PRELAB ASSIGNMENT

1. **Purpose** of experiment

2. **Structures of Plant Pigments.** Highlight with colored pens or pencils the portion of the molecule that determines the class of the pigment. The classes are listed in Table 1 and the background article on plant pigments highlights the structures of these classes. Also include a diagram of your column set up.

3. **Procedure Outline.** Make sure you have an explanation for every step in your procedure outline.

4. **Chemical Data Table.**

5. **Read Background Information.**

6. **PreLab Exercise.**
   (a) Give one advantage and one disadvantage of using 8g of alumina instead of 4.5 g as in Procedure 2 of the experiment.
   (b) What is meant by the expression “TLC is used to monitor the progress of the reaction”?
   (c) What is the purpose of tapping the column as you add the alumina? If you didn’t do this, what might be the effect on your separation?

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>Colors Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotenoid</td>
<td>Carotenes</td>
<td>Golden</td>
</tr>
<tr>
<td>Porphyrin</td>
<td>Pheophytin</td>
<td>Olive Green</td>
</tr>
<tr>
<td>Porphyrin</td>
<td>Chlorophyll a</td>
<td>Blue Green</td>
</tr>
<tr>
<td>Porphyrin</td>
<td>Chlorophyll b</td>
<td>Yellow Green</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>Lutein</td>
<td>Yellow</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>Xanthophylls</td>
<td>Yellow</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>Anthocyanin</td>
<td>Red, Blue, or Purple (pH...</td>
</tr>
</tbody>
</table>
INTRODUCTION

Thus far you have utilized two techniques to isolate pure organic compounds: recrystallization and distillation. Column chromatography is another valuable isolation technique that is often employed when very small quantities of sample are available. This separation method is very similar in principle to thin layer chromatography, and can be used for samples of many different sizes. TLC is used to determine an effective solvent system for the separation and then column chromatography is used to separate and isolate the desired compound(s). This laboratory assignment demonstrates the utility of column chromatography to separate natural products such as leaf pigments. **You will need to bring leaf samples with you to complete this lab.**

The orange and red pigments in fruits and vegetables such as tomatoes and carrots are hydrocarbons known as carotenoids. The two most common carotenoids, which incidentally are precursors to Vitamin A, are lycopene and β-carotene, the structures of which are shown below.

![Chemical structures of Vitamin A, Lycopene, and β-carotene]

The carotenoid pigments also occur in the leaves of plants but they are not obvious because of the presence of other pigments.

The green pigments in leaves are principally from chlorophyll-a and chlorophyll-b, which have the structures shown below.
Chlorophyll-b differs from chlorophyll-a in that it has a formyl (-CHO) group in place of a methyl group (CH₃), at the position shown in the above structure.

The leaves of plants contain not only the chlorophylls a and b, but also other pigments whose colors are masked by the chlorophylls in live, healthy leaves. The other pigments become visible in the fall when the leaf dies and the chlorophylls rapidly decompose. Among the other pigments are the carotenoids, the two commonest being lycopene and \_carotene, both of which are precursors to Vitamin A.

**PROCEDURE: ISOLATING PLANT PIGMENTS BY COLUMN CHROMATOGRAPHY**

**Extraction of Plant Pigments.** Working in a hood, rip two leaves into pieces and then add them to a mortar with 22 mL of acetone, 3 mL of petroleum ether and a spatula tip of calcium carbonate (CaCO₃). Grind the leaf pieces with a pestle until the solvent in the mixture becomes highly colored. Filter the pigment mixture into a separatory funnel. Add 20 mL of petroleum ether and 20 mL of saturated, aqueous NaCl solution into the separatory funnel. Remove the water layer and wash the organic layer 4 times with 5 mL of distilled water. Dry the organic layer with 4 spatula tips of anhydrous sodium sulfate (Na₂SO₄). Gravity filter into an Erlenmeyer flask and concentrate the organic layer to 3 mL by blowing a stream of nitrogen into the flask.

**Packing the Column.** While concentrating the organic layer set up the microscale column located in your red kit. Refer to Figure 8.1 on page 196 for the set up of the microscale column. You will be using silica gel as your stationary phase, and the slurry method to pack the column. The solvent system you will use for the separation is a 7:3 mixture of petroleum ether and acetone. Review the polarity of these solvents using Figure 7.8 on page 176. Also review the polarity of silica gel as a stationary phase using Figure 7.5 on page 174.

Before assembling your 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, use a new bottom plug from your Organic Lab Equipment Kit. Finish assembling the chromatography column as depicted in Figure 8.1. Be sure to clamp the column securely.
and vertically to a ring stand. Then place an Erlenmeyer flask or small beaker under the stopcock of the column to catch any dripping solvent if you should spring a leak while packing the column. It is important to get into the habit of always having a beaker or flask under the column to catch any solvent that may accidentally drip out of the tip of your column.

Weigh out approximately 3 g of silica in a 50 mL beaker and then add 10 mL of the 7:3 petroleum ether/acetone solvent mixtures. While stirring the mixture, pour it onto column as carefully as possible using a spatula to scrape out the solid as you pour the liquid. The most important thing to remember when you are packing a column is to make sure you are creating an evenly distributed and packed stationary phase that is devoid of cracks, air bubbles and channeling. If any silica gel remains on the sides of the column use a minimum amount of the solvent mixture to wash it into the column. Once the column is packed, open the stopcock and allow the solvent level to drop to the top of the silica, but do not allow the solvent layer to go below this point. Allowing the solvent level to drop 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 poor separation. After the column is packed, carefully add a layer of sand (0.5- 1 cm) to the top of the column to minimize the disruption of the flat surface of the column when you need to add additional solvent.

**Preparing for fraction collection.** Tare and label at least 10 test tubes and a 25 mL Erlenmeyer flask for sample collection. Remember you will never leave your column unattended when it is draining solvent and you will always keep a collection flask under the stopcock to prevent the accidental loss of any sample.

**Adding the sample:** Add 10 drops of the concentrated plant pigment extract to the top of the sand using a pipette. Be careful not to disturb the top surface of the column. It is important to use a minimum amount of solvent to dissolve the pigments, if too much solvent is used the mixture will elute too rapidly and poor separation will result. A thin horizontal band of sample is best for an optimal separation. Drain some liquid from the column until the product is well within the silica layer. Add more solvent to the top of the column being very careful not to disturb the surface.

**Sample Collection.** Begin collecting solvent for your first fraction in the 25 mL Erlenmeyer flask until you see the bands of color just above the stopcock of the column. When you reach this point you will begin collecting smaller fractions based on color making sure you have at least one small fraction between colors. If a yellow colored band remains on the column that is not eluting with the petroleum ether acetone solvent mixture, allow the solvent to run down to just the top of the column and then add a solvent mixture of 70:30 isopropanol/water. The compounds left behind maybe flavonoids that require a more polar solvent mixture to elute them from the column. Collect this polar mixture as one fraction. If a red or blue colored band still remains on the column you can elute it with a saturated solution of sodium bicarbonate.

**TLC of sample fractions.** You will need to run a TLC on the first fraction (colorless fraction), and each colored fraction. See Table 1 for the color of each pigment fraction.
Evaporate the solvent by blowing nitrogen over each sample. Remember you can spot at least three fractions on one TLC plate. If each colored fraction contains one component then you can begin the Spectrometer 20 analysis to confirm the identification of the plant pigment. If more than one spot exists in a fraction consult with your TA to determine a plan of action.

**Spectrometer 20 Analysis.** Look up the wavelengths for the pigments you have isolated. Make up a solution using the same techniques for UV/Vis. You can find this information on the back page of your Lab Guide. Fill one cuvet with your solution and one with your solvent only. You will use this for your background. Locate the instructions on the front, right hand side of the Spectrometer 20. Allow the Spec. 20 to warm up 15 min prior to testing your pigments. Set the Spec. 20 at a slightly lower wavelength than you expect to see absorbencies (using dial 3). Put in the background sample and adjust the transmittance to 100% (using dial 5). Now put in your sample solution. Record the wavelength, % transmittance and absorbance by making a table in your notebook. Take out your sample, and then adjust the wavelength in increments of 10. Each time you must put in your background and reset the transmittance to 100%. Continue this until you are slightly above the maximum wavelength for your compound.

**Cleaning-up.** When you are done with the column, pour the excess solvent into the proper waste container, pull out the bottom, and leave the “wet” column out in the beaker in your desk. The column will dry out by the next lab, and the dry used silica can then be easily emptied out into the waste container labeled “silica gel waste”.

**FINAL REPORT**

Make a computer generated table for all isolated pigment fractions using the data collected from the Spectrometer 20. Attach all TLC plates and tabulate the Rfs for all spots. Discuss the order of elution of your pigments and whether or not they followed the order in Table 1 above. Create a table of plant pigments that you have isolated in this lab and include the name, class of compound, color of the band, the Rf and UV max for each isolated compound. Discuss the success of the method in separating the pigments and whether or not you achieved the expected outcomes.

**References:**

P. Keusch, University of Regensburg, “Separation of Plant Pigments by Column Chromatography (CC), Organic Chemistry Demonstration Experiments on Video Chemistry Visualized
www.uniregensburg.de/Fakultaeten/nat_Fak_IV/Organische_Chemie/Didaktik/Keusch/D-TLC-e.htm