8. Column Chromatography

This is the last technique experiment in the Introductory Organic Lab. More importantly it is the first synthetic experiment in the course and therefore the PreLab, InLab and PostLab reports will now include more parts and be more extensive. For the PreLab, don’t forget to include the chemical reaction equation and the following additional items:

6. Chromatographic behavior comparison of the starting material and the product
7. Spectral features comparison of the starting material and the product
8. Explanation of product isolation and purification or the “work-up”

Examples of these items are shown in Section 3.2 in The Laboratory Notebook.

PreLab exercise:

1. Predict the order of elution of fluorene and fluorenone from an alumina chromatography column. Explain your answer in terms of each compound’s structure and polarity and its interaction with the alumina stationary phase

2. Upon development, the fluorene standard usually shows a spot due to fluorene and a second spot which has a much lower Rf value. What is the second component and why does it form? (Hint: use the information in the following introduction.)

Introduction

Column Chromatography is another common and useful separation technique in organic chemistry. This separation method involves the same principles as TLC, but can be applied to separate larger quantities than TLC. Column chromatography can be used on both a large and small scale. The applications of this technique are wide reaching and cross many disciplines including biology, biochemistry, microbiology and medicine. Many common antibiotics are purified by column chromatography.

To understand to uses of this separation technique, we can use the last experiment as an example. In the TLC experiment, we separated and analyzed the different components that make-up over-the-counter painkillers. The technique of TLC was useful in determining the type and number of ingredients in the mixture, but it was not helpful for collecting the separated components. We could only separate and visualize the spots. If we needed to collect the separated materials, column chromatography could be used. We could load 100 mg of a crushed Anacin tablet on a column made up of a silica stationary phase and separate the aspirin from the caffeine and collect each of these compounds in separate beakers. Column chromatography allows us to separate and collect the compounds individually. In this experiment, Column Chromatography (abbreviated CC) will be used to separate the starting material from the product in the oxidation of fluorene to fluorenone and TLC will be used to monitor the effectiveness of this separation.

Choosing a Stationary Phase

As with TLC, alumina and silica are the two most popular stationary phases in column chromatography. For these common phases, the partitioning works in an analogous manner. The more polar sample will be retained on the stationary phase longer. Thus the least polar compound will elute from the column first, followed by each compound in order of increasing polarity.
Although the interactions between the mobile and stationary phase are based on the same principles for CC and TLC, be careful when predicting the order of elution. Since the direction of the solvent flow in TLC moves up and in CC the solvent flows down, it appears that the order is “upside-down”. In TLC the more polar molecules will have lower $R_f$ values, but in CC they will be retained longer on the column. Remember this when considering the polarities of the stationary phase as well as the polarity of the compounds being separated when predicting the order of elution.

Stationary phases for CC can come in a variety of sizes, activities, acidic and basic variations for both alumina and silica. The types of stationary phase chosen are determined experimentally, or often based on results from a previous TLC experiment. The type of adsorbent, the size of the column, the polarity of the mobile phase as well as the rate of elution all affect the separation. These conditions can be manipulated to get the best separation for your mixture.

Choosing Solvents

Solvent systems for use as mobile phases in CC can be determined from previous TLC experiments, the literature, or experimentally. Normally, a separation will begin by using nonpolar or low polarity solvent, allowing the compounds to adsorb to the stationary phase, then slowly switching the polarity of the solvent to desorb the compounds and allow them to travel with the mobile phase. The polarity of the solvents should be changed gradually. On a macroscale, the mixing of two solvents can create heat and crack the column leading to a poor separation.

Some typical solvent combinations are ligroin-dichloromethane, hexane-ethyl acetate and hexane-toluene. Often an experimentally determined ratio of these solvents can sufficiently separate most compounds. 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.

Apparatus

Columns can be as thin as a pencil to a diameter of several feet in industrial processes. They can separate milligram to kilogram quantities of materials. In this experiment, we will be separating a mixture of approximately 50 mg, so a small column can be used. Figure 8.1 shows the typical set-up we will be using during this experiment. It is essential to have several clean tared Erlenmeyer flasks, reaction tubes, beakers, test tubes or vials available to collect the solvent and compounds as they elute. Once you have the general set-up prepared, you can move on to packing the stationary phase in the column.

Packing the Column

There are several acceptable methods when packing a column. These include dry packing (there are two versions of dry packing discussed here) and the slurry method. The slurry method normally achieves the best packing results, but there are several occasions when the dry packing method works just as well if not better.

Dry packing is the method of choice for a microscale column. Begin by filling the column with a nonpolar solvent. Slowly add the powdered alumina or silica 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.

For the second dry pack method, the stationary phase is deposited in the column before the solvent. In this case fill the column to the intended height with the stationary phase and then slowly...
add the nonpolar solvent. The solvent should be added slowly as to avoid uneven channeling. This method is typically used with alumina only, since silica gel expands and does not pack well with this dry method.

The slurry method is often used for macroscale separations. Combine the solid stationary phase with a small amount of nonpolar solvent in a beaker. Thoroughly mix the two until a consistent paste is formed, but is still capable of flowing. Pour this homogeneous mixture into the column as carefully as possible using a spatula to scrape out the solid as you pour the liquid. The slurry method normally gives the best column packing, but is also a more difficult technique to master. Whether the dry or slurry method is chosen, the most important aspect of packing the column is creating an evenly distributed and packed stationary phase. As mentioned, cracks, air bubbles and channeling will lead to a poor separation.

Once the column is loaded, open the stopcock and allow the solvent level to drop to the top of the packing, but do not allow the solvent layer to go below this point. Allowing this solvent level to go below the stationary phase, (known as letting the column to “run dry,”) should always be avoided. Since it allows air bubbles and channel formation to occur leading to a poor separation.

**Adding the Sample**

Once the packing is complete, the sample can be loaded directly to the top of the column. Normally, a *minimum* amount of a polar solvent, 5-10 drops, is used to dissolve the mixture. The solution is then carefully added to the top of the column using a pipet without disrupting the flat top surface of the column. A thin horizontal band of sample is best for an optimal separation. After the sample is loaded, a small layer of white sand is added to the top of the column. This will help to keep the top of the column level when adding solvent eluent. Once the mixture is added and the protective layer of sand is in place, continuously add the solvent eluent while collecting small fractions at the bottom of the column. Using a pipet to add the first bit of solvent on top of the packing, sample, and sand will minimize disturbance of the column and diluting the sample. Collecting small fractions (1-3 mL) is important to the success of your column separation.
Fractions that are too small can always be pooled together; however, if the collected fractions are too large, you may get more than one compound in any particular fraction. If this occurs, the only way to complete the separation is to redo the chromatography. Since column chromatography is time consuming, collecting large fractions is discouraged.

**Monitoring the Column**

If the mixture to be separated contains colored compounds, then monitoring the column is very simple. The colored bands will move down the column along with the solvent and as they approach the end of the column, collect the colors in individual containers. Use the color as your guide. However, most organic molecules are colorless. In this case, the reaction must be monitored by TLC. Spot each fraction on a TLC plate. Four or five fractions can be spotted on a single TLC plate. Develop the plate and use the observed spot or spots to determine which compound is in each of the collected fractions. Spotting some of the starting material or the product (if available) on the TLC plate as a standard will help in the identification.

**Isolating the Separated Compounds**

Once you believe all the materials have been removed from the column, the colors of the materials or TLC results should indicate which fractions contain the compound(s) you are interested in isolating. Combine the like or same fractions and evaporate the solvent. The pure separated compound will be left behind. Recrystallization may be used to further purify a solid product. However, on a milligram scale, there is usually not enough material to do this.

**Flash Chromatography**

Column chromatography is often very time consuming. Allowing the solvent to elute through the column one drop at a time takes patience. One method to speed up the process is to use Flash Chromatography. This method uses a pressure of about 10 psi of air or nitrogen to force the mobile phase through the column. Because the rate of the mobile phase is increased, in general, this method gives a poorer separation. However, by using a finer grade of alumina or silica, flash chromatography can be used to increase the speed without lowering the quality of the separation.

**Reverse-Phase Chromatography**

HPLC (High Performance Liquid Chromatography) is a variation on the traditional liquid chromatographic methods. High pressure pumps are used to force solvent through a tightly packed column connected to a variety of different types of very sensitive detectors. Modern HPLC is used extensively in biochemistry to separate cellular components such as proteins, lipids, and nucleic acids. Mixtures of these types require aqueous mobile phases such as methanol-water or acetonitrile-water and these liquids do not work well on normal silica or alumina stationary phases. Instead of these polar phases, very nonpolar ones, called “reverse-phase” packing are used. These are manufactured by bonding lots of hydrocarbon molecules to the surfaces of a silica gel particles so that the silica gel is like a very nonpolar “grease ball.” In this situation, the order of elution will be exactly opposite the behavior on an alumina or silica column. On a reverse-phase column, the more nonpolar materials will adhere to the stationary phase (or like material) longer and the polar compounds will elute first.
Chiral Separations

Many compounds can be separated using typical chromatographic stationary phases and solvents. These separations depend on the difference in polarity of the molecules to be separated. However, how would you separate two compounds with the identical polarity, such as enantiomers? This separation technique is of great importance in the pharmaceutical industry where the FDA controls the amounts of impurities, including enantiomers, in prescribed drugs. For example, Thalidomide, a drug administered in the 60’s has two enantiomers. This drug was used as a sedative and an anti-depressant, but was found to cause abnormalities in the fetuses of pregnant women. Although this drug was pulled from the market due to the resulting birth defects, there is recent literature that suggests that only one of the enantiomers caused the defects. If the enantiomers could be completely separated, Thalidomide might be used as an FDA approved drug and be helpful to people today.

Chiral stationary phases can be used to separate enantiomers. By giving the stationary phase a “handedness,” one enantiomer will be specifically retained on the column. These columns are very expensive and specific to the particular type of separation, but have led to great achievements in separation science.

There are many, many different types of chromatographic methods including gel electrophoresis and size exclusion chromatography that have not been discussed here. Hopefully, with the basic chromatographic background provided, you can apply this knowledge to the many different types of chromatography used in many different professions today.
Column Chromatography Experiment

The 9-position of fluorene is unusually reactive for a hydrocarbon. The protons on this carbon atom are acidic by virtue of being doubly benzylic and consequently this carbon can be oxidized by several reagents, including dioxygen. In the past, a very powerful and versatile oxidizing agent Cr(VI), in the form of chromium trioxide, was used to carry out this oxidation. Although chromium compounds are facile oxidizing agents, they are not practical in today’s environmentally conscious climate due to chromium’s toxicity. One alternate approach is to use oxygen from the atmosphere as the oxidant. The oxidation is carried out utilizing methyltricapryl ammonium chloride, commonly known as Stark’s catalyst or Aliquat 336, as a phase transfer catalyst.

In the presence of this catalyst, the hydroxide will be capable of deprotonating the acidic fluorene protons, creating a carbanion which can attack oxygen in the air. A hydroperoxide is formed which looses water to give the ketone. Using this phase transfer catalyst and atmospheric oxygen, chromium is eliminated resulting in less hazardous chemical waste.

You should plan to carry out the synthetic reaction (following the reaction by TLC) in the first lab and the column chromatography in the second. The chromatography must be completed in the second lab period. It is a lengthy procedure, and therefore it’s important to start it as soon as possible after coming to lab.

Step 1••

Oxidation of Fluorene to Fluorenone

Caution!!

Caution: NaOH is a strong base and is very corrosive. Wash hands immediately if contact occurs.

To a 10 mL Erlenmyer, add 5 mL of 10M NaOH and 70 mg of fluorene while stirring with a 1/2 inch stir bar. Add 5 mL toluene and stir until all the solid has dissolved. (Observe the color and identify which layer is organic and which is aqueous?) Add approximately 3 drops Stark’s catalyst (aliquat) to the solution. Stir vigorously, but without splashing the solution on your neighbor. Observe the progress of your reaction by TLC. Develop the TLC plate using 20% dichloromethane in ligroin and use the UV lamp to visualize your products. Also spot the plate with the fluorene standard which is on the side shelf. When approximately half of your fluorene has been converted to fluorenone, (which can take as little as 10 min depending on the amount of stirring), pour the reaction mixture into a separatory funnel rinsing the beaker with an additional 5 mL of toluene which is added to the separatory funnel. Separate your organic layer from the aqueous layer. Wash the organic layer with 5% HCl (3 x 5 mL), saturated NaCl (3 x 5 mL) (The notation “3 x 5 mL” means wash the organic layer 3 times with 5 mL portions.) Dry the remaining solution over anhydrous sodium sulfate in a 125-mL Erlenmeyer flask until it no longer clumps together. Allow the product to dry for 5 or 10 min before decanting the toluene from the solid sodium sulfate, transferring the toluene to a 100 mL tared beaker. Wash the solid sodium sulfate with 3 mL of toluene, adding this to the first portion of extract to complete the transfer of product. The crude mixture of fluorene and fluorenone will be separated by column chromatography in the next lab period. The beaker containing the toluene extract can be allowed to stand in your hood (labeled properly) until the next lab period. The toluene will evaporate in the interim. Alternatively, if you have time, reduce the volume of solvent (∼1 mL) by heating gently on a sand bath.
Step 2** Column Chromatography of the Fluorene-Fluorenone Mixture

**Note:** It is important to use a minimum of toluene when loading the column. If too much toluene is used the mixture will run too fast and a poor separation will result.

**Packing the Column.** Before you assemble the column, check the small plug that fits into the bottom of the column to make sure that it has a small fritted disk inside. Next, make sure that the plug fits snugly into the glass column and is not easy to pull out. If it is loose, get a new bottom plug from the stockroom. Finish assembling the chromatography column as depicted in Figure 8.1. 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 ligroin to the bottom of the plastic funnel. Weigh out approximately 4.5 g of Activity III alumina in a small beaker and slowly sprinkle the dry alumina into the ligroin in the column while you tap the column with a pen or pencil and drain off the solvent into a small Erlenmeyer flask, if necessary, to keep it from flowing over the top. This amount of alumina should fill the column to a height of about 10 cm.

**Note:** It is extremely important to never 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.** 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 CH₂Cl₂ 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 a drop more CH₂Cl₂. (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 CH₂Cl₂/toluene solution just barely covers the surface of the alumina. Then, add a few drops of ligroin, 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 to 5 mm layer of sand, then fill the column with ligroin, and collect 3-mL fractions in tared 10x13 mm test tubes, 10 mL Erlenmeyer flasks, 20 mL vials or small beakers. You will probably collect close to 10-3 mL fractions. Since taring 10 beakers or vials is very tedious and wastes time waiting in line for the balances, only tare two small beakers. Once you determine (by TLC) which fractions contain your starting material and product, then combine the “like” fractions and evaporate the solvent.

After you collect each 3-mL fraction in a flask, apply it to a thin-layer chromatography plate by spotting it 2 or 3 times on the plate in the same location. Allow the solvent to completely evaporate from the spot and examine the plate under the UV lamp to determine if there is any material present as evidenced by a dark blue spot. The plates are to be developed as described below. (You will probably see one or two fractions that contain little or no solute(s), these fractions being the first or the middle of the series, followed by fractions that contain the first and second component.) Four or five fractions can be applied and analyzed at the same time using one TLC plate.

Cleaning-up. The aqueous extracts can be poured down the sink with water. Organic waste should be placed in the appropriate waste container.
After spotting and developing each useable 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 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 the benchtop on its side to allow the last traces of heavy solvent vapors to escape. After the flask has cooled to room temperature and if some material has come off the column, 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 “finess,” you can evaporate off the solvent at about the same rate at which you collect fractions, and thus can follow the progress of the chromatography simply by noting the amount of material in each flask. 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, replace the ligroin solvent at the top of the column with 20% dichloromethane in ligroin available on the side shelf. This will speed up the elution of the components.

Once all of the first component has eluted, you can speed up the elution of the second component by using 50% dichloromethane in ligroin. You need to prepare this solution yourself in 10 or 15 mL volumes.

You are to decide when all of the product has been eluted from the column using visual cues and TLC. The thin-layer plates can be developed using 20% dichloromethane in ligroin. Combine fractions that are identical as determined by TLC analysis. Remember to obtain dry weights for the product and for the recovered (unreacted) fluorenone.

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 out in a beaker in your desk. The column will dry out by the next lab, and the dry, used alumina can then be easily emptied out into a waste bin.

Final Report

Tape your developed TLC plates in your notebook with the wide transparent tape from the dispenser on the side shelf. Mark all compound spots on all plates. Include the percent yield of your pure fluorenone and the percent recovery of fluorene as well as a short discussion on the interpretation and quality of your TLC and CC results.

PostLab Questions:

1) What would be the effect of collecting fractions of larger volume when carrying out this experiment?

2) Once the chromatographic column has been prepared, why is it important to allow the level of the liquid in the column to drop to the level of the alumina before applying the solution of the compounds to be separated?

InLab and PostLab Sections - Final Report:

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