

# **EXPERIMENT 10**

## **Chromatography**

### **TLC and Column Chromatography**

# TLC and Column Chromatography

## General Principles of Chromatography

- Chromatography is defined as the **separation** of a mixture of two or more different compounds or ions by **distribution** between **two phases**, one of which is **stationary** and the other is **mobile** (moving).
- Various types of chromatography are possible, depending on the **nature of the two phases** involved:
  - solid—liquid** (column, thin-layer, and paper chromatography),
  - liquid—liquid** (high performance liquid chromatography),
  - gas—liquid** (vapor-phase, gas chromatography) .
- **We will study two methods : Thin layer chromatography, (TLC), and column chromatography.**

# TLC and Column Chromatography

## Thin-layer chromatography (TLC )

### Introduction

TLC is a widely used analytical technique.

### Properties of TLC:

- Simple
- Inexpensive
- Fast
- Efficient
- Requires only milligram quantities of material.

### TLC is useful for:

- Determining **the number of compounds** in a mixture.
- Characterizing if two compounds are **identical**.

# TLC and Column Chromatography

## Thin-layer chromatography (TLC) :

### Introduction

- In TLC (glass, metal, or plastic) plates are coated with a thin layer of adsorbent, which serves as the **stationary phase**.
- The stationary phase is usually **polar—silica gel** ( $\text{SiO}_2 \cdot x\text{H}_2\text{O}$ ) the most widely used.
- The **mobile phase** is a pure solvent or a mixture of solvents; the appropriate composition of the mobile phase depends on the polarities of the compounds in the mixture being separated.
- Most nonvolatile solid organic compounds can be analyzed by thin-layer chromatography.
- TLC does not work well for many liquid compounds because their volatility can lead to loss of the sample by evaporation from the TLC plate.

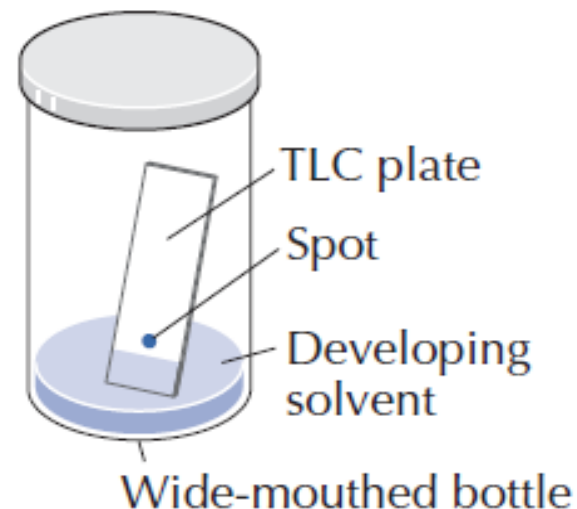
# TLC and Column Chromatography

## Thin-layer chromatography (TLC )

### • Overview of TLC Analysis

#### To carry out a TLC analysis:

- A small amount of the mixture being separated is **dissolved** in a suitable solvent and **applied or spotted** onto the adsorbent near one **end of a TLC plate**.
- The plate is **placed in a closed chamber**.
- The edge nearest the applied spot immersed in a shallow layer of the mobile phase called the **developing solvent**.
- The solvent rises through the stationary phase **by capillary action**, a process called **developing the chromatogram**.
- As the solvent ascends the plate, the sample is distributed between the mobile phase and the stationary phase



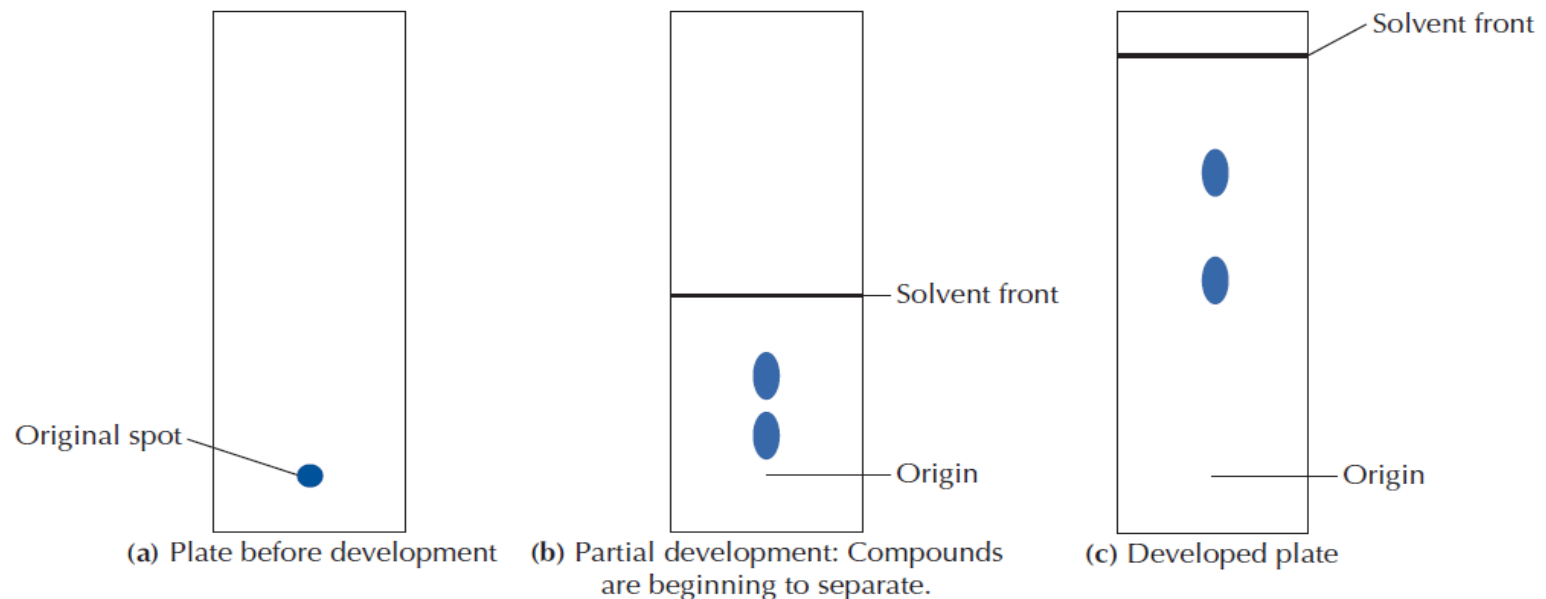
Developing chamber containing a thin-layer

# TLC and Column Chromatography

## Thin-layer chromatography (TLC ):

### Overview of TLC Analysis :

- The more tightly a compound binds to the adsorbent, the more slowly it moves on the TLC plate .
- When silica gel is the stationary phase, the developing solvent moves **nonpolar** substances up the plate **most rapidly**.
- As the chromatogram develops, **polar** substances travel up the plate



Steps in development of a TLC plate.

# TLC and Column Chromatography

## Thin-layer chromatography (TLC ):

### Overview of TLC Analysis :

- The TLC plate is removed from the developing chamber when the **solvent front** (leading edge of the solvent) is 1-1.5 cm from the top of the plate.
- The position of the solvent front is **marked immediately**, before the solvent evaporates, with a pencil line.
- The plate is then left to **dry**.
- The compounds are **visualized** by one of the methods available.

# TLC and Column Chromatography

## Thin-layer chromatography (TLC):

### Plates for Thin layer Chromatography :

- Thin-layer chromatographic plates consist of a solid support, such as **glass**, **metal**, or **plastic** with a thin layer of an adsorbent coating the solid surface, which provides the stationary phase.
- **Silica gel** ( $\text{SiO}_2 \cdot x\text{H}_2\text{O}$ ) is the most commonly used **general-purpose adsorbent** for partition chromatography of organic compounds.
- **Aluminum oxide** ( $\text{Al}_2\text{O}_3$ , also called alumina) can also be used as a **polar adsorbent**.
- ***The more polar the compound, the more strongly it binds to silica gel or alumina.***
- A special type of silica gel adsorbent—used for reverse-phase chromatography—has a **nonpolar surface that adsorbs less polar compounds more strongly than polar compounds**.

### Plastic-backed silica gel plates :

- Usually the least expensive.
- The adsorbent is bound to the plastic by solvent-resistant polyvinyl alcohol, which binds tightly to both the adsorbent and the plastic.
- Precoated plastic plates impregnated with **a fluorescent indicator** are also available; **these plates facilitate the visualization of many colorless compounds** with a UV lamp



# TLC and Column Chromatography

## Thin-layer chromatography (TLC ):

### Sample application

- The sample must be dissolved in a volatile organic a very dilute (1–2%) solution works best. Because the atmosphere in the developing chamber must be saturated with solvent vapor, the solvent needs a high volatility so that it will evaporate easily at room temperature.

Anhydrous reagent-grade acetone or ethyl acetate is commonly used.

### Preparing the plate.

- Before spotting a TLC plate, measure 1.0 cm from the bottom edge of the plate and *lightly* mark both edges with a 0.3-cm or shorter pencil mark .

### Spotting a TLC Plate.

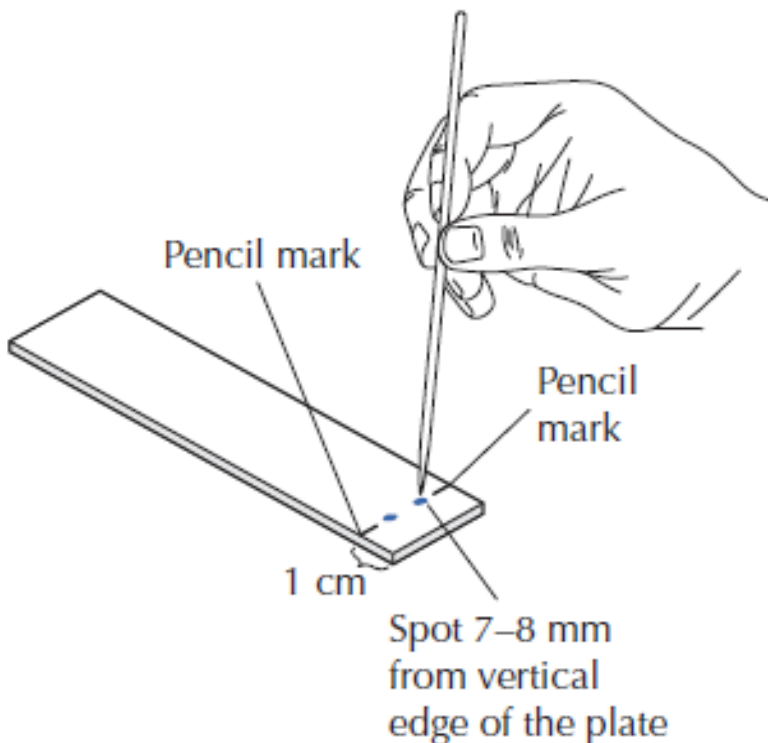
- Tiny spots of the dilute sample solution are carefully applied with a micropipette (*microcapillary*) near one end of the plate. It is important **not to overload** the plate with too much sample, which **leads to large tailing** spots and poor separation.

# TLC and Column Chromatography

## Thin-layer chromatography (TLC ):

### Applying the samples.

- The microcapillary is filled by dipping one end of the microcapillary into the solution to be analyzed. Hold the microcapillary vertically and apply the sample **by touching the microcapillary gently and briefly to the plate**. It is important to touch the microcapillary to the plate very lightly so that no hole is gouged in the adsorbent and to remove it quickly so **that only a very small drop is left on the adsorbent**.

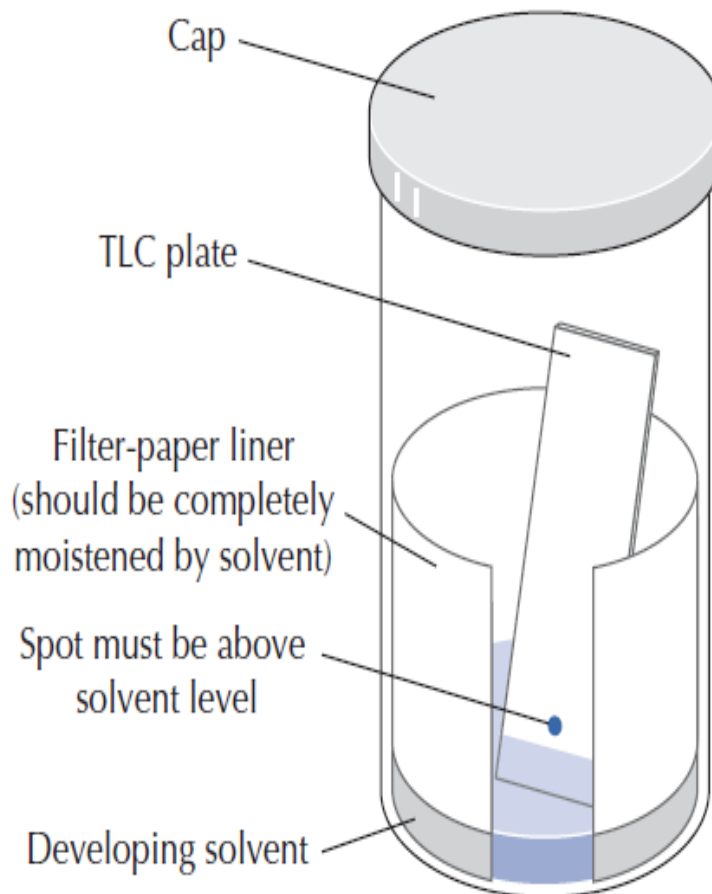


# TLC and Column Chromatography

## Thin-layer chromatography (TLC):

### Development of a TLC plate

- Development is carried out in a closed developing chamber containing a developing solvent.
- To ensure good chromatographic resolution, the developing chamber **must be saturated with solvent vapors** to prevent the evaporation of solvent from the TLC plate as the solvent rises up the plate.
- Inserting a piece of filter paper three-quarters of the way around the inside of the developing chamber helps to **saturate its atmosphere with solvent vapor**.
- If the spots on the plate are **below the solvent level**, spots leach into the solvent thereby, **ruining** the chromatogram.



# TLC and Column Chromatography

## Thin-layer chromatography (TLC ):

### How to carry out TLC. Development

- Place the TLC plate inside the developing chamber and allow the solvent to move up the plate. The adsorbent will become **visibly moist**. **Do not lift or disturb the chamber while the TLC plate is being developed.**
- When the solvent front is 1–1.5 cm from the top of the plate, **remove** it from the developing chamber and **immediately mark** the adsorbent at the solvent front with **a pencil**. The final position of the solvent front must be marked before any evaporation occurs.
- Allow the developing **solvent to evaporate** from the plate before **visualizing** the results.

# TLC and Column Chromatography

## Thin-layer chromatography (TLC):

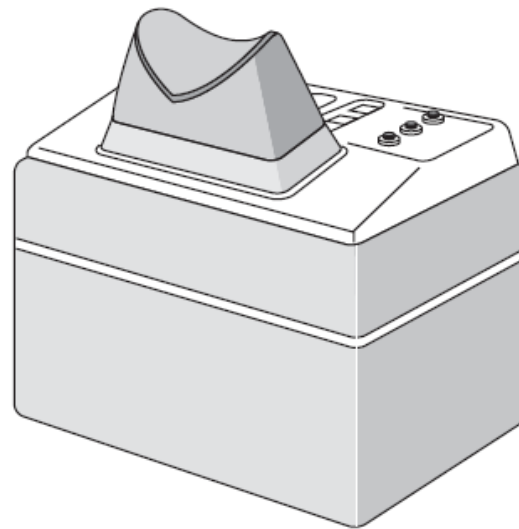
### Visualization techniques

- **Colored compounds** usually can be **seen directly** on the TLC plate, but **colorless compounds** require **indirect methods** of visualization such as:
  - **Fluorescence** (*use of ultraviolet radiation source*)

The separated compounds appear as **dark spots on the fluorescent** field because the substances forming the spots usually quench the fluorescence of the adsorbent

- **Iodine visualization.** Colorless organic compounds absorb iodine ( $I_2$ ) vapors. A plastic wash bottle containing a thin layer of iodine crystals is used for this visualization method.

*Once the spots on the chromatogram are visualized, we can **analyze** the chromatogram.*



Using an ultraviolet lamp with dark box

# TLC and Column Chromatography

## Thin-layer chromatography (TLC ):

### Analysis of TLC Plate

- Under a **constant set of conditions**, a given compound always travels a fixed distance relative to the distance traveled by the solvent front. This ratio of distances is called the ***R<sub>f</sub>*** (*ratio to the front*) and is expressed as a **decimal fraction**:

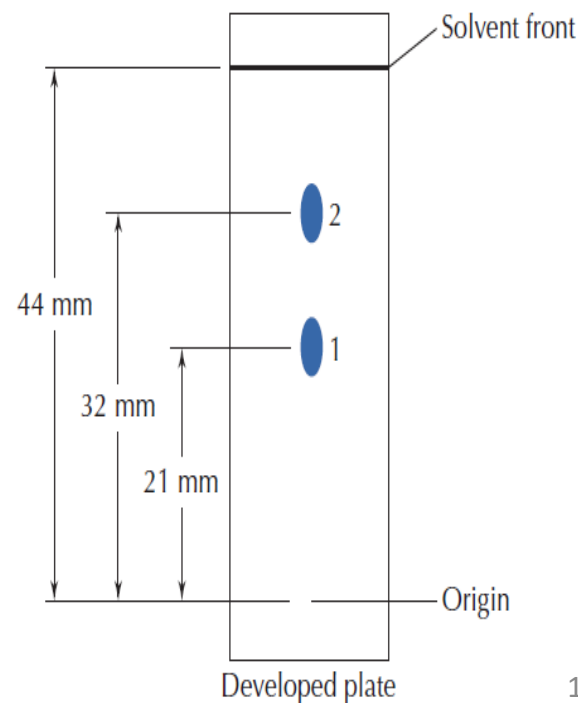
$$R_f = \frac{\text{distance traveled by compound}}{\text{distance traveled by developing solvent front}}$$

- The *R<sub>f</sub>* value for a compound depends on its structure as well as the adsorbent and mobile phase used. It is a **physical characteristic** of the compound.

### **Calculation of an *R<sub>f</sub>* Value:**

$$\text{Compound 1: } R_f = \frac{21 \text{ mm}}{44 \text{ mm}} = 0.48$$

$$\text{Compound 2: } R_f = \frac{32 \text{ mm}}{44 \text{ mm}} = 0.73$$



# TLC and Column Chromatography

## Thin-layer chromatography (TLC ):

### Analysis of TLC Plate

The  $R_f$  for a compound is a **constant** from one experiment to the next only if the **chromatography conditions** below are also **constant**:

- solvent system
- adsorbent
- thickness of the adsorbent
- amount of material spotted
- temperature

# TLC and Column Chromatography

## Thin-layer chromatography (TLC ):

### Experimental procedure

Substances to be tested: ( 1% solutions in acetone)

- o-nitroaniline,
- p-nitroaniline,
- o-nitro phenol,
- p-nitro phenol
- A mixture containing all the above compounds.
- Unknown sample **containing two of the tested compounds.**

### Main steps in procedure:

#### Step 1:

- 1) Prepare the developing container for TLC which is a 400 mL beaker with a watch glass on the top.
- 2) Pour solvent (ethyl acetate: petroleum ether (30:70)) into the chamber to a depth of about 0.5 cm.
- 3) Line part of the inside of the beaker with filter paper.
- 4) Let it stand while you prepare your TLC plate.



# TLC and Column Chromatography

## Thin-layer chromatography (TLC): Experimental procedure

### Step 2: Preparation of the TLC plate

Two 5.0x10.0 cm TLC plates are needed for each group.

- 1) Measure  $\sim 1.0$  cm from the **bottom** of the plate.
- 2) Using a pencil, **draw a line** across the plate at the **1.0 cm** mark (taking care not to disturb the adsorbent). This is the line on which you will spot the plate with samples to be tested.
- 3) Under the line, mark lightly letters or numbers for each sample.
- 4) **Leave about 1 cm between spots.** (the first plate will be used to spot the **four compounds** to be tested, the second will be used to spot the **mixture** and the **unknown**).

### Step 3: Spotting the TLC plate

- 1) Dip a **microcapillary** into a 1% solution of *p*-nitroaniline in acetone and then **gently** touch the end of it onto the proper location on the TLC plate. Don't allow the spot to become too large.
- 2) Spot the other compounds (*o*-nitroaniline, *p*-nitrophenol, and *o*-nitrophenol) on the same plate.

Use a new capillary for each compound.

- 3) Spot the mixture and the unknown on the second plate.

# TLC and Column Chromatography

## Thin-layer chromatography (TLC): Experimental procedure

### Step 4: Developing the plate

- 1) Place the first TLC plate in the **developing** beaker, cover the beaker with the watch glass, and leave it undisturbed on your bench top.
  - *The solvent will rise up the TLC plate by capillary action.*
  - *Make sure the solvent level lies below the spots.*
- 2) Allow the solvent to move upward until it is about **one centimeter** below the top of the plate.
- 3) Remove the plate from the beaker and immediately **mark** the solvent front with a pencil.
- 4) Allow the first plate to dry.
- 5) Use the **same developing beaker** to develop the spots on the second TLC plate.
- 6) Allow the second TLC plate to dry.

# TLC and Column Chromatography

## Thin-layer chromatography (TLC ): Experimental procedure

### Step 5: Visualize the spots

- *If there are any colored spots, circle them lightly with a pencil.*
- Since most samples are not colored ,so they can be **visualized** with a **UV lamp**.
- 1) Place the **first TLC** plate in the **dark box of the ultraviolet lamp** and circle the spots you see.
- 2) Do the same with the **second TLC plate**.

### Calculations:

- **Calculate the  $R_f$  value of each of the four separated compounds spotted on first TLC plate .**
- **Calculate the  $R_f$  values for each of the spots on the second TLC plate.**
- **Use the  $R_f$  values of each of the compounds to identify your unknown and **state the components** of your unknown.**
- **Show all steps in calculations in your laboratory report.**

# TLC and Column Chromatography

## Column Chromatography

### Introduction

- Column chromatography is a preparative method for **separating** and **isolating** compounds from mixtures. It is used for obtaining compounds from **natural sources** or **purifying products** from reaction mixtures.
- Column chromatography is an **upside-down** version of **TLC**. Instead of having a thin layer of adsorbent attached to a solid support, a column is filled with a larger amount of adsorbent and the **mixture is loaded on top of it**.
- TLC relies on **capillary** forces for moving the solvent, in column chromatography an eluent is allowed to percolate through the column by **gravity**. As the eluent is moving down the column it carries the soluble compounds with it.
- Compounds having **strong** interactions with the **adsorbent** move more **slowly** than compounds having weaker interactions with the adsorbent.

# TLC and Column Chromatography

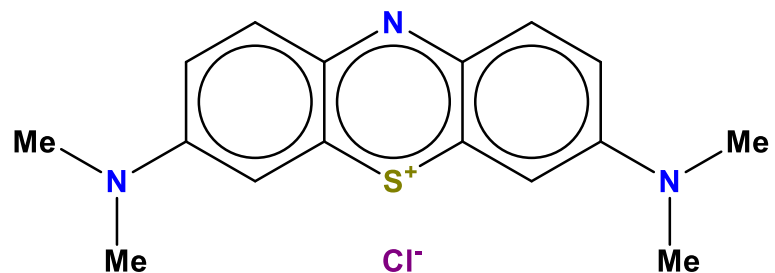
## Column Chromatography

### Introduction

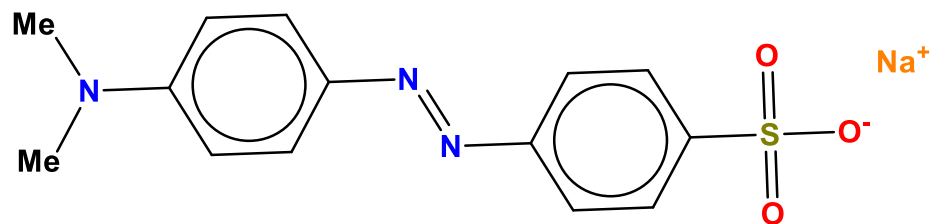
- Under the **right conditions** the compounds will separate in **distinctive bands** and each band will come out of the column individually. The bands are collected and the solvent is evaporated to give a clean compound.
- *In this experiment a mixture of methylene blue and methyl orange dyes will be separated through a column packed with alumina.*
- *Methyl blue is not very polar compared to methyl orange, this means the first collected band is the methylene blue once it is eluted with ethanol, and the second band will be the methyl orange if eluted with more polar mobile phase(water).*

# TLC and Column Chromatography

## Column Chromatography



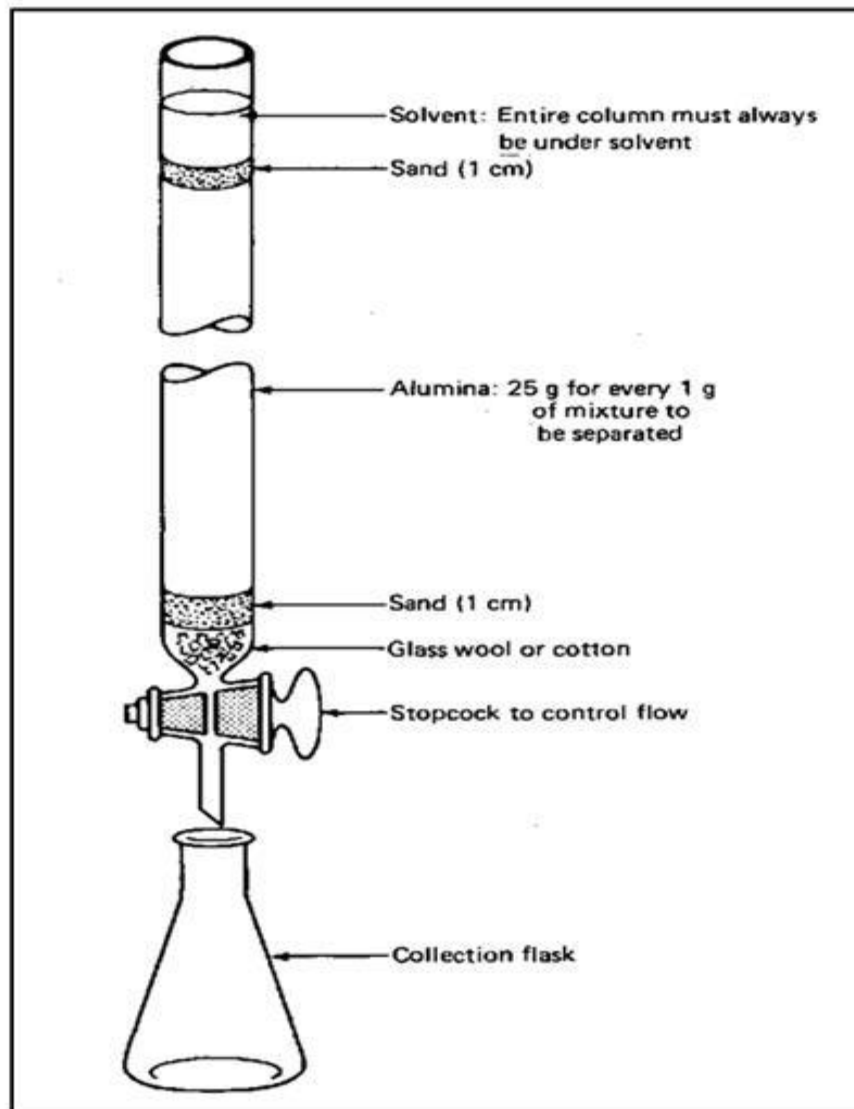
**Methylene blue**



**Methyl orange**

# TLC and Column Chromatography

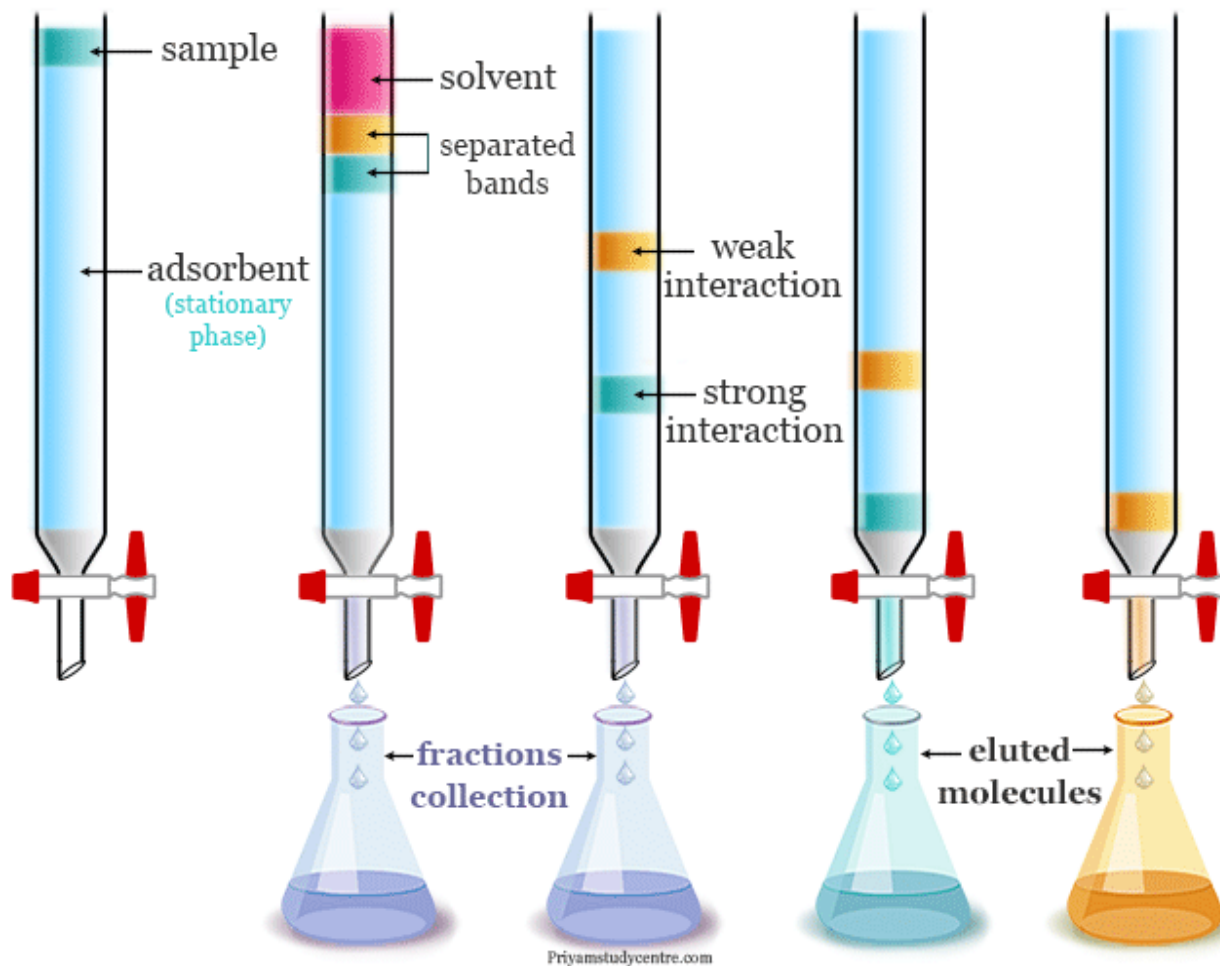
## Column Chromatography



# TLC and Column Chromatography

## Column Chromatography

### SEPARATION BY COLUMN CHROMATOGRAPHY





# TLC and Column Chromatography

## Column Chromatography

1. **Clamp** the "column" on a burette clamp.
2. Close the **flow controller (stopcock)**.
3. Add a small amount of glass wool to the bottom of the column and a **tiny layer of sand**.
4. **Tap** the column so that the sand becomes level.
5. **Fill** the column with ethanol, trying not to disturb the sand bed.
6. Add about (20- 25) grams of **aluminum oxide**.
7. Open the stopcock so that there is a **slow drip**.
8. **Slowly** add the aluminum oxide to the ethanol in the column. Allow it to settle and occasionally tap the column to release any trapped air.
9. When the column is filled, open the stopcock to a low rate and allow the ethanol to drop to a level such that it is just **above the aluminum oxide**.
10. Using a 3 mL syringe, gently add **0.5 mL** of the dye mixture (methylene blue and methyl orange mixture, found in the hood).
11. Allow the dye mixture to **percolate slowly** (open the stopcock to a low setting) into the column and stop the flow when it is just above the aluminum oxide bed.

# TLC and Column Chromatography

## Column Chromatography

12. Add **some ethanol** to the top of the column and allow this to percolate when it is just above the aluminum oxide bed.
13. Repeat **step 12** one more time.
14. **Fill** up the area above the column with ethanol
15. Open the stopcock to a **moderate** rate and allow the first dye to elute off the column into a container ( a 50 or 100 mL beaker). Fill the reservoir with more ethanol if need be to keep the flow going.
16. When the **first** dye appears to be nearly off, switch containers and collect an **intermediate** fraction.
17. When all of the first dye is eluted, fill the reservoir with **distilled water** and switch to a third container. Elute until the entire second dye is eluted.

- **Report and discuss what you have observed and separated in your laboratory report.**