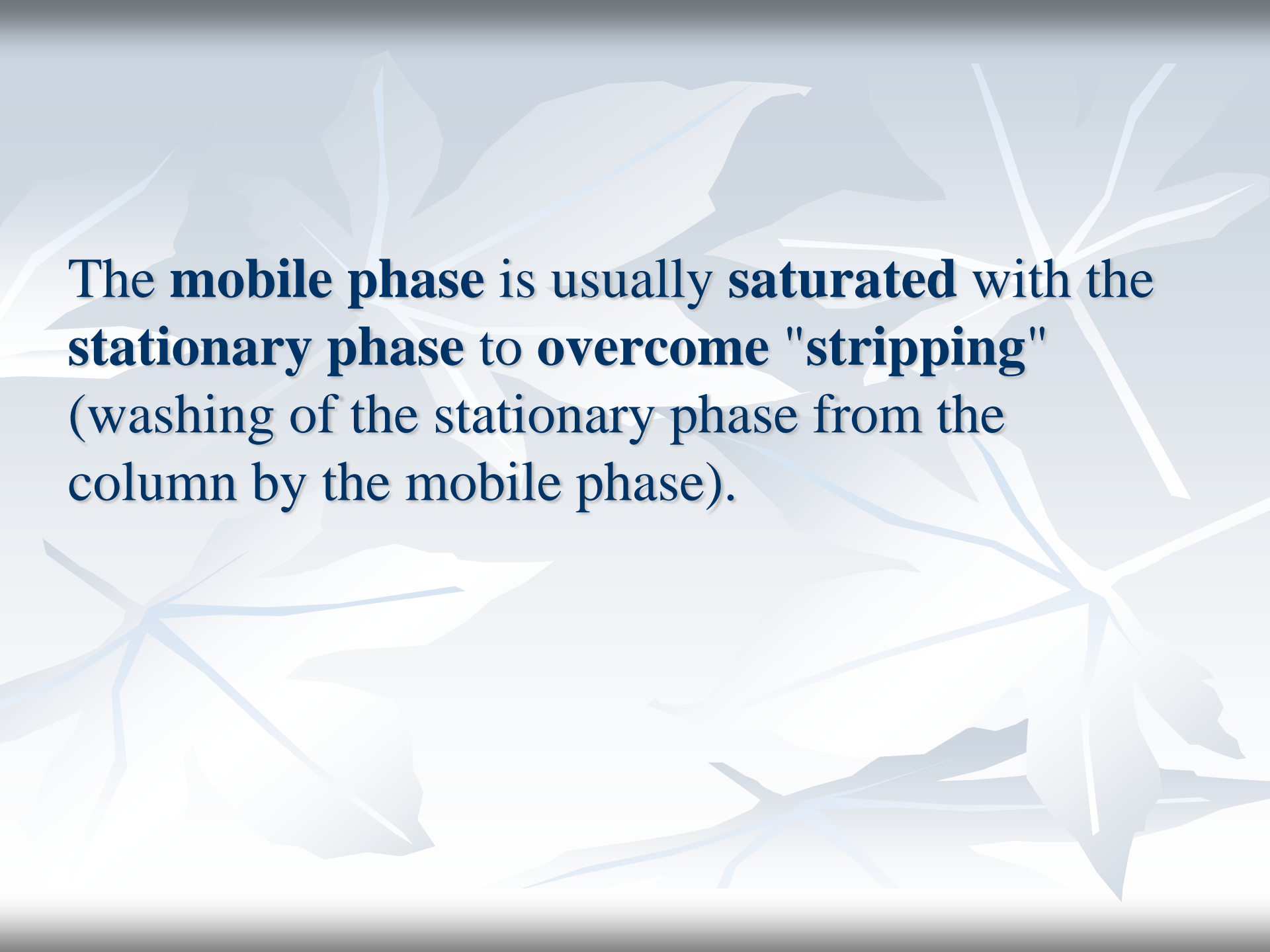
Silica gel, diatomaceous earth (as kieselguhr, celite etc.)
& cellulose.

Selection of the mobile phase

The **liquid stationary & mobile phases** should have a considerable difference between their solvent strength parameters.

Pure water > Methanol > Ethanol > Propanol > Acetone > Ethyl acetate > Ether > Chloroform > Dichloromethane > Benzene > Toluene > Carbon tetrachloride > Cyclohexane > Hexane > Pentane.

e.g. if the **stationary phase** is **water**, **pentane** would be the **eluent** of choice.



The mobile phase is usually saturated with the stationary phase to overcome "stripping" (washing of the stationary phase from the column by the mobile phase).

Gel Permeation Chromatography (GPC)

This type is also known as:

Size Exclusion Chromatography (SEC)

Molecular Exclusion Chromatography (MEC)

Molecular Sieve Chromatography (MSC)

Gel Filtration Chromatography (GFC)

Gel Chromatography.

Stationary phase

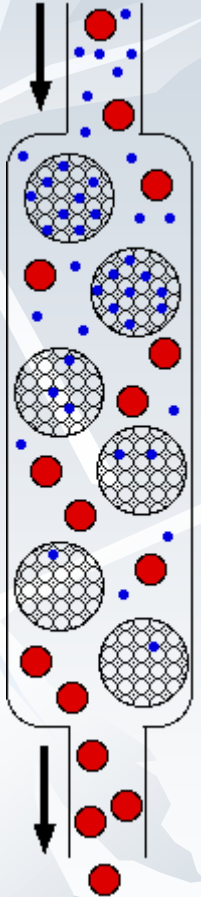
Porous polymeric matrix: formed of spongy particles, with pores completely filled with the liquid mobile phase (gel).

The gels (polymers) consist of **open, three-dimensional networks** formed by cross-linking of long polymeric chains.

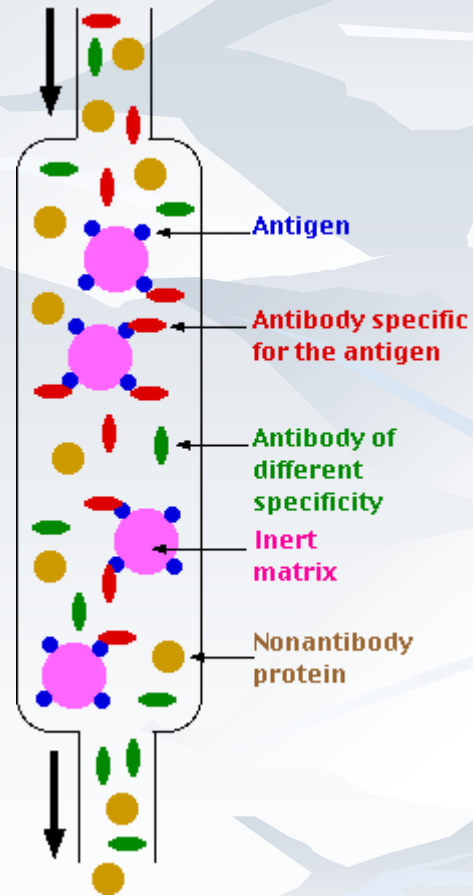
The **pore size** varies with the degree of cross-linking.

The **diameter of the pores is critical** as separation is based on that **molecules above certain size are totally excluded from the pores** because they can not enter the gel.

The **interior of the pores is reached**, partially or wholly, by smaller molecules.



Affinity Chromatography



Mobile phase

Mobile phase is a liquid as **water** or **dilute alcohol**

Separation mechanism

Based on difference between the solutes molecular weights.

Molecules will distribute themselves outside & inside the pores according to their size.

Larger are excluded, **medium sized** enter half-way & **smallest** permeate all the way.

The **retention volume V_o** of a substance is inversely proportional to the molecular weight (M. Wt) of the solute.

$$V_o \approx 1 / M.wt$$

V_o = retention volume

M.wt = Molecular Weight

Applications of GPC to natural products

Determination of M. wt. of peptides, proteins & polysaccharides.

Desalting of colloids e.g. desalting of albumin prepared with 2% $(\text{NH}_4)_2\text{SO}_4$.

Separation of mixture of mono- & polysaccharides.

Separation of amino acids from peptides & proteins.

Separation of proteins of different molecular weights.

Separation of mucopolysaccharides & soluble RNA.

Separation of myoglobin & haemoglobin.

Separation of alkaloids & purification of enzymes.

Ion Exchange Chromatography

Principle

Process by which **ions** of an **electrolyte solution** are brought into contact with an **ion exchange resin**.

The **ion exchange resin** is an insoluble polymer consisting of a "**matrix**" (Lattice or framework) that **carries fixed charges** (not exchangeable) and mobile **active ions** "**counter ions**" which are **loosely attached to the matrix**.

In **water**, the **counter-ions** move more or less **freely in the framework** & can be **replaced by ions of the same sign present in the surrounding solution**.

The "**matrix**" (framework) of a "**cation exchanger**" is considered as a crystalline non-ionized "**polyanion**" & the **matrix of an "anion exchanger"** as a non-ionized "**polycation**".

Cation Exchangers

Active ions (counter ions) are cations.

The polar groups attached to the matrix are acidic (sulphonic acids, carboxylic acids, phenols, phosphoric acids) e.g. a cation exchanger in the free carboxylic acid form:



X = Frame work (matrix)

-COO⁻ = Fixed charge (anionic),

Non-exchangeable

H⁺ = Counter ion (cation), Exchangeable

They are usually (but not always) supplied in the Na⁺ form: **X-COO⁻Na⁺**

or $\overline{\text{Na}^+}$, Where $\overline{\quad} = \text{Matrix}$

e.g. exchange with CaCl₂ aqueous solution



Anion Exchangers

Active ions (counter ions) are anions.

The polar groups attached to the matrix are tertiary or quaternary ammonium groups (basic).

e.g. Anion exchanger in the quaternary ammonium form:



X = Framework (matrix)

-NR₃⁺ = Fixed charge (cationic)

Non exchangeable

-OH⁻ = counter ion (anion), Exchangeable

They are supplied as the chloride rather than the hydroxide as the chloride form is a more stable. Represented as: $\mathbf{X.NR_3^+Cl^-}$

or $\overline{\text{Cl}^-}$ (where, $\overline{\quad}$ is the matrix)

e.g. exchange with Na_2SO_4 solution



Regeneration of the resin

Ion exchange process is generally reversible e.g in the following:



The cation exchanger could be exhausted after exchanging all Na^+ for Ca^{++} , the exchanger could be regenerated (loaded again with Na^+) by contacting it with excess Na^+ ions e.g. a solution of NaCl .

Types of Exchangers

Ion Exchangers

These are either **cation** or **anion exchangers** of either **organic** or **inorganic** nature.

A- Inorganic ion exchangers

Common clays, soils, minerals e.g. zeolites used for "softening water".

Disadvantage: low ion-exchange capacity.

Advantages:

Great resistance to high temperatures.

High volume capacity.

Great selectivity towards simple inorganic ions.

B- Organic exchangers

They may be natural or synthetic.

Preparation of organic synthetic ion exchangers :

Polycondensation of phenols, sulpho- & carboxy-derivatives with formaldehyde → cationic exchangers.

Polycondensation of aromatic amines with formaldehyde → anionic exchangers.

These techniques yield products linear in structure & relatively soluble in water which are now replaced by resin materials based on styrene divinyl benzene copolymers and polyacrylate.

Applications of Ion Exchange Chromatography

1- Water softening:

Removal of Ca^{2+} , Mg^{2+} & other multivalent ions causing hardness of water by filtration through a layer of strong cation resin.

2- Water demineralization:

Removal of cations & anions dissolved in water. Usually carried by the two step technique in which two columns of strongly acid cation exchanger in $[\text{H}^+]$ form & strongly basic anion exchanger in $[\text{OH}^-]$ form are used in sequence.

3- Neutralization:

Cationic exchanger in $[\text{H}^+]$ can be used to neutralize alkali hydroxide & anionic exchanger in $[\text{OH}^-]$ form to neutralize the acidity.

4- Separation of electrolytes from non-electrolytes.

5- Separation of carbohydrates & their derivatives:

Uronic acids separated on anion exchanger.

Sugars converted into ionized form by using borate & separated on strong anion exchanger.

Hexosamines separated on strong cation exchanger.

THANK YOU

