Objectives

1) To select a suitable solvent system for use in separation by thin-layer chromatography.
2) To identify the compounds in a mixture by comparing their $R_f$ values with reference compounds.
3) To separate a two-compound mixture using column chromatography and calculate recovery percentages for each compound.

Introduction

The term chromatography refers to several related techniques for analyzing, identifying, or separating mixtures of compounds. All chromatographic techniques have a two-part operation in common. In each technique a sample mixture is placed into a liquid or gas, called a mobile phase. The mobile phase carries the sample through a solid support, called the stationary phase, which contains an adsorbent or another liquid. The different compounds in the sample mixture move through the stationary phase at different rates, due to different attractions for the mobile and stationary phases. Thus, individual compounds in the mixture separate as they move through the stationary phase. The separated compounds can be collected or detected, depending on the particular chromatographic technique involved.

Thin-layer chromatography (TLC) is a simple and inexpensive analytical technique that can quickly and efficiently separate quantities of less than ten micrograms of material. TLC has many applications in the organic laboratory. TLC is used for the rapid analysis of reagent and product purity, or to quickly determine the number of compounds in a mixture. Also, by comparing an unknown compound’s behavior to the behaviors of known standard compounds, mixture compounds can be tentatively identified.

Chemists frequently use TLC to follow the progress of a reaction by monitoring the disappearance of a reactant or the appearance of a product. Also, TLC is often used for selecting a suitable solvent before attempting a larger scale column chromatographic separation. Then, during the column chromatography experiment, TLC is frequently used to monitor the separation.
In TLC, capillary action allows a liquid (mobile phase) to ascend a solid (stationary phase) coated on a support plate. A sample of the compound mixture is applied near the bottom of a dry TLC plate, as shown in Figure 1(a). The plate is placed into a developing chamber, a covered container with a shallow layer of mobile phase liquid in the bottom. As the mobile phase ascends the plate, the mixture compounds move along the plate to different extents, due to differences in their relative attractions for the mobile and the stationary phase. After the separation is complete, the TLC plate is called a chromatogram, as shown in Figure 1(b).

**Figure 1** A TLC plate (a) labeled for identification and spotted, (b) as a chromatogram

During the TLC process, the solid stationary phase, called the adsorbent, adsorbs the mixture compounds. As the mobile phase or the eluent travels up over the adsorbent, the compounds within the mixture move at different rates. A reversible and continuous competitive attraction between the eluent and the adsorbent for the mixture compounds causes this rate difference. Compounds with less attraction to the adsorbent move rapidly with the eluent. Compounds with more attraction to the adsorbent move slowly with the eluent. Because TLC adsorbents are typically very polar, the more polar is a compound in the mixture, the more strongly it adheres to the adsorbent and the more slowly it moves. Similarly, intermolecular attractions between the eluent and the compounds determine the solubility of the compounds in the mobile phase. In general, the more polar the eluent, the more rapidly a given compound moves. Polar compounds, which are strongly attracted to the adsorbent, require polar eluents to attract them away from the adsorbent.
Determination of Retention Factor or Rate of Flow (Rf) Value

The ratio of the distance that a compound moves to the distance that the eluent front moves is called the retention factor, denoted as Rf. A calculation for Rf is shown in Equation 1.

\[
R_f = \frac{\text{distance traveled by compound, mm}}{\text{distance traveled by eluent front, mm}} \quad \text{(Eq. 1)}
\]

For example, in Figure 2 the stock sample compound moved distance A while the eluent front traveled distance S. If distance A is 25 millimeters (mm) and distance S is 55 mm, then the Rf is calculated as shown in Equation 2.

\[
R_f = \frac{A}{S} = \frac{25 \text{ mm}}{55 \text{ mm}} = 0.45 \quad \text{(Eq. 2)}
\]

The chromatographic behavior of individual compounds in reproducible as long as the stationary and mobile phases and the temperature are kept constant. Therefore, an Rf can be used for identification purposes.

When a compound is strongly attracted to the adsorbent and does not travel very far from the origin, or point of application, the Rf is small. An increase in eluent polarity would probably increase the attraction of the compound for the eluent. As a result, the compound would move farther up the plate, resulting in a larger Rf.

Identical Rf values for a known compound and an unknown compound on the same chromatogram suggest that the known and the unknown compounds are the same. However, two different compounds can have the same Rf in a given eluent. Additional evidence that two samples are the same compound can be obtained by comparing their mobilities in several
eluent systems of varying polarities. Two different compounds are unlikely to have the same $R_f$ in eluents of different polarities, while two different samples of the same compound will have the same $R_f$ in every eluent.

**Choosing Adsorbents and Eluents**

Alumina ($\text{Al}_2\text{O}_3$) and silica gel ($\text{SiO}_2 \cdot \text{xH}_2\text{O}$) are the most commonly used adsorbents in TLC and column chromatography. However, for use in TLC a binder such as calcium sulfate is added to these adsorbents to hold them onto the plate. For this reason, commercially prepared adsorbents may not be used interchangeably between TLC and column chromatography.

The eluents are liquid organic compounds of various structures and polarities, as shown in Table 1. The more polar an eluent, the greater is its eluting power, that is, its ability to move compound over the adsorbent surface.

![Diagram showing eluting power from least polar to most polar](image)

**Table 1** Approximate polarities of eluents used in chromatography

<table>
<thead>
<tr>
<th>Least Polar</th>
<th>Increasing Eluting Power</th>
<th>Most Polar</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclohexane</td>
<td>petroleum ether</td>
<td>methanol</td>
</tr>
<tr>
<td>hexane</td>
<td>toluene</td>
<td>acetone</td>
</tr>
<tr>
<td>dichloromethane</td>
<td>ethyl acetate</td>
<td>ethanol</td>
</tr>
</tbody>
</table>

Combining eluents of low polarity with those of high polarity allows the preparation of mixed eluents of practically any eluting power. For example, the eluting power of a 1:1 mixture of hexane and ethyl acetate would be between the eluting powers of pure hexane and pure ethyl acetate. Eluent selection is usually a matter of trial and error until a separation or desired mobility is achieved.

A TLC experiment has three general stages: spotting, developing, and visualizing.

**Spotting a plate**

The original is marked, usually by drawing a thin line across the bottom of the plate with a pencil as shown in Figure 3. The sample compound or mixture should be dissolved in a volatile solvent such as acetone or dichloromethane. A glass capillary tube is used to apply a small amount of sample solution onto the plate, keeping the sample in as small an area as possible. With practice, spots with diameters of 1-2 mm can be produced.
After the solvent evaporates, additional sample solution can be applied to the same spot. Application of too much sample can lead to “tailing” and poor separation, as shown in Figure 3. Varying amounts of a sample can be spotted on the same plate to determine which application gives the best results.

**Developing a plate**

To develop the chromatogram, a piece of filter paper is placed along the walls of the developing chamber which contains a shallow layer of the appropriate eluent. The paper acts as a wick that adsorbs the eluent and ensures that, when the chamber is closed, its atmosphere is saturated with eluent vapor, minimizing evaporation from the plate.

When the spotted plate is placed into the chamber, the origin marked on the plate must be higher than the level of the eluent, to prevent the sample from dissolving from the plate into the eluent layer. When the eluent reaches a point approximately 5 mm from top of the plate, the plate is removed from the chamber. The point that the eluent has reached is called the **eluent front** (or solvent front) and is immediately marked with a pencil, as shown in Figure 3. The plate is dried by allowing the eluent to evaporate from the plate.

If the eluent front is allowed to reach the top of the plate, the mixture compound may continue to move along the plate. An $R_f$ obtained under these circumstances is not valid.

**Visualizing the compound**

Upon development, a successful separation of colored compounds will reveal distinct spots, indicating that the mixture compounds have separated, as shown in Figure 3. To make separated colorless compounds observable to the eye, the spots are treated in some way to make them visible. The process is called **visualization**.

Some compounds are fluorescent and can be visualized by viewing the TLC plate under an ultraviolet (UV) lamp. Frequently, the adsorbent contains a chemically inert fluorescent
material. When viewed under UV light, compounds that absorb UV light appear as dark spots that may be outlined with a pencil.

Another simple method for visualizing organic compounds is to place the chromatogram in a chamber containing iodine (I₂) crystals and vapor. The I₂ vapor forms a colored complex with many compounds and allows detection of their spots. The spot location must be marked immediately because the I₂ will eventually sublime from the plate.

In some instances, a reagent such as phosphomolybdic acid, potassium permanganate, or ninhydrin solution is sprayed on the plate. These reagents can react with the colorless compound of the plate and give a colored product.

**Adsorption column chromatography** is a another technique that uses a solid stationary phase, the **adsorbent**, packed in a glass column, and a solvent, the mobile phase, that moves slowly through the packed column. A solvent used as a mobile phase is called an **eluent**.

In an adsorption column chromatography experiment, a mixture of compounds is added to the eluent. As the eluent moves through the column, the stationary phase and the mobile phase interact with the compound in the mixture. The difference in attraction of the compounds to the stationary and mobile phases result in the compounds moving at different rates through the packed column, separating from one another.

A compound attracted more strongly by the mobile phase will move rapidly through the column, and elute from, or come off, the column dissolved in the eluent. In contrast, a compound more strongly attracted to the stationary phase will move slowly through the column.

Alumina and silica gel are the most commonly used adsorbents for adsorption column chromatography. Alumina is generally suitable for chromatography of less polar compounds. Silica gel gives good results with compounds containing polar functional groups.

Even within a mixture of relatively low polarity compounds, the more polar compounds of the mixture bind tightly to the adsorbent; less polar ones bind more loosely. Separation occurs when an eluent of low to moderate polarity is passed through the column. Less polar compounds of the mixture readily dissolve in the eluent and move through the column. More polar compounds have a stronger attraction to the adsorbent than to the moving eluent. When differences in attraction are great enough, the compounds can be separated.

A mobile phase consisting of a single eluent or a mixture of eluents may be sufficient to separate and elute each compound of a mixture. However, if the mixture to be separated
includes compounds with a wide range of polarities, two types of eluents or two ratios of mixed eluents may have to be used. In such a case, the eluent of lower polarity is used first. Table 2 shows the relative polarity of various eluents used as mobile phases.

Table 2  Relative polarities of various eluents

<table>
<thead>
<tr>
<th>Compound</th>
<th>Increasing polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexane</td>
<td></td>
</tr>
<tr>
<td>tetrachloroethane</td>
<td></td>
</tr>
<tr>
<td>benzene</td>
<td></td>
</tr>
<tr>
<td>toluene</td>
<td></td>
</tr>
<tr>
<td>trichloroethane</td>
<td></td>
</tr>
<tr>
<td>dichloroethane</td>
<td></td>
</tr>
<tr>
<td>diethylether</td>
<td></td>
</tr>
<tr>
<td>tert-butyl methyl ether</td>
<td></td>
</tr>
<tr>
<td>ethyl acetate</td>
<td></td>
</tr>
<tr>
<td>acetone</td>
<td></td>
</tr>
<tr>
<td>ethanol</td>
<td></td>
</tr>
<tr>
<td>methanol</td>
<td></td>
</tr>
<tr>
<td>water</td>
<td></td>
</tr>
</tbody>
</table>

Preparing Adsorption Chromatography Columns

A typical column has a bed height ten times the column diameter. The slurry pack is a satisfactory method for packing adsorption chromatography columns. The slurry used is a mixture of adsorbent and the chosen eluent. The column is packed by closing the stopcock, filling the column half full with the eluent, and adding the adsorbent-eluent slurry. During the addition of the slurry, the stopcock is opened to allow eluent to drain slowly through the column as the adsorbent bed packs.

Air bubbles must not be allowed to form in the bed. Such bubbles cause the bed to be irregular and interfere with the uniform movement of the mixture compounds through the column. Once the bed is packed, the mixture sample which is dissolved in a minimum amount of a moderately polar eluent is loaded into the column. Separation of the mixture compounds is then achieved by adding eluent to the top of the column bed. Eluent is collected in fractions as it elutes from the bottom of the column.

If the separation requires more than one eluent, the column is packed using the less polar eluent. For example, 4:1 hexane/ethyl acetate is less polar than 2:1 hexane/ethyl acetate. If both eluent systems are to be used, the column is packed with 4:1 hexane/ethyl acetate.

Each compound forms a band when a mixture is placed on the column. As a compound is attracted to the eluent, that band moves through the column. The column must be vertical at
all times, as shown in Figure 4(a). If the column is not kept upright, Figure 4(b), bands may elute unevenly, preventing effective separation.

**Figure 4**
(a) A vertical column allows good separation of compounds
(b) a non-vertical column causes non-horizontal bands and poor separation.

Colored bands are visible as they move through the column. If the bands are not visible, the eluent is collected in small fractions. Each fraction is tested to determine whether it contains any compound. TLC is the most commonly used method for this purpose.

**Experimental Procedure**

**Part A: Mobile phase determination by TLC method**

1) Obtain four TLC plates from the supply room. By using a **pencil, not pen**, lightly draw a line across the short side of each plate, on the silica gel side approximately 1 cm from the bottom. Be careful not to scratch the silica gel as you are drawing the line.

2) Use small capillary tubes to spot solutions of benzil and benzoin along the line. Keep a gap (0.8-1 cm) between the two spots. When spotting the solutions, gently and quickly touch the capillary to the surface of the plate so that the spots are not too large. Also, write a letter above or below to indicate what is spotted at each position (e.g. “A” for benzil and “B” for benzoin) as shown in Figure 5.

3) Set up a TLC chamber as shown in Figure 6. Put a piece of filter paper in a 100 mL beaker. Place a small amount of n-hexane in this beaker. The liquid should cover the bottom of the beaker but the surface should be below the pencil line when the plate is placed in the beaker (that is, less than 1 cm in depth). The filter paper lining is used to saturate the atmosphere within the beaker with solvent fumes.
4) Place one spotted TLC plate in the TLC chamber, cover with a watch glass and allow the solvent to move through the plate until it is approximately 0.5-1 cm from the top. Do not disturb the chamber while the plate is being developed!!!

5) Remove the plate from the chamber and mark the solvent front with a pencil. Allow the plate to dry for a few minutes. Place it under short-wave ultraviolet light (254 nm) and circle dark spots appearing on the plate under the UV light with pencil.

6) Repeat step 2) to 5) for other three TLC plates but each time change the mobile phase from 100% n-hexane to a mixture of n-hexane and ethyl acetate (EtOAc) at the volume ratios of 4:1, 2:1 and 1:1, respectively.

7) From the results, decide which solvent system would be most appropriate for the separation of benzil and benzoin (PART B). Report the result to your instructor.

PART B: Separation of a mixture by column chromatography

1) Prepare 50 mL of the solvent of choice (from PART A) in an Erlenmeyer flask.

2) Weigh 0.1 g of solid mixture (benzil+benezoin) in a test tube. Record the exact weight and dissolve with a minimum amount of dichloromethane (~1-1.5 mL).

3) Prepare a silica gel column as shown in Figure 7. Plug the column with cotton and affix it to a clamp stand. Place a beaker under the outlet.

4) In a clean and dry Erlenmeyer flask, mix silica gel (5 g) with the solvent (~20 mL) (CAUTION: Silica gel dust is very harmful if inhaled). Then, slowly transfer the slurry into the column using a glass funnel until the silica gel level is about 10-12 cm (when settle). If necessary, gently tap the side of the column with a rubber tube during the packing process to compact the silica gel.
5) Allow solvent to drain into the beaker. Use extra solvent to remove silica gel that may stick to the inner wall of the column and allow the solvent to drain into the beaker.

6) When the solvent reaches the silica gel surface, slowly add the mixture solution into the column using a dropper. The flat surface of silica gel should be minimally disturbed.

7) Allow sample to adsorb onto the silica gel, and gently rinse the inside wall of the column with 1-2 mL of solvent.

8) When the solvent reaches the top of the column again, carefully add more solvent to the column. Do not let the column dry out during the eluting process.

9) Collect 15-20 fractions (3 mL each) in test tubes and label as 1, 2, 3,.....

10) Analyze each of your collected fractions by TLC (Figure 8).
11) Combine the fractions containing pure benzoin in a ceramic evaporating dish and place the dish on a steam bath until a solid or thick oil is obtained.
12) Allow the dish to cool to room temperature and collect the solid into a pre-weighed plastic bag.
13) Calculate the weight and recovery percentage of isolated benzoin.
14) Repeat step 11) to 13) with fractions containing pure benzil. Discard the fractions containing mixture of the two compounds in a proper waste bottle.

**Laboratory Safety Precaution**

1) Wear safety goggles and lab coat at all times while working in the laboratory.
2) Hexane and ethyl acetate are irritating and flammable. Dichloromethane is irritating and toxic.
3) Wash your hands thoroughly with soap or detergent before leaving the laboratory.