EXPERIMENT 10

Chromatography

TLC and Column Chromatography

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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.

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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 ($SiO_2.xH_2O$) 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.

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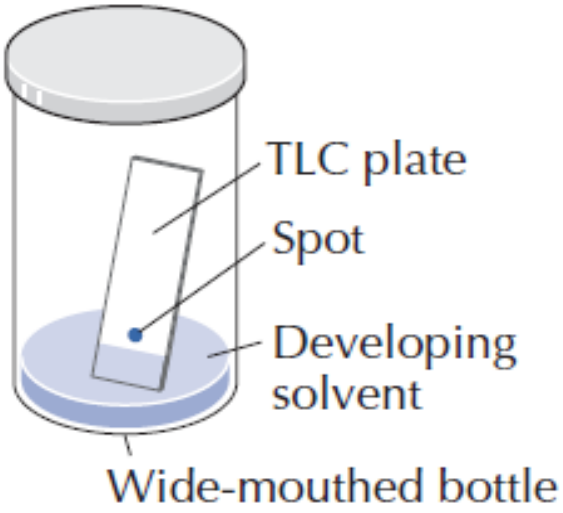
Thin-layer chromatography (TLC)

- **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

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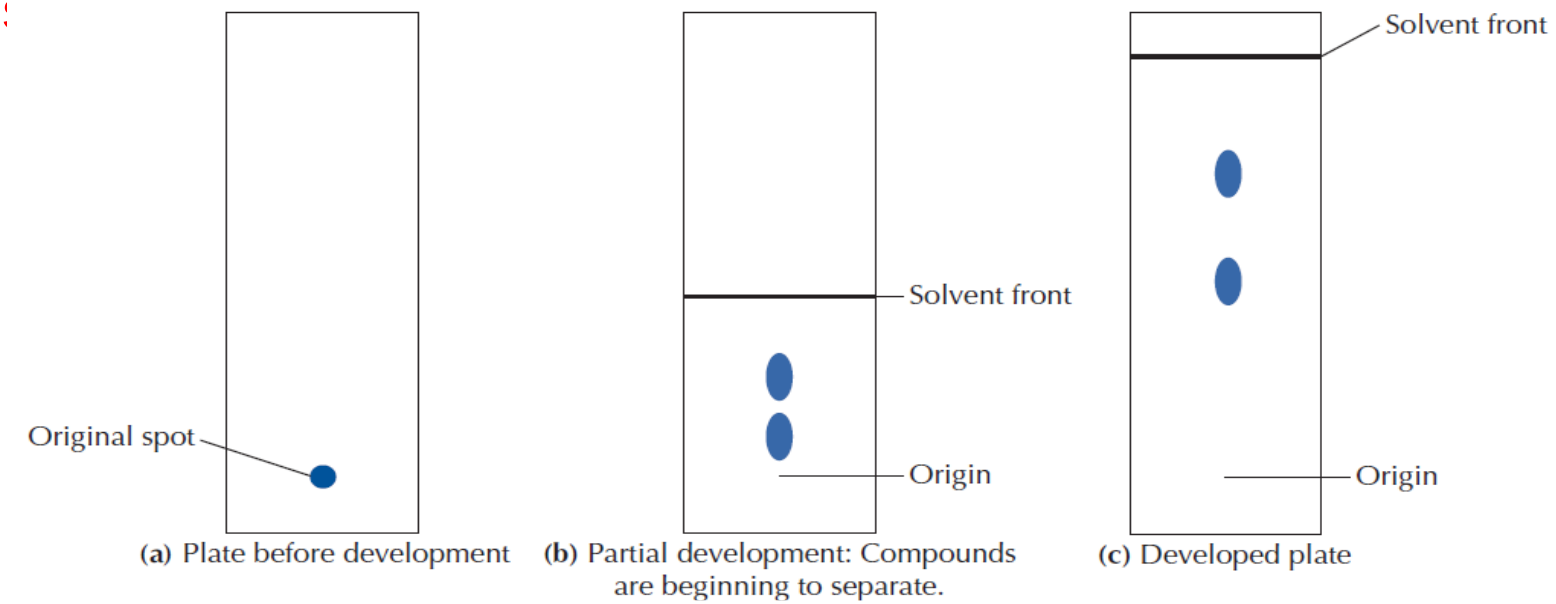
Thin-layer chromatography (TLC):

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.

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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.

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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 ($SiO_2.xH_2O$) is the most commonly used general-purpose adsorbent for partition chromatography of organic compounds.
- Aluminum oxide (Al_2O_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

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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.

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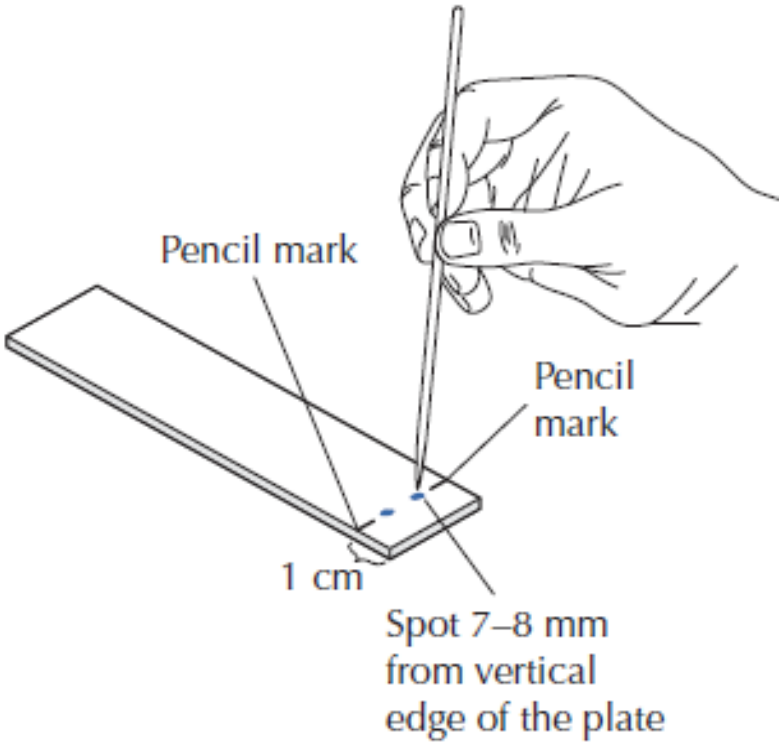
Thin-layer chromatography (TLC):

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.



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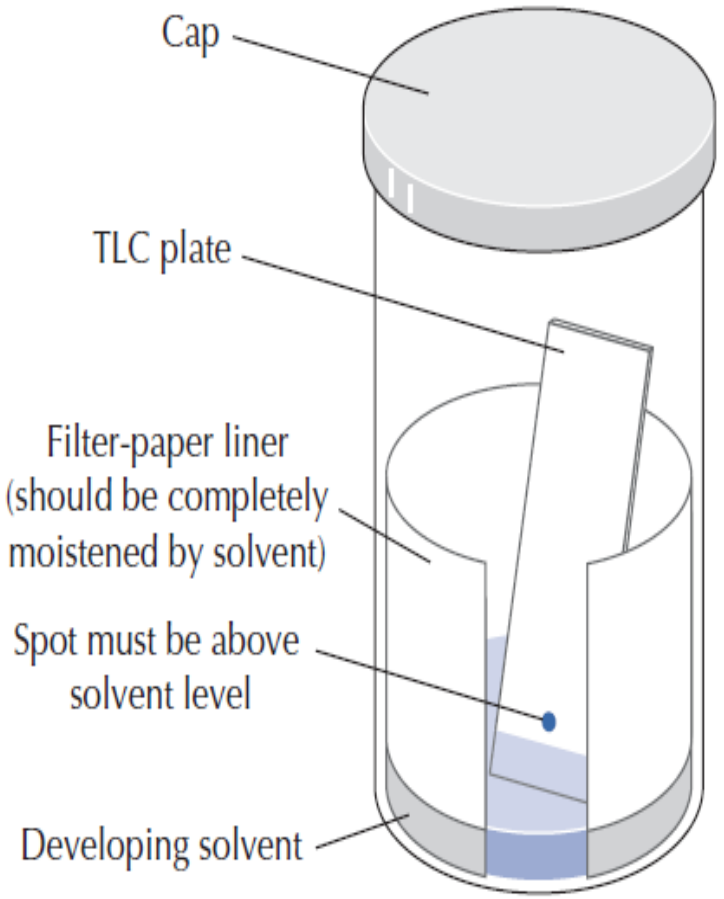
Thin-layer chromatography (TLC):

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.



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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.

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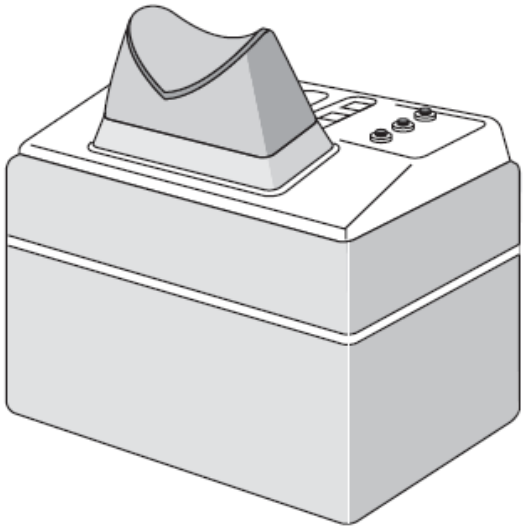
Thin-layer chromatography (TLC):

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 absorbs iodine (I_2) vapors. Aplastic wash bottle containing a thin layer of iodine crystals is used for this visualization method.



Using an ultraviolet lamp with dark box

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

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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 ***Rf*** *(ratio to the front)* and is expressed as a decimal fraction:

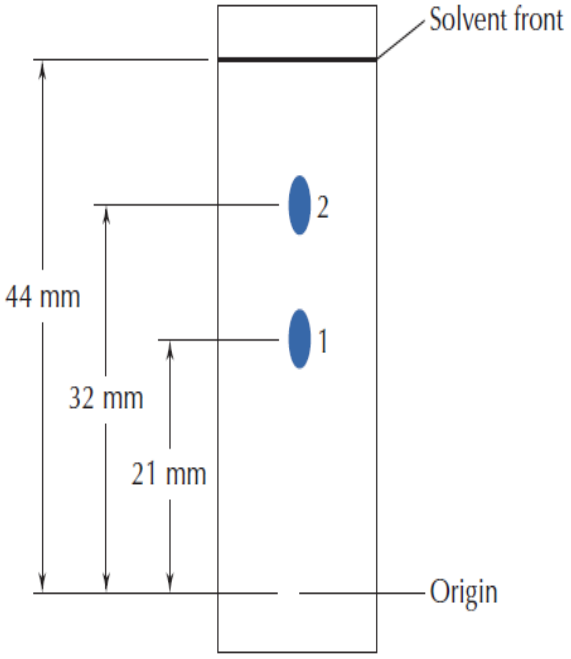
$$R_f = \frac{\text{distance traveled by compound}}{\text{distance traveled by developing solvent front}}$$

- The *Rf* 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 Rf Value:

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

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



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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.

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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 ~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 onitrophenol) on the same plate.

Use a new capillary for each compound.

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

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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.

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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.***

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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 filed 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).*

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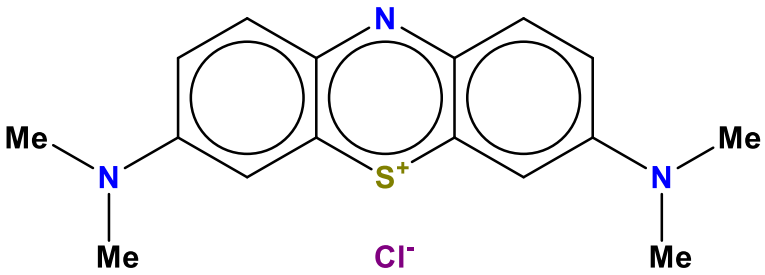
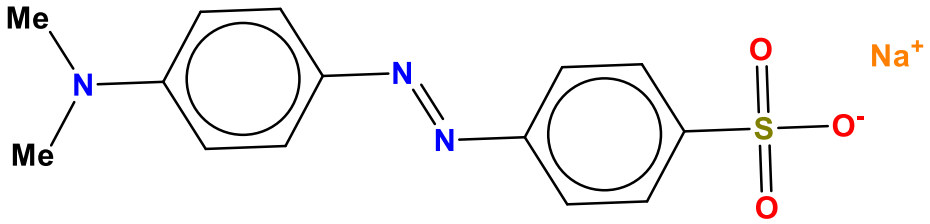
Column Chromatography



Methylene blue



Methyl orange

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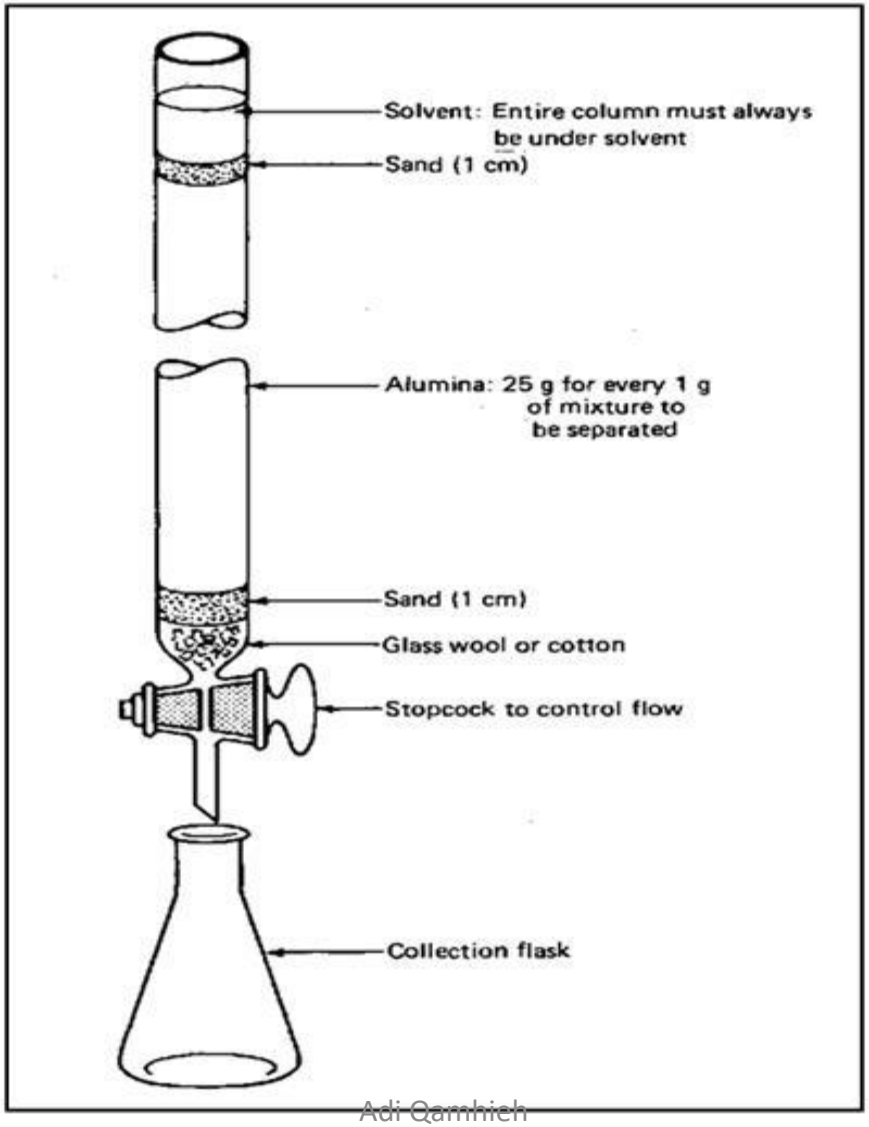
Column Chromatography



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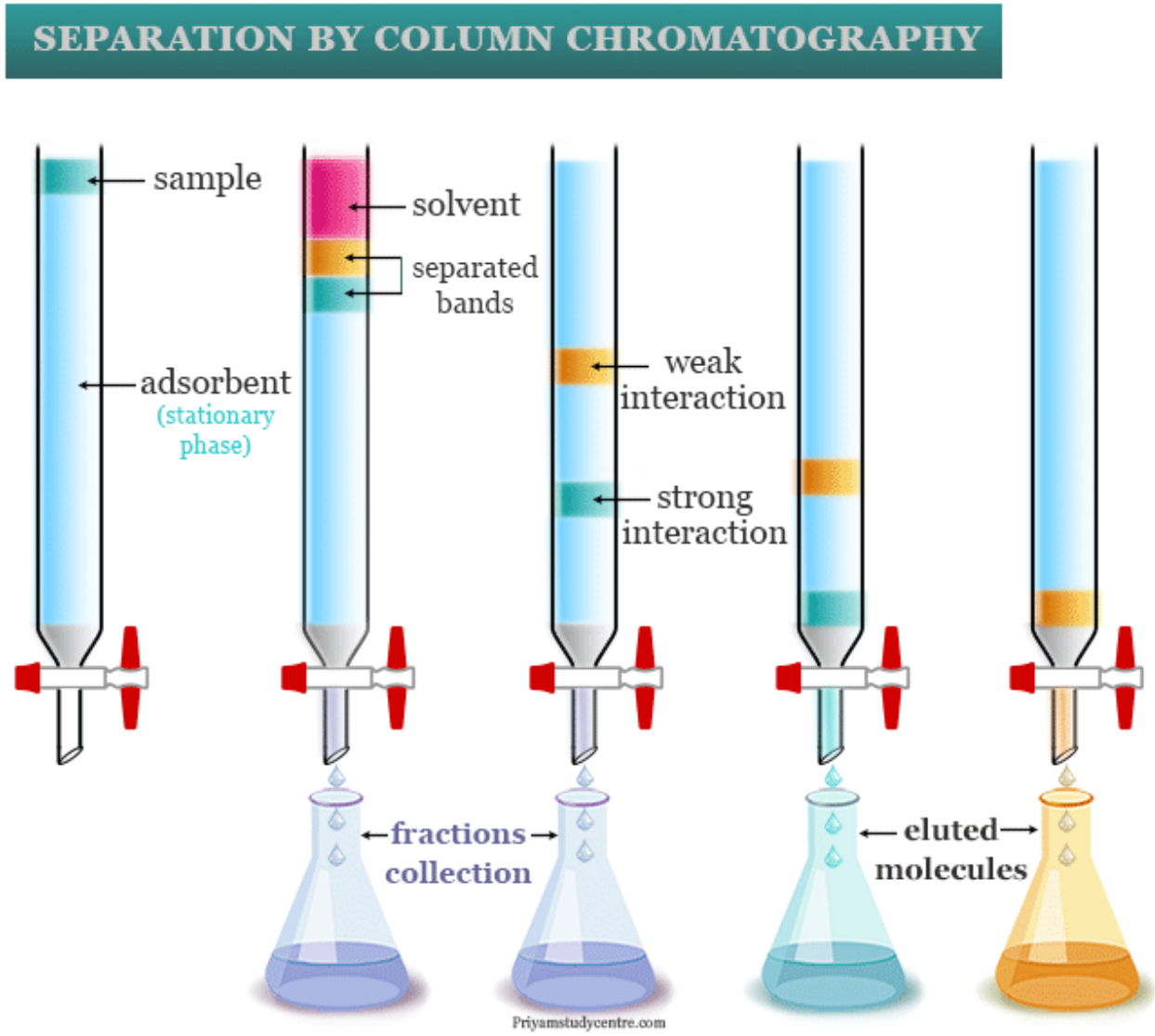
Column Chromatography



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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 an 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.***