Chromatography

Part-I: Thin layer and Paper Chromatography

Presented by:
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- Chroma means “color” and graphein means to “write”
- 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 differential distribution of analytes between two phases

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

Classification

A. 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

B. 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

C. According to the force of separation:

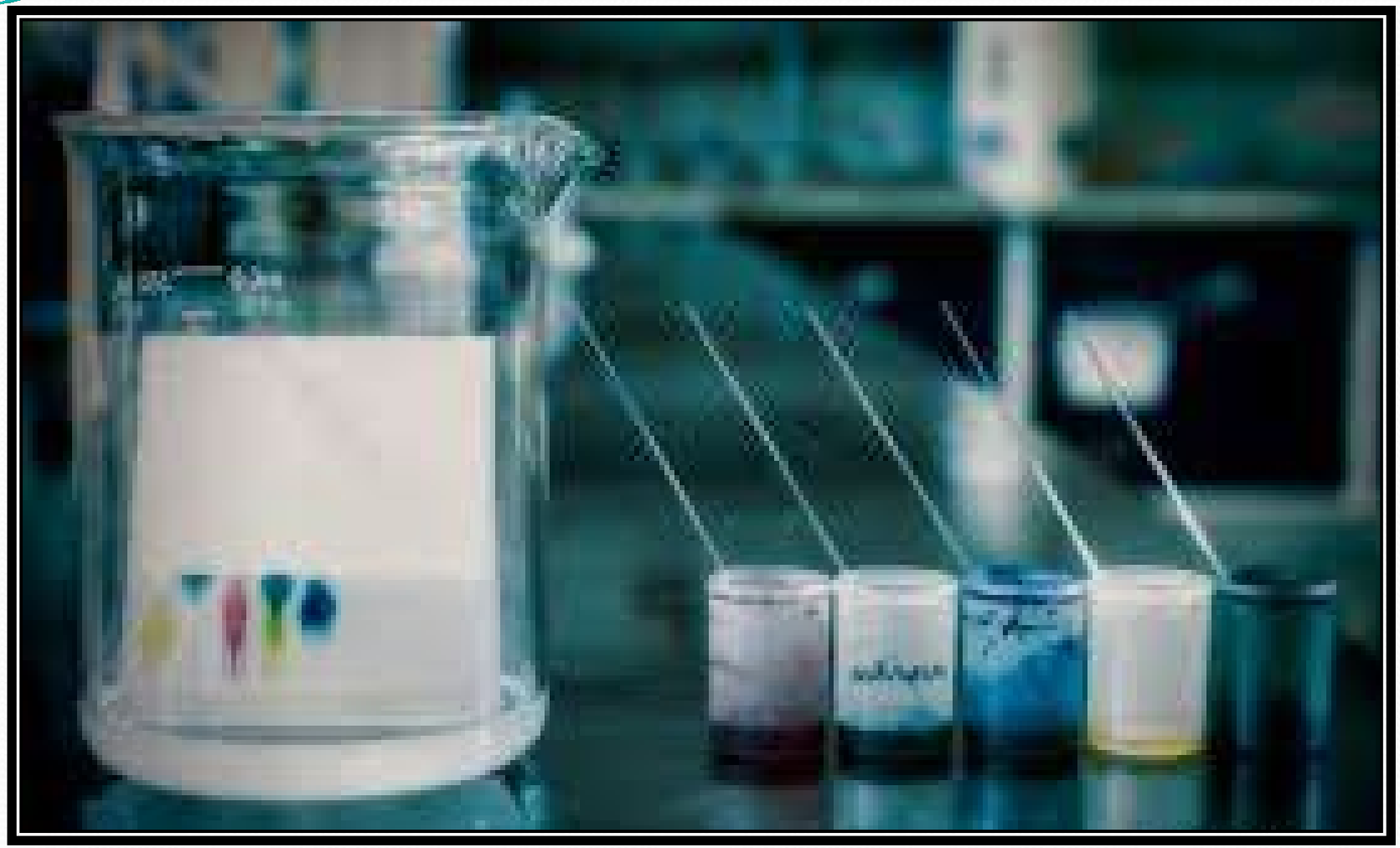
1- Adsorption chromatography.

2- Partition chromatography.

3- Ion exchange chromatography.

4- Gel filtration chromatography.

5- Affinity chromatography.



Thin Layer Chromatography

Thin layer chromatography (TLC)

- is a method for identifying substances and testing the purity of compounds.
- is a useful technique because it is relatively quick and requires small quantities of material.
- is Cheaper and requires less skill as compared to advanced chromatographic methods

- **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.**

Selection of Stationary Phase

For choosing the stationary Phase (Adsorbent), the following characters are considered

- Solubility of compound e.g, hydrophilic or lipophilic
- Nature of substance to be seperated i.e whether it is acidic, basic or amphoteric
- Adsorbent particle size
- Adsorbent should not adhere to glass plate
- Reactivity of compound with the solvent or adsorbent
- Chemical reactivity of compounds with binders

Stationary phases for thin-layer chromatography

Stationary phase	Predominant sorption process	Use
silica gel	adsorption or partition	general
modified silica gels	adsorption or partition	similar to bonded phase HPLC
alumina	adsorption or partition	general
cellulose powder	partition	inorganic, amino acids, nucleotides, food-dyes
kieselguhr	partition	sugars
modified celluloses e.g. DEAE and CM	ion-exchange	nucleotides, phospholipids
Sephadex gels	exclusion	macromolecules

Preparation of chromatoplates

- Glass plates or flexible plates are commonly used for adsorbent.
- Size used depends on type of separation to be carried out, the type of chromatographic tank and spreading apparatus available.
- The standard sizes are 20 x 5 cm, 20 x 10 cm or 20 x 20 cm .
- The surface should be flat without irregularities.
- The standard film thickness is 250um

Methods for application of adsorbent.

- Pouring
- Dipping
- Spraying
- Spreading.

- **Pouring:** The adsorbent of finely divided and homogeneous particle size is made into slurry and is poured on a plate and allowed to flow over it so that it is evenly covered.

- **Dipping :** This technique is used for small plates by dipping the two plates at a time, back to back in a slurry of adsorbent in chloroform or other volatile solvents. Exact thickness of layer is not known and evenness of layer may not be good.

- **Spraying :** Slurry is diluted further for the operation of sprayer. But this technique is not used now a days as it is difficult to get uniform layer.

- **Spreading :** All the above methods fail to give thin and uniform layers. Modern methods utilize the spreading devices for preparation of uniform thin layers on glass plates. Commercial spreaders are of two types (a) Moving spreader, (b) Moving plate type.

  gives layer thickness from 0.2 to 2.0 mm.

Activation of plates

- After spreading plates are allowed to dry in air and further dried and activated by heating at about 100° c for 30 mins.

- By removing the liquids associated with layer completely, the adsorbent layer is activated

Mobile phase

- If the stationary phase is hydrophilic such as silica gel G, various mixtures of hydrophobic solvents such as benzene, cyclohexane and chloroform provide satisfactory mobile phases.
- The organic solvent mixture of low polarity is used Highly polar solvents are avoided to minimize adsorption of any components of the solvent mixture. Use of water as a solvent is avoided as it may loosen the adhesion of a layer on a glass plate.
- The solvents listed in elutropic series are selected as per requirement.

Choice of the mobile phase

The choice of the mobile phase depends upon the following factors:-

1. Nature of the substance to be separated
2. Nature of the stationary phase used
3. Mode of chromatography (Normal phase or reverse phase)
4. Separation to be achieved- Analytical or preparative.

Mobile phase

- A mixture of an organic solvent and water with the addition of acid, base or complexing agent to optimize the solubility of the components of a mixture can be used.
- For example, good separations of polar or ionic solutes can be achieved with a mixture of water and n-butanol. Addition of acetic acid to the mixture allows more water to be incorporated and increases the solubility of basic materials, whilst the addition of ammonia increases the solubility of acidic materials.

Solvent Systems

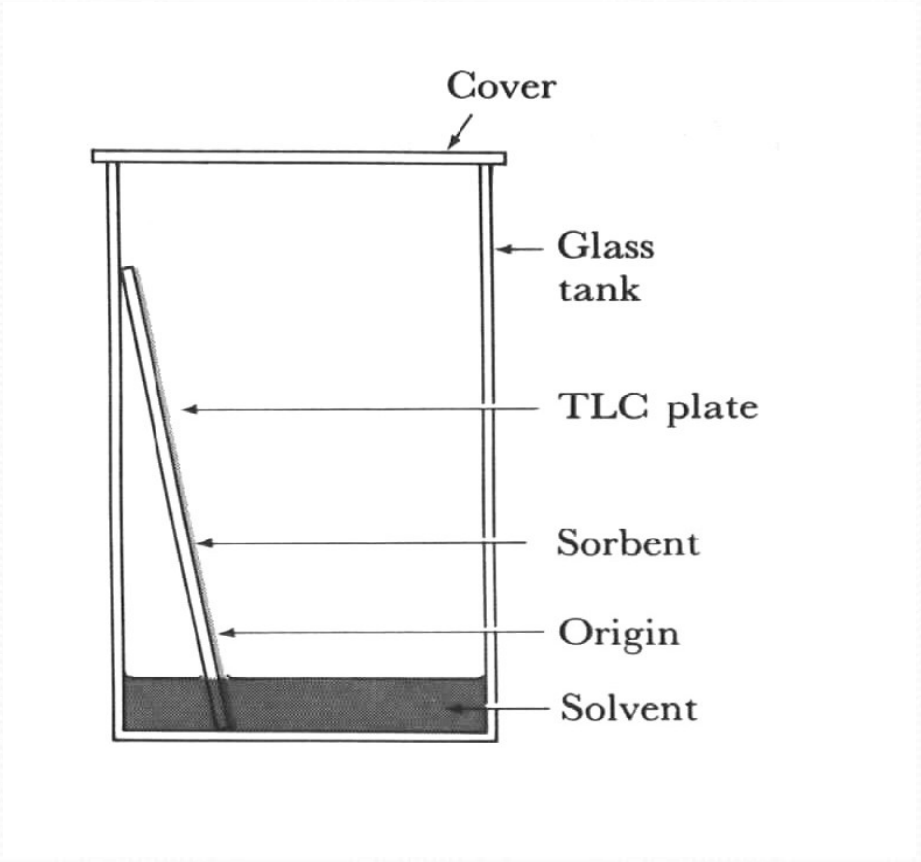
The solvent system performs the following main tasks:

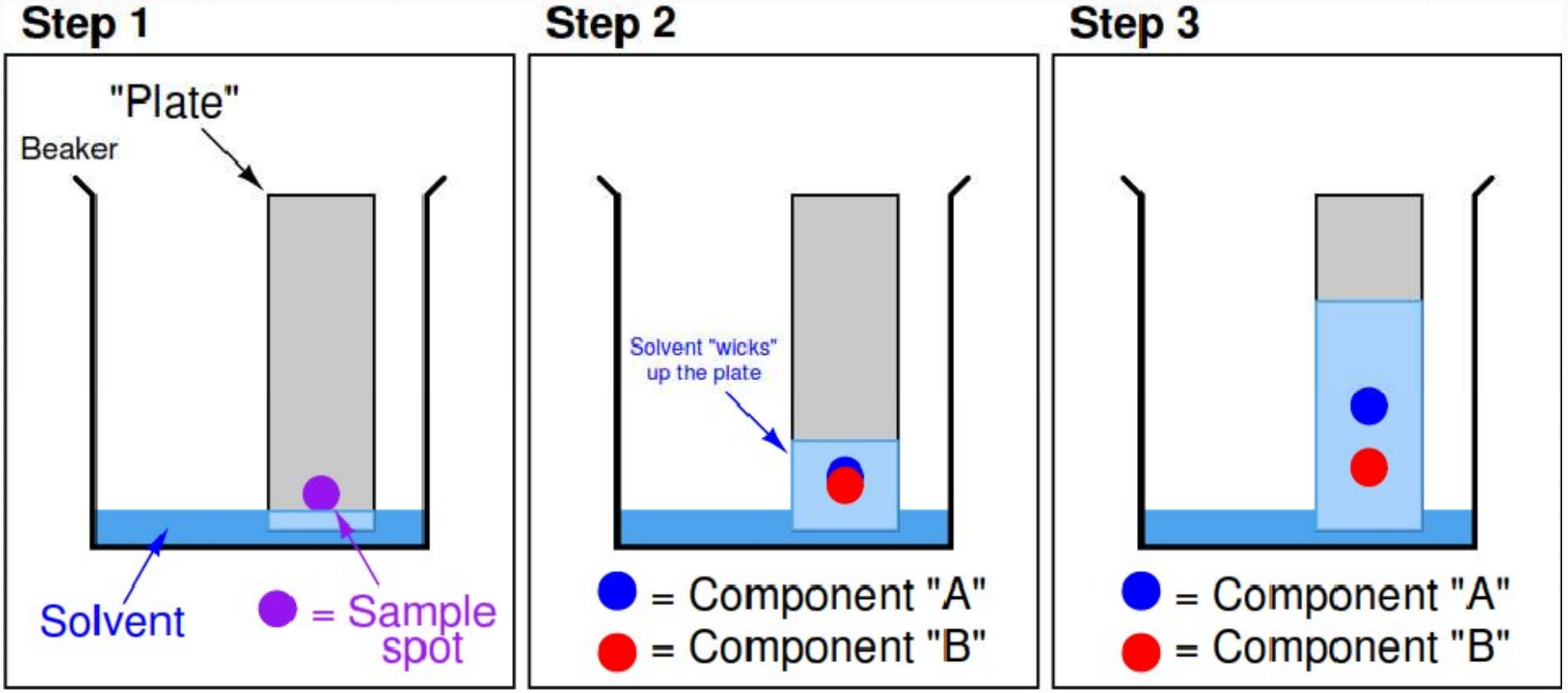
- To dissolve the mixture of substances,
- To transport the substances to be separated across the sorbent layer,
- To give Rf values in the medium range, or as near to this as possible,
- To provide adequate selectivity for the substance mixture to be separated.

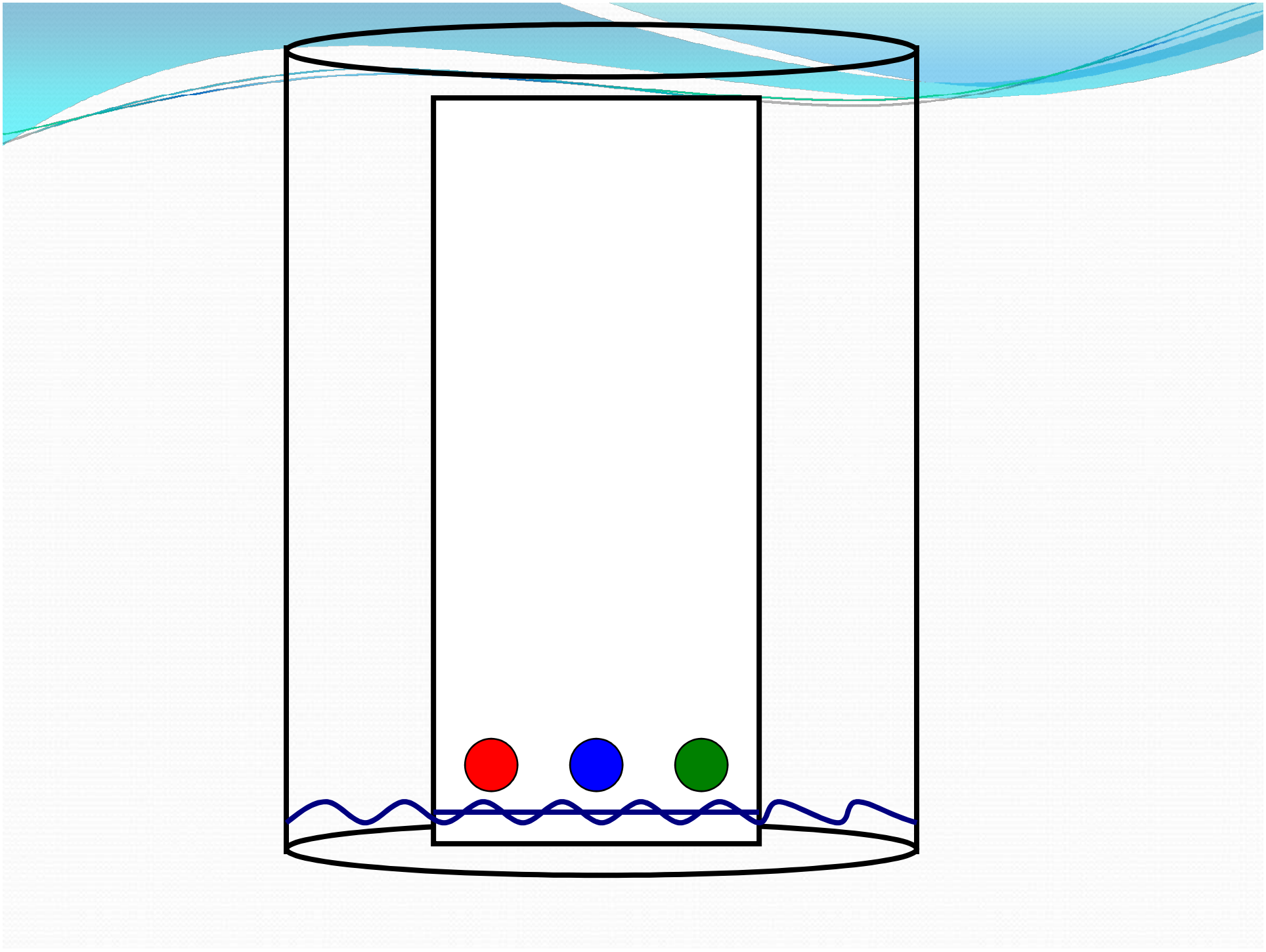
They should also fulfill the following requirements:

- Adequate purity,
- Adequate stability,
- Low viscosity,
- Linear partition isotherm,
- A Vapor pressure that is neither very low nor very high,
- Toxicity that is as low as possible

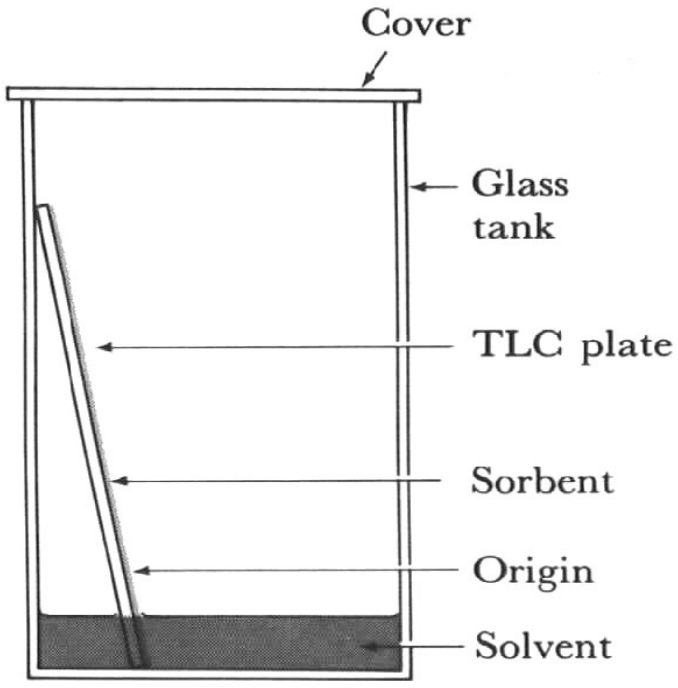
Thin Layer Chromatography (TLC)

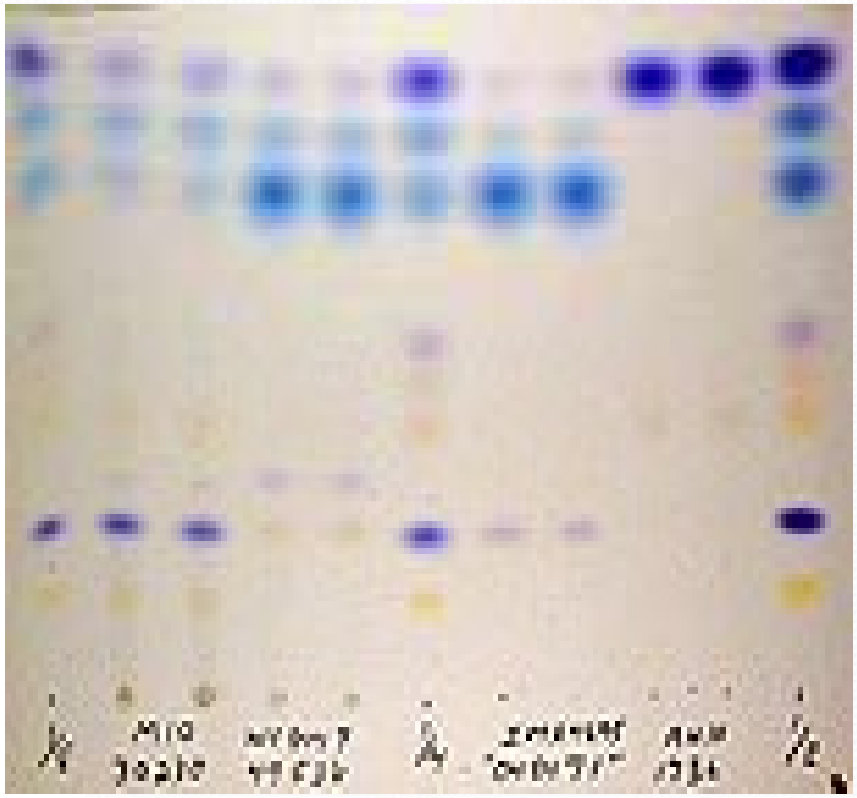



Step 1
"Plate"
Beaker
Solvent
= Sample spot
Step 2
Solvent "wicks" up the plate
= Component "A"
= Component "B"
Step 3
= Component "A"
= Component "B"



TLC





The Experimental Techniques of TLC: Steps

1. **Preparing the Chamber**
2. **Preparing the Plates for Development**
3. **Developing the Plates**
4. **Identifying the Spots (visualization)**
5. **Interpreting the Data**

The Experimental Techniques of TLC: Details

1. 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.

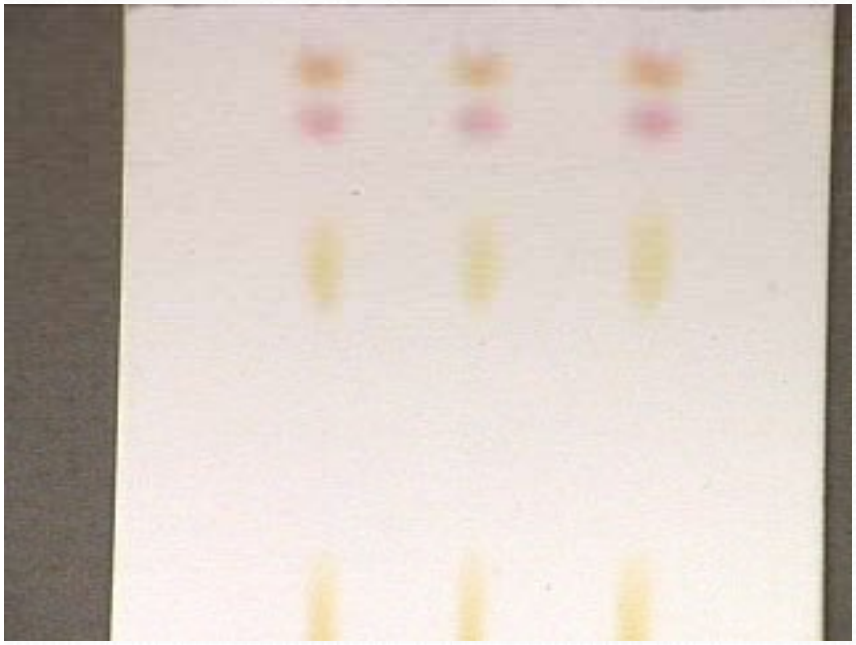
2. Preparing the Plates for Development

- 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.

3. 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.

4. 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.

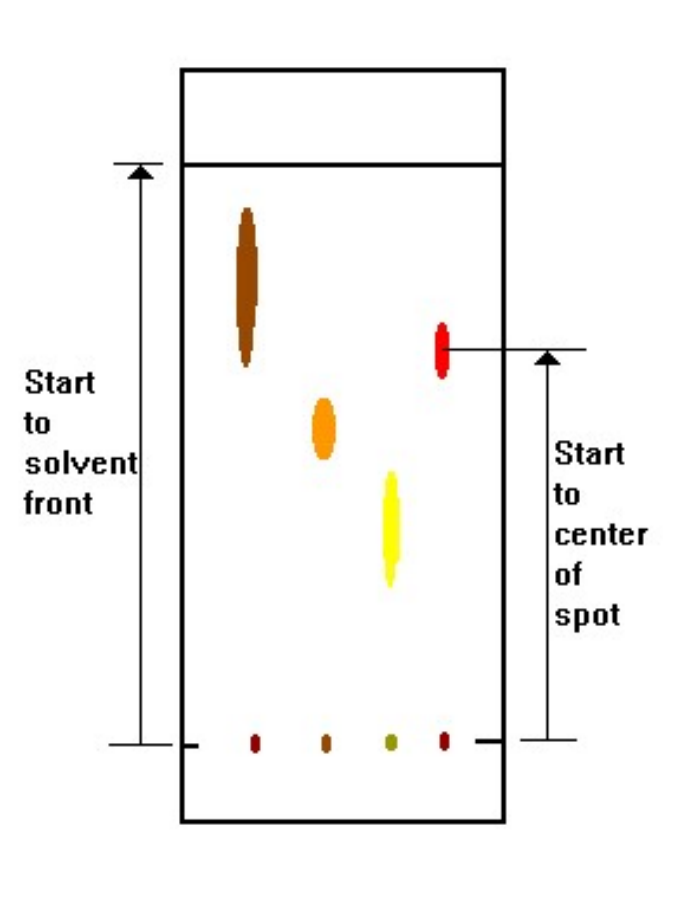
Visualizing Agents

- For Alkaloids: Dragendorff's reagent
- For Cardiac glycosides: Antimony trichloride
- For Sugar: Aniline phthalate
- For Amino acids: Ninhydrin

5. 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**

Applications of TLC

1. **AS A CHECK ON PROCESS**:

- Used for checking other separation procedure and purification processes.
- Checking of distillation fractions and for checking the progress of molecular distillation
- Checking the progress of an organic reaction

2. **IN ORGANIC CHEMISTRY**

- Isolation and separation of individual components of a mixture.
- For checking the purity of samples.
- As a purification process.
- Examination of reactions

- For identifying organic compounds e.g.
  a) Acids - Eg: phenol carboxylic acid .
  b) Alcohols
  c) Glycols
  d) Alkaloids – Purine alkaloids
  e) Amino acids , proteins , peptides
  f) Antibiotics – Tetracycline's separated on thin layer silica gel G by using solvents like 10% citric acid, n-butanol, methanol etc.

3. **SEPARATION OF INORGANIC IONS**

- Used for separating cationic, anionic, purely covalent species and also some organic derivatives of the metals.

4. **ANALYSIS OF COMPONENTS OF FOOD STUFFS**
Examples: Fruits, milk, orange juice, wine , Eggs.

5. SEPARATION OF AMINO ACIDS

- Separation of amino acid in a mixture by development in 2 directions. The sample is placed in one corner of a square plate and development was performed in ascending direction.

6. SEPARATION OF VITAMINS

- Mobile phase- 80% cyclohexane+20% di ethylene Spraying reagent – 20% antimony(III)chloride solution in $CHCl_3$ After spraying,

➢ **Vit A:** Blue immediately with rapid fading

➢ **Vit D3:** Gradually changes yellow to orange.

➢ **Vit E:** becomes visible when the plate is heated at 100^{o}c for 5-6 minutes.

7. APPLICATIONS OF TLC IN QUANTITATIVE ANALYSIS

- Two basic techniques for the quantitative analysis includes,

1) Analysis of fractions on a plate

2) Determination of fraction after elution from coating material.

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 (mobile phase).

The stationary Phase and papers used

- Whatman filter papers of different grades like No.1, No.2, No.3, No.4, No.20, No.40, No.42 etc. are used.
- In general this paper contains 98-99% of α-cellulose, 0.3 ±1% β-cellulose.
- **Factors that governs the choice of paper:**
  - Nature of Sample and solvents used.
  - Based on Quantitative or Qualitative analysis.
  - Based on thickness of the paper.
- Moisture / Water present in the pores of cellulose fibers present in filter paper acts as stationary phase & another mobile phase is used as solvent in general Paper Partition Chromatography

Types of Papers:

- **Modified Papers:** acid or base washed filter paper, glass fiber type paper.
- **Hydrophilic Papers:** Papers modified with methanol, formamide, glycol, glycerol etc.
- **Hydrophobic papers:** acetylation of OH groups leads to hydrophobic nature, hence can be used for reverse phase chromatography.
- Impregnation of silica, alumna, or ion exchange resins can also be made.

Choice of the Solvent

- The commonly employed solvents are the polar solvents, but the choice depends on the nature of the substance to be separated.

- If pure solvents do not give satisfactory separation, a mixture of solvents of suitable polarity may be applied.

Types of Solvents/ Mobile Phase

- Pure solvents, buffer solutions or mixture of solvents Examples-
- **Hydrophilic mobile phase:**
  - Isopropanol: ammonia: water 9:1:2
  - Methanol : water 4:1
  - N-butanol : glacial acetic acid : water 4:1:5
- **Hydrophobic mobile phases:**
  - dimethyl ether: cyclohexane
  - kerosene: 70% isopropanol

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

- The isolation depends on partition coefficient of the solute.

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

General Procedure

1- Choice of paper and solvent to be used.

2- Preparation of chamber.

3- Application of the sample.

4- Equilibration of paper.

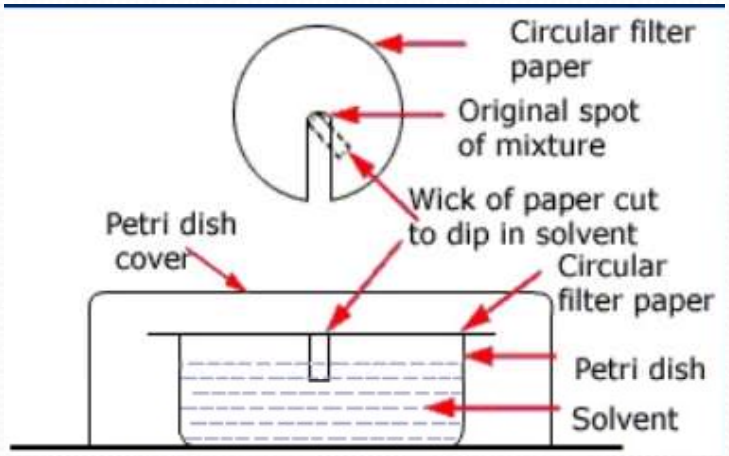
5- Development.

6- Detection.

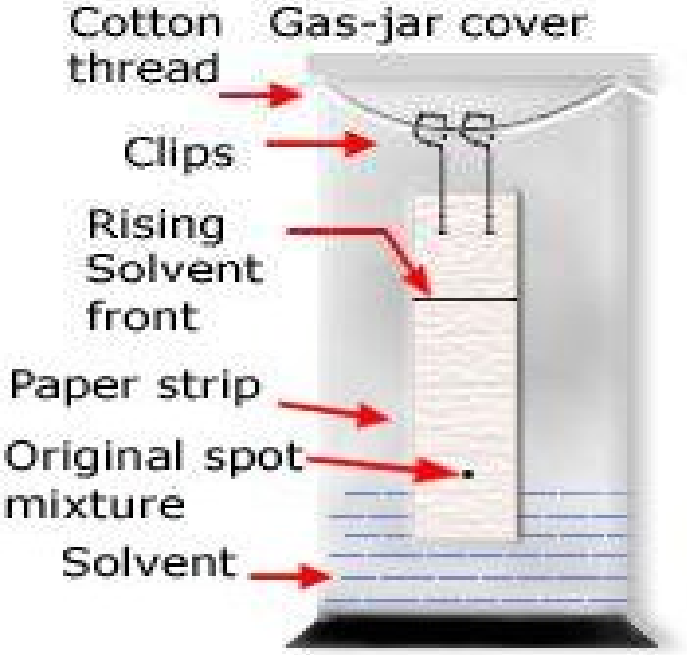
7- Identification of substances: R_f Value

Techniques of development with various flow directions

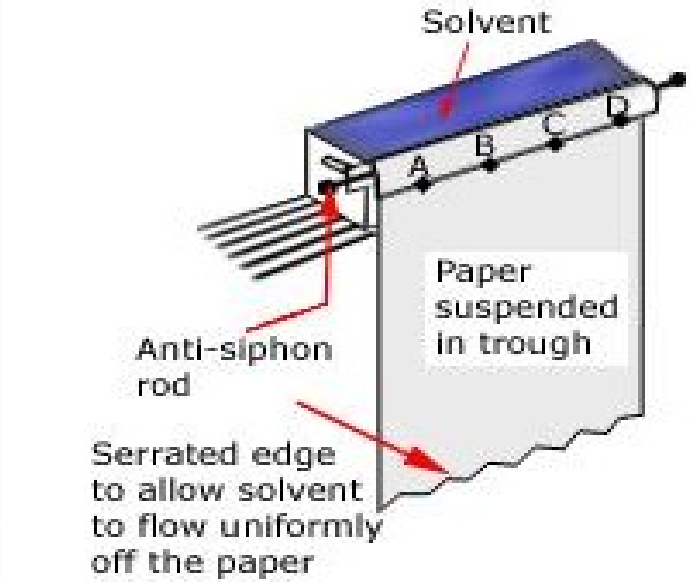
Radial development



Ascending development



Descending development



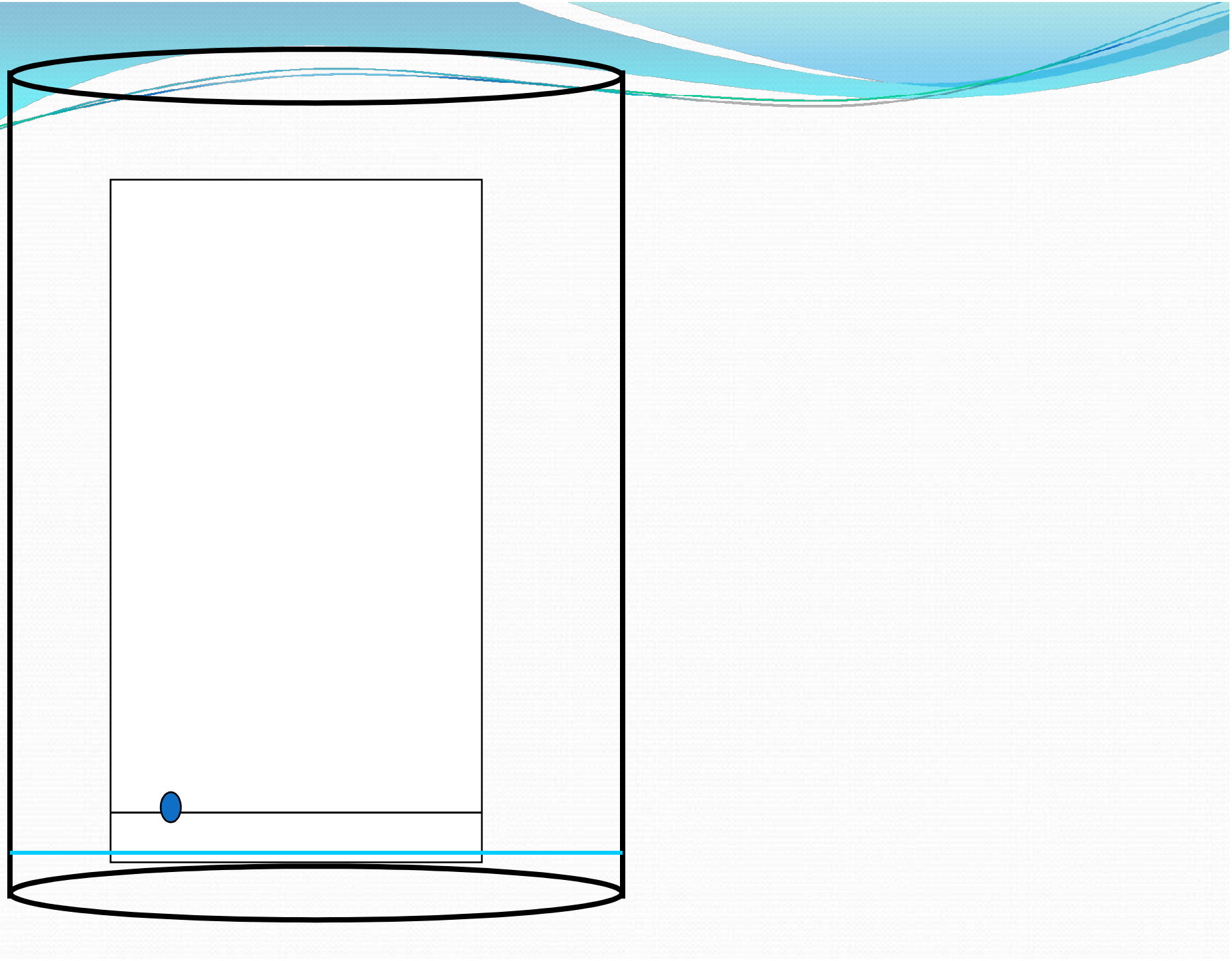
Multiple chromatography

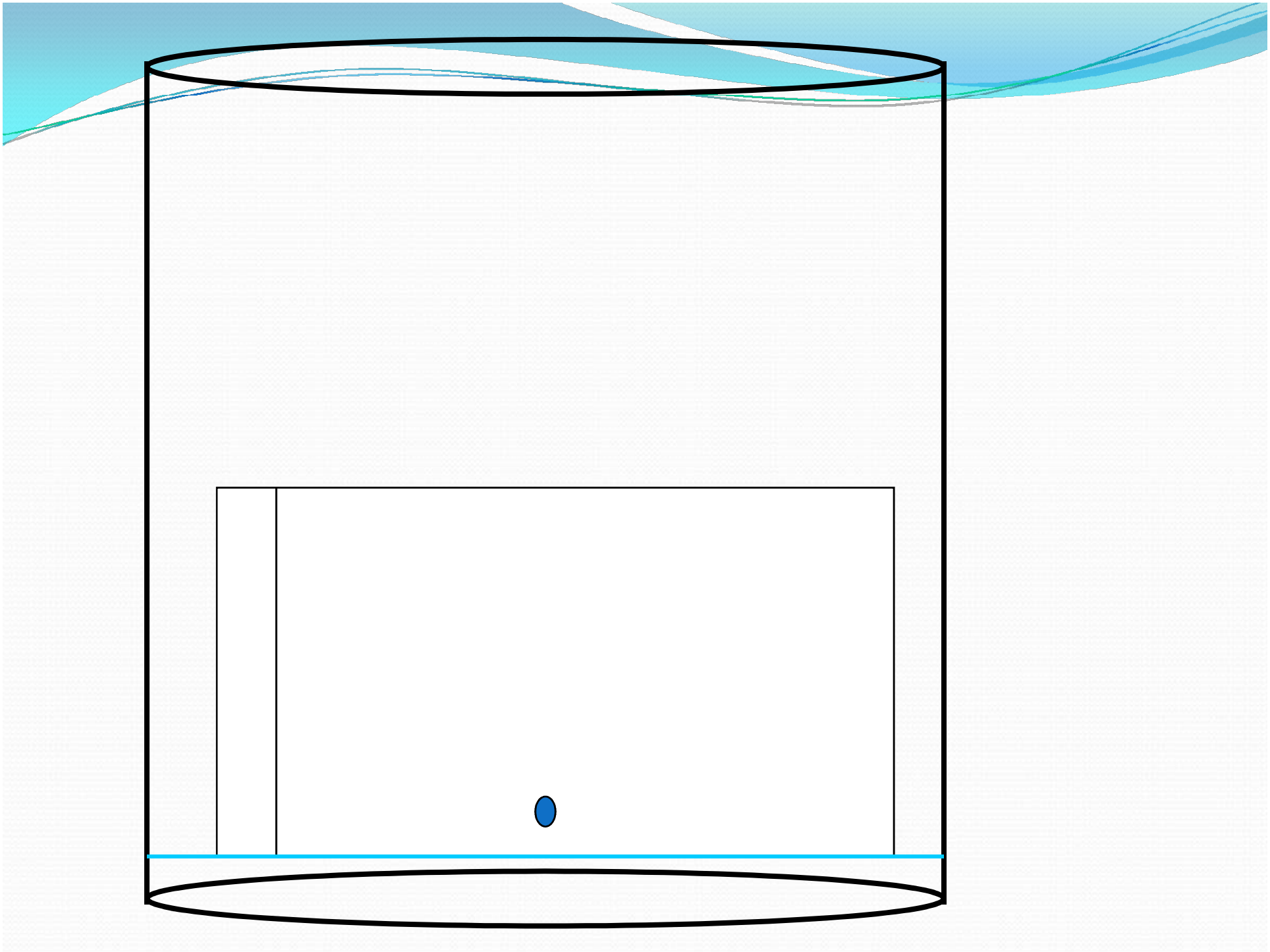
- Multiple chromatography includes all procedures in which the development is repeated after one development is completed.

- **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.

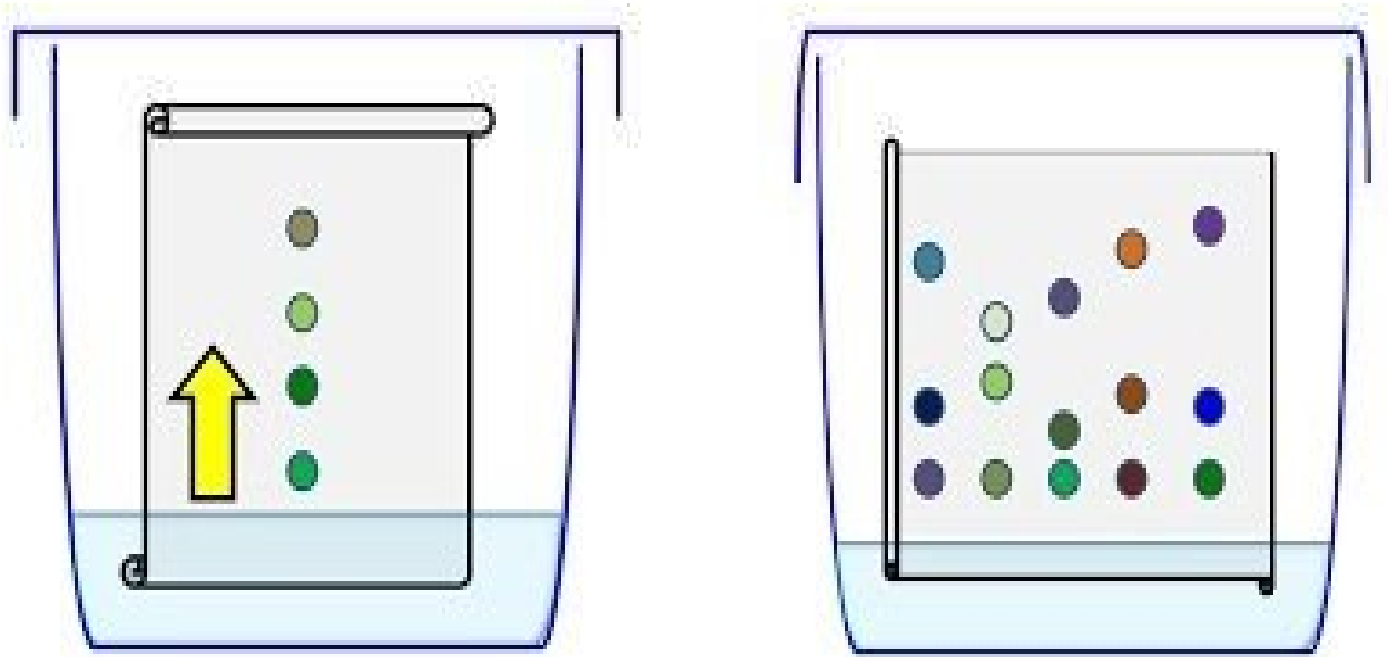
B- two-dimensional chromatography:

- 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^{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.





Two dimensional development



Applications

- Two-dimensional TLC can provide improved resolution and consequently is very advantageous in the separation of complex mixtures.
- Used for the separation of a large number of compounds that cannot be separated in a single dimension TLC experiment e.g. Pharmaceuticals, petroleum products etc.

Thank You

.....Keep Learning!