COLUMN CHROMATOGRAPHY
EXTRACTION OF PIGMENTS FROM SPINACH
(THIS LABORATORY PROCEDURE WAS PROVIDED BY Dr. V. WAGHULDE.)

**Purpose:** To separate plant pigments from spinach leaves using column chromatography.

The leaves of plants contain a number of colored pigments generally falling into two categories, chlorophylls and carotenoids. Chlorophylls \(a\) and \(b\) are the pigments that make plants look green. These highly conjugated compounds capture the (non-green) light energy used in photosynthesis.

**Carotenoids** are part of a larger collection of plant-derived compounds called terpenes. These naturally occurring compounds contain 10, 15, 20, 25, 30 and 40 carbon atoms, which suggest that there is a compound with five carbon atoms that serves as their building block. Their structures are consistent with the assumption that they were made by joining together isoprene units, usually in a "head to tail" fashion. Isoprene is the common name for 2-methyl-1,3-butadiene. The branched end is the "head" and the unbranched is the "tail." That isoprene units are linked in a head to tail fashion to form terpenes is known as the isoprene rule.

![Isoprene](image)

Carotenoids are tetraterpenes (eight isoprene units). Lycopene, the compound responsible for the red coloring of tomatoes and watermelon, and \(\beta\)-carotene, the compound that causes carrots and apricots to be orange, are examples of carotenoids.

**\(\beta\)-Carotene** is also the coloring agent used in margarine. When ingested, \(\beta\)-carotene is cleaved to form two molecules of vitamin A and is the major dietary source of this vitamin. Vitamin A, also called retinol, plays an important role in vision.

![\(\beta\)-Carotene](image)

Spinach leaves contain **chlorophyll \(a\) and \(b\)** and **\(\beta\)-carotene** as major pigments as well as smaller amounts of other pigments such as xanthophylls; these are oxidized versions of carotenones and phenophytins, which look like chlorophyll except that the magnesium ion (\(\text{Mg}^{2+}\)) has been replaced by two hydrogen ions (\(\text{H}^+\)).
In this experiment we will isolate and separate the spinach pigments using differences in polarity to effect the separation. Since the different components are colored differently, we can follow this separation visually. Notice that since β-carotene is a hydrocarbon it is very nonpolar.

Both chlorophylls contain C—O and C—N bonds (polar groups) and also contain magnesium bonded to nitrogen - forming a bond so polar that it is almost ionic. The structures of the chlorophylls are given below. Both chlorophylls are much more polar than β-carotene. If you look carefully you can see that the two chlorophylls differ only in one spot. Chlorophyll a has a methyl group (-CH₃) in a position where chlorophyll b has an aldehyde (-CHO); look on left side of structures below. This makes chlorophyll b slightly more polar than chlorophyll a. After we isolate the pigment mixture from the leaves in a hexane solution, we will use the difference in polarity to separate the various pigments using column chromatography. We will analyze the original extract and the pigment fractions using thin layer chromatography, which also separates based on polarity.
Column chromatography is performed by packing a glass tube with an absorbent as shown in (Survival Manual p. 243-Figure 29.4). A column may be packed 'wet' by pouring solvent-adsorbent slurry into the tube, or 'dry' by filling it with dry adsorbent. The mixture to be purified is then dissolved in a small amount of the appropriate solvent and added carefully to the top of the column, so as not to disturb the packing. The column is developed by adding more solvent to the top and collecting the fractions of eluent that come out of the bottom in separate test tubes. For 'flash' column chromatography, moderate air pressure is used to push the solvent through the column. The success of the separation and the contents of the fractions can be determined by spotting the fractions along with the initial mixture on TLC.

A column may be developed with a single solvent mixture or a with a polarity gradient (a solvent system which gradually increases in polarity.) For example, a column may be developed first with a low-polarity solvent, such as hexane, and as fractions are collected the developing solvent is changed to 70:30 hexane: acetone, then 50:50 hexane: acetone. (The solvent is changed by adding more to the top of the column). A polarity gradient is used for mixtures of compounds with very different polarities.

**Solvents.** A common non-polar solvent for chromatography is hexane. It can be used with a variety of polar solvents, some of which are listed below in order of increasing polarity: chloroform, ethyl acetate, methylene chloride, and methanol.

**NOTE: DO NOT LET THE TOP OF THE COLUMN RUN DRY UNTIL YOU ARE FINISHED.**

**Procedure:**

1. Weigh 1.0 g of dry spinach leaves. Remove any stems and veins from the leaves before you weigh.
2. Using mortar and pestle, grind the spinach leaves to a fine paste by adding 1-2 mL of acetone. If the acetone evaporates, then add an additional 1 mL.
3. Transfer the paste using a Pasteur pipette along with acetone to a centrifuge tube without cap and centrifuge the mixture.
4. Transfer the liquid portion (after centrifuging) using a Pasteur pipette to another centrifuge tube WITH cap.
5. Add 3 mL of hexane and 3 mL of water to the above liquid and cap the centrifuge tube. Mix the contents and centrifuge again.
6. Remove the organic layer using a Pasteur pipette and place it in a clean, dry test tube.
7. Repeat steps 5 and 6 with the remaining aqueous layer.

**Drying organic layer**

8. Prepare a drying column using a short stem Pasteur pipette by carefully pushing a small piece of cotton down to the narrow part. Use a piece of stiff wire or the tip of another pipet to push the cotton in place.
9. Clamp the pipette with a thermometer clamp onto a ring stand after adding about 1 g of anhydrous sodium sulfate.
10. Pass the organic layer through the column, draining into your smallest round bottom flask. Rinse the column with an additional 1-2 mL hexane.
11. Concentrate the organic layer by using a rotoevaporator. Seal the flask and place it temporarily in your lab drawer. This is your extract.

**Column Chromatography**

12. Label 3 clean and dry test tubes: • hexane, • 70:30 hexane : acetone, and • pure acetone. Add 8 mL of each solvent or mixture to the respective test tubes. Look in the hood to find these prepared solvents or solvent mixtures.
13. Label 4 more clean and dry test tubes, 1-4, for collection of various color bands from the column.
14. Prepare a column using a short stem disposable Pasteur pipette by carefully pushing a small piece of cotton down to the narrow part.
15. Clamp the pipette with a thermometer clamp after adding about 1.5 g of alumina. Place the clean, dry test tube # 1 under the column to collect the solvent.
16. Add hexane to the column so as to pack the alumina and remove any air gaps in the column. Continue addition of hexane till you see the hexane coming out of the column.
17. Once the hexane level drains to just above the alumina, load the column by adding your extract to the top of the column. Avoid allowing the column to go dry throughout the procedure. Remember to save some sample of original extract for TLC.
18. Immediately begin eluting the column by adding more hexane. Continue addition of hexane and try to observe the yellow band separating from the green extract. This band is very light in color and moves relatively quickly!
19. Continue the addition of hexane until you get the yellow band near the bottom of the column. Change to test tube #2 to collect the yellow band.
20. Change the solvent to the 70:30 hexane : acetone mixture. This should move the green band through the column. Change to test tube #3 after you have collected the entire yellow band.
21. When the green band approaches the bottom of the column, switch tubes and collect it in test tube # 4. If the band does not move with this solvent, then use acetone.
22. Concentrate the fractions by using a stream of air and hot water bath (40 °C). Stopper the test tube and place in the drawer.
23. Run one TLC plate to demonstrate your separation. You should spot the extract you saved as well as fraction 2 (yellow) and fraction 4 (green). Use 70:30 hexane : acetone as the elution solvent. Be sure to mark the solvent front when you remove the plate from the solvent chamber.
24. Calculate the Rf and note the colors of various TLC spots, especially the phenoxytin, which are gray in color and will disappear fast from the TLC plate. Remember to attach the TLC plate to your notebook.
**Data/Results:**
Distance traveled by solvent__________cm

<table>
<thead>
<tr>
<th>Extract</th>
<th>Color</th>
<th>Distance of spot (cm)</th>
<th>R_f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Carotene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. phenophytin A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. phenophytin B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. chlorophyll A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. chlorophyll B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. xanthophylls 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. xanthophylls 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. xanthophylls 3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Yellow
1. 
2. 

Green
1. 
2. 
3. 

**Draw a picture of your TLC plate as it appears next to the data table. Label all spots on the drawing. Include the elution solvent system.** Be sure to clearly label the drawing as extract, yellow band or green band and label the colors of as many of the separated spots as possible. Identify as many spots as you can. Determine which pigments were present in the yellow band and which were present in the green band from your column. In the crude extract, you may be able to see the following components (in order of decreasing R_f values):
- Carotenes (1 spot) (yellow-orange)
- Phenophytin a (gray, may be nearly as intense as chlorophyll b)
- Phenophytin b (gray, may not be visible)
- Chlorophyll a (blue-green, more intense than chlorophyll b)
- Chlorophyll b (green)
- Xanthophylls (possibly 3 spots: yellow)

Depending on the leaf sample, the conditions of the experiment, and how much sample was spotted on the TLC plate, you may observe other pigments. These additional components can result from air oxidation, hydrolysis, or other chemical reactions involving the pigments discussed in this experiment.
Conclusion:

In your conclusion you should address how well the column separated the various pigments. Include the color and $R_f$ of all the pigments in original extract. Explain the contents of individual color bands based on color and $R_f$ with comparison to the original extract.

Questions:

1. The column chromatography procedure for the separation of a polar and a non-polar compound calls for sequential elution with the following solvents: hexane, 70:30 hexane : acetone, acetone and 80:20 acetone : methanol. Why it is important to follow this solvent order?

2. What would happen to the $R_f$ value of the pigments if you were to increase the relative concentration of acetone in the developing solvent?

3. During a mixture separation with column chromatography, why must the level of the solvent be kept above the top of the stationary phase once the procedure is started?

4. Why did $\beta$-carotene move through the column faster than other components (mostly green and gray), regardless which solvent is used to elute them? [Use structural ideas in your answer and consider the column absorbent.]