Column Chromatography: Fluorenone, Cholesteryl Acetate, Acetylferrocene, and Plant Pigments

PRELAB EXERCISE: Compare column chromatography and thin-layer chromatography (TLC) with regard to the (1) quantity of material that can be separated, (2) time needed for the analysis, (3) solvent systems used, and (4) ability to separate compounds.

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids when carrying out microscale experiments. It becomes expensive and time consuming, however, when more than about 10 g of material must be purified. Column chromatography involves the same chromatographic principles as detailed for TLC in Chapter 8, so be sure that you understand those before doing the experiments in this chapter.

As discussed in Chapter 1, organic chemists obtain new compounds by synthesizing or isolating natural products that have been biosynthesized by microbes, plants, or animals. In most cases, initial reaction products or cell extracts are complex mixtures containing many substances. As you have seen, recrystallization, distillation, liquid/liquid extraction, and sublimation can be used to separate and purify a desired compound from these mixtures. However, these techniques are frequently not adequate for removing impurities that are closely related in structure. In these cases, column chromatography is often used. The broad applicability of this technique becomes obvious if you visit any organic chemistry research lab, where chromatography columns are commonplace.

Three of the five experiments in this chapter involve synthesis and may be your first experience in running an organic reaction. Experiments 1 and 2 involve the synthesis of a ketone. In Experiment 3 an ester of cholesterol is prepared. Experiment 4 demonstrates the separation of colored compounds. Experiment 5 involves the isolation and separation of natural products (plant pigments), which is analogous to Experiment 2 in Chapter 8 but on a larger scale.
The most common adsorbents for column chromatography—silica gel and alumina—are the same stationary phases as used in TLC. The sample is dissolved in a small quantity of solvent (the eluent) and applied to the top of the column. The eluent, instead of rising by capillary action up a thin layer, flows down through the column filled with the adsorbent. Just as in TLC, there is an equilibrium established between the solute adsorbed on the silica gel or alumina and the eluting solvent flowing down through the column, with the less strongly absorbed solutes moving ahead and eluting earlier.

Three mutual interactions must be considered in column chromatography: the activity of the stationary adsorbent phase, the polarity of the eluting mobile solvent phase, and the polarity of the compounds in the mixture being chromatographed.

**Additional Principles of Column Chromatography**

**Adsorbents**

A large number of adsorbents have been used for column chromatography, including cellulose, sugar, starch, and inorganic carbonates; but most separations employ alumina \([\text{Al}_2\text{O}_3]\) or silica gel \([\text{SiO}_2]\). Alumina comes in three forms: acidic, neutral, and basic. The neutral form of Brockmann activity grade II or III, 150 mesh, is most commonly employed. The surface area of this alumina is about 150 m²/g. Alumina as purchased will usually be activity grade I, meaning that it will strongly adsorb solutes. It must be deactivated by adding water, shaking, and allowing the mixture to reach equilibrium over an hour or so. The amount of water needed to achieve certain activities is given in Table 9.1. The activity of the alumina on TLC plates is usually about III. Silica gel for column chromatography, 70–230 mesh, has a surface area of about 500 m²/g and comes in only one activity.

**TABLE 9.1 - Alumina Activity**

<table>
<thead>
<tr>
<th>Brockmann activity grade</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent by weight of water</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

**Solvents**

Solvent systems for use as mobile phases in column chromatography can be determined from TLC, the scientific literature, or experimentally. Normally, a separation will begin with a nonpolar or low-polarity solvent, allowing the compounds to adsorb to the stationary phase; then the polarity of the solvent is slowly switched to desorb the compounds and allow them to move with the mobile phase. The polarity of the solvent should be changed gradually. A sudden change in solvent polarity will cause heat evolution as the alumina or silica gel adsorbs the new solvent. This will vaporize the solvent, causing channels to form in the column that severely reduce its separating power.

Several solvents are listed in Table 9.2, arranged in order of increasing polarity (elutropic series), with \(n\)-pentane being the least polar. The order shown

**TABLE 9.2 - Elutropic Series for Solvents**

<table>
<thead>
<tr>
<th>(n)-Pentane (least polar)</th>
<th>Petroleum ether</th>
<th>Cyclohexane</th>
<th>Hexanes</th>
<th>Carbon disulfide</th>
<th>(t)-Butyl methyl ether</th>
<th>Dichloromethane</th>
<th>Tetrahydrofuran</th>
<th>Dioxane</th>
<th>Ethyl acetate</th>
<th>2-Propanol</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Acetic acid (most polar)</th>
</tr>
</thead>
</table>
in the table reflects the ability of these solvents to dislodge a polar substance adsorbed onto either silica gel or alumina, with \( n \)-pentane having the lowest solvent power.

As a practical matter, the following sequence of solvents is recommended in an investigation of unknown mixtures: elute first with petroleum ether (pentanes); then hexanes; followed by hexanes containing 1%, 2%, 5%, 10%, 25%, and 50% ether; pure ether; ether and dichloromethane mixtures; followed by dichloromethane and methanol mixtures. Either diethyl ether or \( t \)-butyl methyl ether can be used, but \( t \)-butyl methyl ether is recommended. Solvents such as methanol and water are normally not used because they can destroy the integrity of the stationary phase by dissolving some of the silica gel. Some typical solvent combinations are hexanes-dichloromethane, hexanes-ethyl acetate, and hexanes-toluene. An experimentally determined ratio of these solvents can sufficiently separate most compounds.

**Compound Mobility**

The ease with which different classes of compounds elute from a column is indicated in Table 9.3. Molecules with nonpolar functional groups are least adsorbed and elute first, while more polar or hydrogen-bonding molecules are more strongly adsorbed and elute later. The order is similar to that of the eluting solvents—another application of “like dissolves like.”

**Sample and Column Size**

Chromatography columns can be as thin as a pencil for milligram quantities to as big as a barrel for the industrial-scale separation of kilogram quantities. A microscale column for the chromatography of about 50 mg of material is shown in Figure 9.1; columns with larger diameters, as shown in Figures 9.2 and 9.3, are used for macroscale procedures. The amount of alumina or silica gel used should generally weigh at least 30 times as much as the sample, and the column, when packed, should have a height at least 10 times the diameter. The density of silica gel is 0.4 g/mL, and the density of alumina is 0.9 g/mL, so the optimum size for any column can be calculated.

**Packing the Column**

**Microscale Procedure**

Before you pack the column, tare several Erlenmeyer flasks, small beakers, or 20-ml vials to use as receivers. Weigh each one carefully and mark it with a number on the etched circle.

Uniform packing of the chromatography column is critical to the success of this technique. Two acceptable methods for packing a column are dry packing and slurry packing, which normally achieve the best results. Assemble the column as depicted in Figure 9.1. To measure the amount of adsorbent, fill the column one-half to two-thirds full; then pour the powder out into a small beaker or flask. Clamp the column in a vertical position and close the valve. Always grasp the
valve with one hand while turning it with the other. Fill the column with a non-polar solvent such as hexanes almost to the top.

- **Dry Packing Method.** This is the simplest method for preparing a microscale column. Slowly add the powdered alumina or silica gel through the funnel while gently tapping the side of the column with a pencil. The solid should “float” to the bottom of the column. Try to pack the column as evenly as possible; cracks, air bubbles, and channels will lead to a poor separation.

- **Slurry Packing Method.** To slurry pack a column, add about 8 mL of hexanes to the adsorbent in a flask or beaker, stir the mixture to eliminate air bubbles, and then (this is the hard part) swirl the mixture to get the adsorbent suspended in the solvent and immediately pour the entire slurry into the funnel. Open the valve, drain some solvent into the flask that had the adsorbent in it and finish transferring the slurry to the column. Place an empty flask under the column and allow the solvent to drain to about 5 mm above the top surface of the adsorbent. Tap the column with a pencil until the packing settles to a minimum height. Try to pack the column as evenly as possible; cracks, air bubbles, and channels will lead to a poor separation.

The slurry method normally gives the best column packing, but it is also the more difficult technique to master. Whether the dry packing or slurry packing
**Procedure:**
You will be separating the mixture that results from the reaction below:

![Chemical Reaction]

In this particular experiment, the oxidation is incomplete and you have a mixture of fluorene and fluorenone to separate.

**Objectives:**
- Separate the two components by column chromatography.
- Identify each component
- Determine the mass of each component

**Step 1:** Weigh out ~50 mg of the mixture. Use a balance outside of hood to eliminate the indicated mass from being affected by the air flow.

Follow the procedure from Williamson as follows:
get a new bottom plug from the stockroom. Finish assembling the chromatography column as depicted in Figure 9.1. Be sure to clamp the column securely and vertically.

Grasp the valve with one hand and turn it with the other. Close the valve and fill the column with hexanes to the bottom of the plastic funnel. Weigh out approximately 4.5 g of activity grade III alumina in a small beaker and slowly sprinkle the dry alumina into the hexanes in the column while you tap the column with a pen or pencil. It may be necessary to drain off some of the solvent to keep it from flowing over the top. This amount of alumina should fill the column to a height of about 10 cm. It is extremely important not to let the column run dry at any time. This will allow air to enter the column, which will result in uneven bands and poor separation.

After all of the alumina has been added to the column, open the stopcock and continue to tap the column as you allow the solvent to drain slowly until the solvent just barely covers the surface of the alumina, collecting the solvent in an Erlenmeyer flask.

Adding the Sample

It is important to use a minimum amount of solvent when dissolving the sample. If too much is used, poor separation will result.

The solvent is drained just to the surface of the alumina, which should be perfectly flat. Dissolve the crude mixture of fluorene and fluorenone in 10 drops of dichloromethane and 10 drops of toluene and add this with a pipette to the surface of the alumina. Be sure to add the sample as a solution; should any sample crystallize, add 1 more drop of dichloromethane. (This is done so that the sample to be added to the column is in the most concentrated solution possible.) Drain some liquid from the column until the dichloromethane-toluene solution just barely covers the surface of the alumina. Then add a few drops of hexanes and drain out some solvent until the liquid just covers the alumina. Repeat until the sample is seen as a narrow band at the top of the column. Carefully add a 4–5 mm layer of sand and then fill the column with hexanes.

Collect 3-mL fractions in a combination of small vials, 10-mL Erlenmeyer flasks, 13 × 100 mm test tubes, vials, and small beakers. You will probably collect close to ten 3-mL fractions. While the chromatography is running, you will be determining the amount of fluorene or fluorenone in each fraction by TLC. Once you determine which fractions contain which compound, you will combine the “like” fractions and evaporate the solvent.

After you collect each 3-mL fraction in a flask, apply it to a TLC plate by spotting it 2 or 3 times on the plate in the same location. Four or five fractions can be applied and analyzed at the same time using one TLC plate. Allow the solvent to completely evaporate from the spot and examine the plate under a UV lamp to determine if there is any material present, as evidenced by a dark blue spot. The TLC plates can be developed using 20% dichloromethane in hexanes. You will probably collect a few fractions that contain little or no material; these fractions are likely to be the first or the middle of the series.
After spotting and developing each fraction on the TLC plate, combine like fractions and immediately start to boil off the solvent on the sand bath in the hood using a boiling stick broken in half. Tilt the flasks and vials on their side as much as possible to allow the heavy vapors to escape. As soon as all the liquid seems to have boiled off, set the flask on its side on the bench top in the hood to allow the last traces of heavy solvent vapors to escape. After the flask has cooled to room temperature, if that fraction contains any material, crystals may appear. If an oily, gooey residue is present, you may have to scratch it with a glass stirring rod to induce crystallization. With good organizational effort, you can do the TLC analysis and evaporate off the solvent at about the same rate at which you collect fractions; thus you can follow the progress of the chromatography simply by noting the amount of material in each flask, vial, or test tube. If, after solvent removal and cooling, the flasks are perfectly clean on careful inspection, they can be used to collect subsequent fractions.

If the yellow band has not moved one third of the way down the column after two fractions have been collected, you can speed up the elution by replacing the hexanes solvent at the top of the column with 20% dichloromethane in hexanes. Once the first component has completely eluted, you can speed up the elution of the second component by using 50% dichloromethane in hexanes. Decide when the product has been completely eluted from the column by using visual cues and TLC. Using a few drops of dichloromethane, wash all the fractions that contain fluorene as determined by TLC analysis into a tared container. Do the same for the fluorenone fractions. Evaporate the dichloromethane and obtain dry weights for the product and the recovered fluorene.

Mark all compound spots on all TLC plates with a pencil. Tape your developed TLC plates in your notebook with wide transparent tape. Calculate the theoretical yield and the percent yield of your pure fluorenone both with and without taking into account the amount of fluorene starting material recovered. Calculate the percent recovery of fluorene.

Cleaning Up. When you are done with the column, pour out the excess solvent into the proper waste container, pull out the bottom, and leave the wet column propped in a beaker in your desk hood. The column will dry out by the next lab, and the dry, used alumina can then be easily emptied out into a waste bin.