Isolation of Chlorophyll and Caretenoid Pigments from Spinach

Introduction

Photosynthesis in plants takes place in organelles called **chloroplasts**. Chloroplasts contain a number of colored compounds (pigments) which fall into two categories, **chlorophylls** and **caretenoids**.

Caretenoids are yellow pigments that are also involved in the photosynthetic process. In addition, chloroplasts also contain several oxygen containing derivatives of carotenes called **xanthophylls.**

In **part A**, you will extract the chlorophyll and caretenoid pigments from spinach leaves using acetone as the solvent. The pigments will be separated by column chromatography using alumina as the adsorbent. Increasingly more polar solvents will be used to elute the various components from the column. The colored fractions collected will then be used to elute the various components from the column. It should be possible for you to identify most of the pigments already discussed on your thin-layer plate after development.

Chlorophylls are the green pigments that act as the principal photoreceptor molecules of plants. They are capable of absorbing certain wavelengths of visible light that are then converted by plants into chemical energy. Two different forms of these pigments found in plants are **chlorophyll a** and **chlorophyll b**. The two forms are identical except that a methyl group in **a** is replaced by an aldehyde in **b**. **Pheophytin a** and **pheophytin b** are identical to chlorophyll a and b, respectively, except that in each case the magnesium ion Mg^{2+} has been replaced by two hydrogen ions $2H^{+}$.

What is **column chromatography**? The IUPAC definition is: "Column chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction." Once you start running a column, don't let it 'run dry.' If the solvent level runs down below the surface of the stationary phase, it will dry out and crack, and your products will *not* elute.

Terminology	
Stationary phase	The solid material in the column.
Mobile phase	The solvent used for the column.
Load/Loading	The sample you put on the column / The process of putting the sample on the column
Eluent/Eluting	The sample that comes off a column / The process of the sample coming off a column
Wash/Washing	A solvent used to clean and moisten the column prior to loading the sample / The process of cleaning and moistening the column.
Fractions	The washes and eluents collected (often in test tubes)

Different stationary and mobile phases can be used depending on what's being separated. The most common stationary phases are silica and alumina. In this lab, you will be using alumina because it is more polar than silica. The most common mobile phases are hexanes (non-polar) and acetone, methanol, ethyl acetate (polar). A solvent (or mixture of solvents) is chosen depending on how polar/non-polar the compounds are. (likes dissolve likes…)

In **part B,** the pigments will be separated on a column packed with alumina. Although there will be many different compounds in your sampler, they usually separate into two main bands on the column. The first band to pass through the column is yellow and consists of the carotenes. This band may be less than 1 mm wide and may pass through the column very rapidly. It is easy to miss seeing the band as it passes through the alumina. The second band consists of all the other pigments discussed earlier. Although it consists of both green and yellow pigments, it appears as a green band on the column. The green band spreads out on the column more than the yellow band, and it separates more slowly. Occasionally, the yellow and green components will separate as the band moves down the column. As the sample elutes from the column, collect the yellow band in one test tube, and the green one in another test tube.

Experimental

Part A. Extraction of the Pigments.

You will be provided a stock solution of freshly ground spinach. This solution is made by blending spinach pulp in an electric blender, and dissolving it in acetone.

Obtain 2.0 mL of the stock solution from the hood in a 15mL centrifuge tube. To this, add 2.0 mL of hexanes, cap it, and shake the mixture thoroughly. Set aside 10mL Hexanes in a graduated cylinder. Then add 2.0 mL of water and shake thoroughly with occasional venting. Centrifuge the mixture to break the emulsion, which usually appears as a cloudy green layer in the middle of the mixture. Remove the bottom aqueous layer with a Pasteur pipet. Using a Pasteur pipet, prepare a column containing anhydrous sodium sulfate to dry the remaining hexanes layer, which contains the dissolved pigments. Put a plug of cotton into a Pasteur pipet, and tamp it into position using a glass rod or a second pipet. Add about 0.5g of powdered or granular sodium sulfate, and tap the column with your finger to pack the material.

Clamp the column in a vertical position and place a dry test tube (12 x 75mm) under the bottom of the column. Label this test tube with an **E** for extract so you don't confuse it with the tubes you will be working with later in this experiment. With a Pasteur pipet, transfer the hexanes layer to the column. When the solution has drained add 0.5 mL of hexanes to the column to extract all the pigments from the drying agent. Evaporate most of the solvent by placing the test tube in a warm water bath (50 – 70C°). Dissolve the remaining residue in 0.5 mL of hexanes. This removes any (polar) acetone from the pigments, which will interfere with the chromatography procedure.

Part B. Column Chromatography

Advance preparation. Before running the column, assemble the following glassware and liquids. Obtain five dry test tubes (12 x 75 mm), and number them 1 through 5. Prepare two dry Pasteur pipets with bulbs attached, one for each solvent. Place 15.0 mL of hexanes and 10.0 mL of acetone into two separate containers. Clearly label them.

To prepare a chromatography column packed with alumina: place a *small loose* plug of cotton in a Pasteur pipet, and push it *gently* into position using a glass rod or a second pipet. Clamp the column in a vertical position so that the bottom of the column is just above the height of the test tubes you will be using to collect the fractions. Add ~1.25 g of alumina to the pipet while tapping the column gently with your finger. When all the alumina has been added, tap the column with your finger for several seconds to ensure that the alumina is tightly packed. Place test tube 1 under the column.

Running the column.

Note: Read and understand the following procedure on running the column. The chromatography procedure must take less than 15 minutes! You ***cannot*** *stop until all the material is eluted from the column. If you take too long, you will not get good separation of the pigments. You must have a good understanding of the whole procedure before beginning.*

Equilibrating the column:

Using a Pasteur pipet, slowly add about 2.0 mL of hexanes to the column. The column must be completely moistened by the solvent. Drain the excess hexanes until the level is down to the top of the alumina. Once you have added hexanes to the alumina, the top of the column must *never* be allowed to run dry. If necessary you may add hexanes at any point to keep this from happening.

When the level of the hexanes reaches the top of the alumina, add about 3/4 (0.4 mL) of the dissolved pigments to the column. Leave the remainder in the test tube for the thin-layer chromatography procedure. (put a stopper on the tube and place it in your drawer) Continue collecting the eluent in test tube 1. Just as the last of the pigment solution penetrates the column, add 1 mL of hexanes and drain until the surface of the liquid again reaches the alumina surface.

Add about 4mL of hexanes to start eluting the yellow band. Continue adding hexanes until the yellow band approaches the bottom of the column. Right before the yellow band begins eluting, switch your collection tube to test tube 2. When the eluent becomes colorless again, place test tube 3 under the column. The total volume of yellow material should be less than 2.0 mL.

Add several mL of acetone as soon as the last of the hexanes run down to the surface of the alumina. Continue adding acetone until the green band approaches the bottom of the column. Right before it begins eluting, switch to test tube 4. Continue adding acetone. When there is little or no green color in the eluent, switch to test tube 5 and stop the procedure.

Using a warm water bath (50-70°C), evaporate the solvent from the tube containing the yellow pigment, the tube containing the green pigment and the tube containing the original pigment solution (2, 4, E). As soon as all the solvent has evaporated from each of the tubes, remove them from the water bath. Do not allow any of the tubes to remain in the water bath after the solvent has evaporated or the heat may cause the pigments to decompose. Stopper the tubes.

Part C. Thin-Layer Chromatography

You will be using 10-cm x 3.3-cm TLC plates. They have a flexible backing but should not be bent excessively. Handle them carefully, or the adsorbent may flake off them. Also, you should only handle them by the edges; the surface should not be touched. Using a lead pencil (not a pen), *lightly* draw a line across the plate (short dimension about 1 cm from the bottom (see figure). Using a centimeter ruler, move its index about 0.5 cm in from the edge of the plate and lightly mark off three 1-cm intervals on the line. These are the points at which the samples will be spotted.

Prepare three micropipets to spot the plate. Your TA will show you how to make these. Make up 10 mL of a 70% hexanes – 30% acetone solution in a 10 mL graduated cylinder. Using this mixture, prepare a TLC **development chamber** using a 250 mL beaker covered with a watchglass or a screw-cap bottle.

Using a Pasteur pipet, add three drops of the 70% hexanes – 30% acetone solution to each of the three test tubes containing dried pigments (2, 4, E). Swirl the tubes so that the drops of solvent dissolve as much of the pigments as possible. The TLC plate should be spotted with three samples: the extract, the yellow band from the column, and the green band. For each of the three samples, use a different micropipet to spot the sample on the plate. For the extract (E) and the green band (4), touch the plate once lightly to keep the spot small. Let the solvent evaporate. The spot should be no larger than 2 mm in diameter and should be fairly dark green. For the yellow band (2) repeat the spotting technique 5-10 times on the same spot, until it is definitely yellow colored. Let the solvent evaporate completely between successive applications, and spot the plate in exactly the same position each time. Save the liquid samples in case you need to repeat.

Development of the TLC Plate. Place the TLC plate in the development chamber. Do not disturb the chamber! Do not move it, etc. When the solvent front is 0.5 cm from the top of the plate, remove it from the chamber and mark the solvent front with a lead pencil. As soon as the plates have dried, outline the spots with pencil, and indicate the colors. Do this quickly since the colors will change and may fade on exposure to air.

Analysis of the Results. In the crude extract, you should be able to see the following components (in order of decreasing R_f values)

Carotenes (1 spot, yellow-orange)
Pheophytin a (gray, may not be nearly as intense as chlorophyll b)
Pheophytin 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 spinach 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. It is very common to observe other pigments in samples of frozen spinach. It is also common to observe components in the green band that were not present in the extract.

Identify as many of the spots in your sample as possible. Determine which pigments were present in the yellow band and which were present in the green band. Draw a picture of the TLC plate in your notebook. Label each spot with its color and its identity if possible. Calculate the R_f values for each spot produced by chromatography of the extract. Staple the TLC plate to your lab report when you turn it in.