Experiment: **Column Chromatography**

**Background**

As you have probably seen in past work, thin layer chromatography (TLC) can be used to separate compounds, primarily based on polarity. Column chromatography utilizes the exact same concepts, but on a larger scale, to allow separation of gram-quantities of material. The purpose of this experiment is to extract organic plant pigments from spinach, separate them by column chromatography, and then analyze the effectiveness of the separation by TLC.

As a review, it is important to remember that any chromatographic separation, such as TLC or GC, involves a stationary phase and a mobile phase. The stationary phase, as the name implies, does not move within the chromatographic system. It is either a solid or a viscous liquid held onto a solid support. The mobile phase, either a liquid or a gas, constantly flows through the particles that make up the stationary phase. Molecules that are more strongly attracted to the stationary phase than to the mobile phase will move more slowly than molecules that are more strongly attracted to the mobile phase. As a result, separation is achieved.

With thin layer chromatography (TLC), which you will also do in this experiment, separation takes place on thin sheets of plastic coated with a thin layer of stationary phase. The stationary phase can be many different solids, but the most common ones are silica gel (SiO$_2$) and alumina (Al$_2$O$_3$). For column chromatography, these same solids, rather than being coated on a thin sheet, are instead packed into a glass column. For TLC, the solvent moves up the plate by capillary action, whereas for column chromatography the solvent moves down the column, flowing under the force of gravity.

Alumina is more polar than silica gel, so it is used primarily for separating relatively nonpolar substances (hydrocarbons, ether, aldehydes, ketones, and alkyl halides) because better separation of components can be achieved. (More polar compounds would potentially bind too tightly to the alumina stationary phase, resulting in little or no migration.) The following sequence
illustrates the general affinity of the functional groups towards the polar stationary phase, where the greater the affinity, the slower the movement of the compound.

Slowest = ions > acids/bases > amides > alcohols > ketones > aldehydes > esters > ethers > halides > unsaturated hydrocarbons > saturated hydrocarbons = fastest

Spinach leaves contain two types of chlorophyll (a and b) and β-carotene, as the major types of pigments. In this experiment we will extract and separate these major classes of pigments. Since these different components are highly colored, we can visually monitor the separation on the column.

In addition to its larger scale of purification, another advantage of column chromatography is the ability to change eluting solvents during the separation. This means a relatively non-polar solvent mixture can be used at the start to elute non-polar compounds and then a more polar solvent mix can be added to move the more polar compounds through the column. In this way, excellent separation of compounds, such as the main pigments in spinach, can be achieved. Once your column chromatography has separated the green-colored and orange-colored pigment bands, a TLC will be run to confirm the quality of the separation.
Pre-lab Preparation

1. Consider the structures of the chlorophylls versus the carotenoids. Which have more polarity? How did you decide?

2. Based on your answer above, which will move fastest on the column/TLC plate? Explain why.

3. Before coming to lab, carefully go over the “Background” section for this experiment and review Thin Layer Chromatography. Draw a diagram here showing how to measure Rf values.

4. Watch the following video showing how to do column chromatography. Your quiz this week may ask questions about this video. Based on your answers, if you are not prepared appropriately for lab, you may be asked to leave without credit for the lab. Column chromatography | Chemistry - YouTube

Experimental Procedure

Safety Considerations

Solvents should be kept in the hood to avoid inhalation.

Gloves should be worn to avoid contact with solvents.

Correct balancing of the centrifuge is needed to safely operate this device.

Part 1: Extraction of Spinach

1. Weigh approximately 2.0 g of dry spinach (blot any water with a paper towel), with stems and veins removed before weighing. Add the spinach leaves to a mortar and then add approximately 1 g of sand and 1 g of magnesium sulfate. Using the mortar and pestle, grind the leaves into a paste.

2. Once the leaves are thoroughly ground, add 2 mL of acetone using your 10 mL graduated cylinder. Transfer the sludge with a short, disposable plastic pipet, with the tip cut off to create a larger opening, to a centrifuge tube. If a lot is left behind, you may rinse the mortar with an additional 2 mL of acetone and add this to the same centrifuge tube and then cap the tube.
3. Create a balancing centrifuge tube using an empty tube and water added to the same height as the liquid in your spinach extraction sample. This centrifuge tube with water will be used to balance the centrifuge.

4. Place your spinach solution and the balancing tube in opposite positions in the centrifuge. If other groups are ready at the same time, you may run your sample with their set. Run the centrifuge for 2 minutes at 5,000 rpm (5 x 1000 on the dial). If the centrifuge begins to wobble or make noise, stop and rebalance the tubes. If necessary, you can use a balance to make sure the tubes are the same mass.

5. Transfer the liquid using a pipet, leaving behind any solids, to a new centrifuge tube. Add 2 mL of hexane, cap the tube and shake to mix. Then add 3 mL of water to the liquid, cap and shake again to thoroughly mix. Centrifuge again. Remember to create a balancing tube with the right amount of water to match your liquid height.

6. Remove the top, hexane layer with a pipette and place in a test tube. Do NOT get any of the bottom water layer. This means you will have to leave some of the green, hexane layer behind to avoid transferring water accidentally.

7. Add approximately 0.5 g of anhydrous sodium sulfate (Na2SO4) to remove any traces of water. After 5 minutes, your sample will be dry enough to pour on to the column, once it is ready. After this drying time, remove a couple of drops and place it in a vial to save for TLC.

Part 2: Column Chromatography

1. First, gather your necessary solvents. Obtain a bottle of hexane, a bottle of 90%;10% hexane:acetone solution and a bottle of 70%;30; hexane:acetone. Then obtain a 125 mL Erlenmeyer flask, 2 test tubes and a test tube rack.
2. Next, prepare your column by adding a very small piece of glass wool to the bottom. Clamp the column in the hood and then add 1 mm of sand using a funnel, then tap the column to level the sand. Next add approximately 2.5 cm of dry silica and tap again to pack it down. Finally, add another 1 mm of sand to the top and tap. It is VERY important not to overfill or you will not have room for the solvent. Place the Erlenmeyer flask under the column to collect waste solvent then open the stopcock and use a funnel to slowly add hexane until it begins to flow out the bottom and then close the stopcock. (Note we are dry packing the column, unlike in the video, where they loaded the column with solid suspended in solvent.) Do NOT let the column run dry from this point on. Whenever the stopcock is open, you will have to continuously add solvent slowly to prevent the sand and silica from being exposed to air.

3. Open the stopcock and allow the hexane level to reach as close to the sand as possible without running dry, then close the stopcock. Take your spinach extract and pour it on to the column. (Make sure you have first set aside two drops in a vial for TLC.) Open the stopcock and allow the extract to load on to the column but close it before it runs dry. Then add another 2 mL of hexane and open the stopcock again to finish loading the pigments. Close before running dry.

4. Next, slowly begin adding the 90:10 hexane:acetone mix to the top of the column. Open the stopcock and allow the sample to elute. Remember to continue adding solvent to
prevent the column from being exposed to air. You may close the stopcock at any time to prevent it running dry.

5. Watch the pigments separate in the column and document what you see in your notebook. Continue to adding the 90:10 mix until the first colored band reaches the stopcock and then change to test tube to collect the first pigment. Once the first pigment is eluted, replace the Erlenmeyer flask. You do not want to collect any mixture of pigments in your test tube containing the first pigment. Any overlap of pigments should be collected in the Erlenmeyer flask. Remember, you can close the stopcock at any time to stop the flow while you consider the next steps.

6. Now switch to a more polar solvent, 70%:30% hexane:acetone, to elute the next band of pigments. When the next colored band reaches the stopcock, change to a test tube to collect it. Continue to add acetone slowly to keep the column wet. When the second band has been collected, you may stop the column and begin TLC analysis. (Note that there may be some residual color in the column from other, smaller types of pigments. These do NOT need to be collected.)

Part 3: TLC Analysis

1. With gloves, prepare a TLC plate by drawing a pencil line approximately 1 cm from the bottom. You will be spotting three solutions on this origin line, so place three vertical tick marks on the origin line so that you know where you will spot the solutions.

2. Using a micropipette for each solution, dip it into the original extract and spot on the plate. You want to achieve a very small diameter, highly colored spot, so that when it elutes the component spots will be small but visible. Repeat this process for the two samples of pigment you collected from the column. Before you place it in the solvent chamber, your spots need to be clearly visible or you will not be able to see it after elution.

3. Once you have three suitable spots, prepare a solvent chamber by adding a small amount of 70%:30% hexane:acetone solution to a large beaker. Make sure the level will be BELOW your origin line on your TLC plate. Add a filter paper to the beaker to ensure that the air will be saturated with solvent, then add your TLC plate, keeping the edges away from the filter paper. Cover with plastic wrap and allow it to elute until it is approximately 2 cm from the top of the plate. Then, remove the plate and immediately mark the solvent front. (While your TLC elutes, empty your column into the waste container and clean it with water and then acetone.)

4. Circle the components you see in each sample and then measure the Rf values for each spot. Review how to do this by consulting the Thin Layer Chromatography lab on the Science Learning Center website or other scientific reference.

5. Draw a diagram of your TLC plate in your observations, including the measurements.
6. Clean up everything and dispose of solid waste in the solid waste container and waste liquids in the appropriate liquid waste container.

### Post-Lab and Report Requirements

1. Describe, in your own words, how to perform column chromatography.

2. Explain why solvents were changed to different compositions during the column chromatography.

3. Did your column successfully separate the two main pigment components in spinach? How do you know?

4. If your compounds are not separating completely on your column, what changes could you make. Propose two possibilities and explain.

Conclusion/Summary: Write a brief statement summarizing what you accomplished.