Isolation of Chlorophyll and Carotenoid Pigments from Spinach

Introduction

We will be extracting plant pigments from frozen spinach, and we will be able to collect at least two distinct colored fractions (maybe more depending on the spinach we use!). Frozen spinach is a good source of β-carotene and the chlorophylls. The structures of these compounds are shown on the last page of this handout. Acetone is a good solvent for extracting these non-polar pigments from the spinach leaves. (As well as some other pigments in smaller amounts. Whether or not you will be able to detect these other pigments will depend on several variable factors.) Unfortunately, acetone is miscible (will mix with) with water as well; so your acetone extract will also contain a good deal of water. As a budding organic chemist, you should be starting to consider water as your enemy! We will separate the organic compounds from your green acetone mixture by extraction with the non-polar solvent hexane. Then we will dry the hexane solution, evaporate the solvent, and separate the plant pigments. We will accomplish this final separation by column chromatography, using alumina as the stationary phase and a mixture of ligroin (a mixture of alkanes with a boiling point of 50-100 ˚C) and ethyl acetate as solvents. These pigments are sensitive to both light and heat, so you must treat them carefully!

Experimental Procedure:

Extraction and isolation of the pigments (**to save some time, the storeroom will do this, and we will only do the TLC and Column Chromatography half of the experiment**.)

Weigh a 10 g sample of spinach (squeeze out the water first!) into a 100 mL beaker. Add 25 mL of acetone and macerate (mix vigorously) the mixture until the leaves lose most of their color to the acetone solution. Place a plug of glass or cotton wool in a short stem funnel, and filter your mixture through this plug into a 125 mL separatory funnel. Press the spinach pulp with a spatula, and rinse the leaves with an additional 5 mL of acetone.

Add 30 mL of hexane and 30 mL of saturated aqueous sodium chloride to this separatory funnel, shake this mixture vigorously with occasional venting, and drain off the aqueous layer. Wash the organic layer two more times, using 30 mL of water each time. You should drain off the aqueous layer after each wash with 30 mL of water! If you have any emulsions during these washings, they may be broken by adding a small amount of saturated aqueous sodium chloride. Transfer your green hexane solution to a 50 mL Erlenmeyer flask and dry with anhydrous magnesium sulfate. Filter this solution into a clean, dry 50 mL Erlenmeyer flask. At this point, you may store this solution overnight by placing a stopper in the flask and wrapping parafilm around the stopper. If you have time, you may evaporate the solvent by placing the solution in a 100 mL beaker and heating the solution on a hot plate (use a boiling chip or stick!) in the hood until only 3-5 mL of the dark green solution remains. Be careful not to evaporate to dryness at this point as you will overheat your pigments, and they will be oxidized and become discolored! You should then store your solution overnight in a refrigerator in a labelled vial.

TLC and Column Chromatography

Spot your dark green hexane solution on a TLC plate. Prepare the TLC plate in the normal fashion. Depending on the concentration of your extract, you may have to make the spot more concentrated by repeatedly touching the plate (try to make your spot as small as possible) on the same spot. You may check the concentration of the spot by visualizing it under a UV lamp before developing the plate. The developing solvent will be ligroin/ethyl acetate (70:30 by volume). In this crude extract you might be able to see the following components (from greatest to smallest R_f values):

β-carotene (yellow-orange)

pheophytin a and pheophytin b (grey - you may or may not be able to see these spots. These have the same structure as the chlorophylls, except that the Mg^{2+} has been replaced with 2 H^+)

chlorophyll a (blue-green, more intense than chlorophyll b)

chlorophyll b (green - same structure as chlorophyll a except one methyl group has been replaced with a CHO group)

xanthophylls (yellow - possibly more than one spot)

Prepare the column as described in the attached handout. We will be using approximately 4 g of alumina for this column chromatography. Use the same 70:30 mixture of ligroin/ethyl acetate as the solvent in preparing your column. While you are packing your column, you should evaporate the solvent from your green hexane extract using a 100 mL beaker on a hot plate in the hood as described above until 1 mL of solution remains. If you evaporate to dryness (be careful!), redissolve the residue in 1 mL of ligroin before beginning the column chromatography.

After placing your crude extract on the column as described in the column chromatography handout, elute the column with the 70:30 mixture of ligroin/ethyl acetate until the yellow/orange band of β-carotene has been collected. Try to collect all of this band in the smallest fraction possible. After the β-carotene has been collected, use pure ethyl acetate as your developing solvent to collect your chlorophylls. Try to collect as many different colored fractions as possible, and use pre-weighed (tared) vials to collect your fractions. You should be able to obtain at least two relatively pure fractions - one of the β-carotene, and one of the chlorophylls. Check the purity of each colored fraction by developing a TLC plate. You can make several spots on one TLC plate. Compare the R_f's of your pure fractions with the TLC of your initial crude extract. Evaporate the solvents from your pure fractions in the hoods, and determine the yields of your pure compounds. Don't forget to account for the mass of your boiling chips! Your yields will be very low, so don't worry when you get a small mass.

Questions:

1. Rationalize the order of the compounds' progress through the column. Focus on how their structural features affect their polarity and how this polarity affects their rate of travel through the column.

2. Why did we change the solvent to pure ethyl acetate mid-way through our column?

Column Chromatography

note: Silica gel can be used instead of alumina in this preparation

1. Determine the amount of alumina you will need by filling your column with alumina. Leave about 1-2 inches of space at the top of the column. See the picture on page 172 of the Williamson text. If your Micro Buchner funnel still has the polyethylene frit, remove this by poking it out.

2. Put the alumina in a beaker and add enough of your solvent to make a slurry of the alumina.

3. Put a small plug of cotton wool in the bottom of your column, followed by a small layer (5-10 mm) of sand. Then fill the column about half full with your solvent.

4. Add your slurry of alumina and solvent to your column. Be careful not to overfill your column.

5. Drain solvent through your column until the alumina is packed and level. No cracks or bubbles!! These will hurt the consistency of the alumina and therefore make your separation less successful. Be careful not to let the level of the solvent dip below the top of your alumina! You will need to drain a fair amount of solvent through your column, but you can re-use the solvent that you drain through.

6. Once the column is packed, leave a small amount of solvent (~ 5 mm) above the level of the alumina, and carefully add some sand (about 5 mm) to the top of the column. Be sure that your alumina is level. Then drain the solvent to the top of the sand layer.

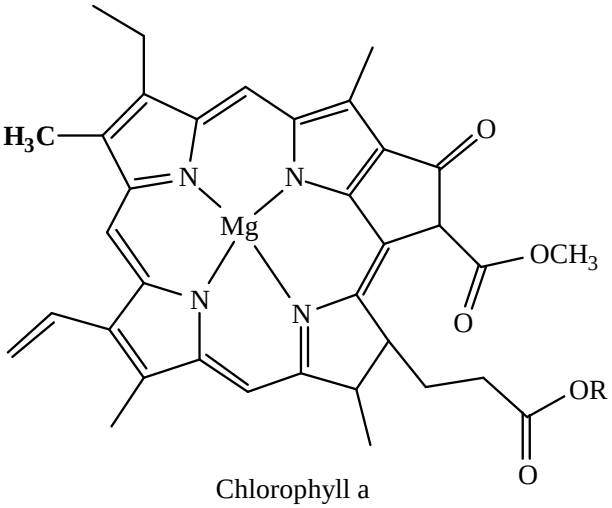
7. Now dissolve your compound in a small amount of solvent (preferably a non-polar solvent - or the solvent you are using in your chromatography), and carefully add it with a disposable pipette to the top of your sand.

8. Drain the solvent with your compound to the top of the sand on your column, carefully add one mL of your solvent to the column, and drain it to the sand again.

9. Now carefully add enough solvent to fill your funnel (don't disturb the alumina), and start draining solvent.

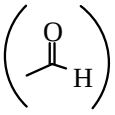
10. Begin collecting fractions from the bottom of your column, and away you go!! Note: a fraction is a separate given amount of solvent that comes off the bottom of the column. Generally, chemists collect separate fractions of a specific volume in vials or test tubes - depending on the size of the column. In this experiment, our compounds will be colored, so you will be able to isolate your compounds in the smallest fraction possible by switching vials when the colored bands come off the bottom of the column.

A couple of extra notes. The best separation occurs when your compound has an Rf of about 0.3. You can determine this with TLC plates before you choose your solvent. You may also use silica gel in place of alumina. We use alumina in this lab.



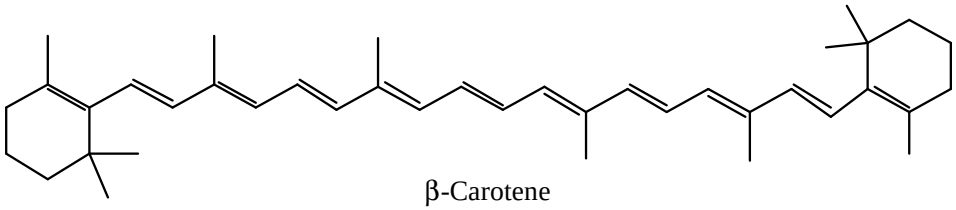
Chlorophyll a

"R" is a 20 carbon chain with only alkanes and alkenes

Chlorophyll b - **CH_3** is an aldehyde 

pheophytin a
pheophytin b

same structures as the chlorophylls except the Mg^{2+} has been replaced with 2 H^+



β-Carotene

Xanthophylls is a general term that encompasses many different compounds. They are molecules of β-carotene that have been oxidized, so they contain many OH groups. Picture the β-carotene above with many OH groups on the rings or the long chain.