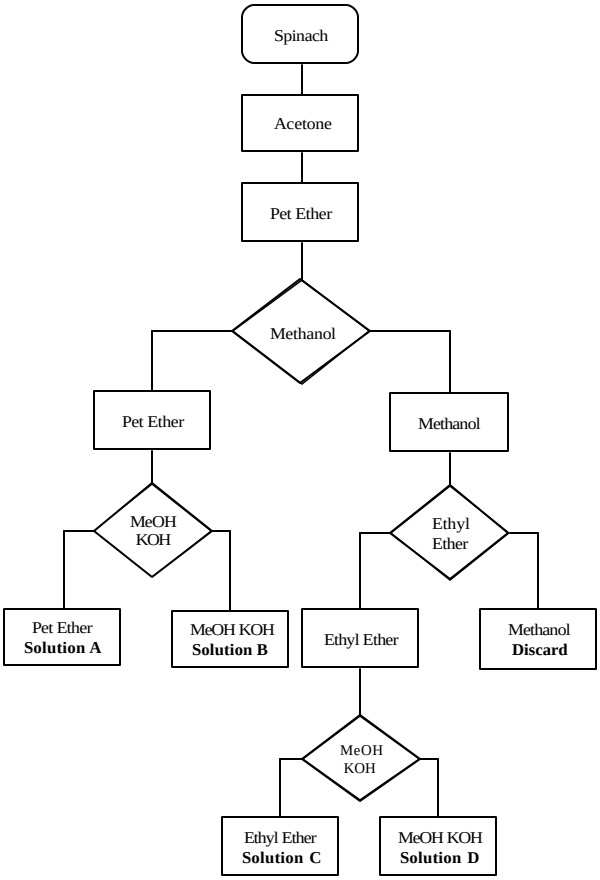
Experiment 10
(Lab Period 11)

Separation of Photosynthetic Pigments

The chloroplasts of spinach, as of most plants, look green because the major photosynthetic pigments, the chlorophylls, look green. However, there are many other pigments present in the chloroplast, primarily the xanthophylls and the carotenoids, which are masked by the chlorophylls. Non-green pigments must be separated from the chlorophylls to be detectable.

In this experiment, a solvent-partitioning technique will be used to attempt to separate four main pigment types in spinach chloroplasts: chlorophyll a, chlorophyll b, carotenoids (like ?-carotene) and xanthophylls (such as lutein). The procedure, outlined below, is designed to yield four solutions, each containing one of these pigment types due to differences with respect to solubility in organic solvents and susceptibility to alkaline hydrolysis. By measuring the absorption spectrum of each of the final four solutions, you will also determine whether the pigments can be distinguished by their abilities to absorb different wavelengths of visible light. The absorbance of each of the solutions will also provide a rough estimate of the relative abundance of the pigments in the extracts.

The following procedure can be diagrammed as:



The following steps will be performed in the separation:

Acetone should extract lipids.

Petroleum ether should remove lipids from the acetone, which mixes with water.

Methanol, with a small proportion of water, should remove slightly more hydrophilic lipids from relatively hydrophobic lipids, which remain in the petroleum ether layer.

Additional **water** should drive the slightly hydrophilic lipids into the **ethyl ether** from the methanol, which (like acetone) mixes with water.

Methanolic KOH should hydrolyze the lipid (phytyl) side-chains of chlorophylls, freeing the water-soluble chlorophyllides. This step should leave any lipid pigments (e.g., carotenes and xanthophylls) in the ether layers, and the light-absorbing portion of the chlorophyll molecules (the chlorophyllides) should move into the lower, aqueous layers.

After studying the procedure and looking at the structures of the different pigments, try to determine which pigment(s) should be present in each of the final four solutions.

Procedure

NOTE: No flames are to be turned on during this laboratory period. These solvents are volatile and highly flammable. Proceed through step 10.b. IN THE FUME HOOD.

Begin the work as a group of four students; use one of your group's chloroplast preparations from last week.

1. Thaw the sedimented chloroplasts and prepare six large screw-capped tubes by labeling them # 1 through # 6. (Label each tube twice; the solvents may smear the label.)

2. Using the graduations on the centrifuge tube, resuspend the thawed chloroplasts by adding 2 ml of 80% acetone to the sediment, mixing well by moving the chloroplasts up and down in a Pasteur pipet. Then add 6 ml of 80% acetone to a total volume of 8 ml and mix well. When color appears evenly distributed in the liquid, even as a sediment begins to form, centrifuge the mixture at 1,000 x g for 5 min. Aspirate the supernatant, transferring 1 ml to a clean 13-mm tube, and the remainder to tube # 1. Close the small tube tightly with Parafilm. Note and record the color of the sediment, then discard it.

3. Measure 12 ml of petroleum ether in a graduated cylinder and add it to tube # 1. Mix well using the Vortex machine. Slowly add 14 ml distilled water, pouring the water down the wall of the tube. Tighten the screw cap and mix gently, then loosen the cap to vent the gas that will be released. Mix again, venting again, and repeat mixing and venting two or three more times. Let the tube stand to allow the layers to separate sufficiently to see whether the color has moved into the upper layer. Mix again as necessary until transfer of color into the upper layer appears as complete as possible.

4. Using a clean Pasteur pipet, aspirate the upper layer to tube # 2. (You are now finished with tube # 1. Place it in the discard rack in the fume hood.) Wash the upper layer by adding 10 ml of distilled water, mixing thoroughly, venting occasionally. Allow the layers to separate then use the same Pasteur pipet to aspirate and discard the lower layer.

5. To the upper layer remaining in tube # 2, add 10 ml of distilled water, mixing to repeat the washing. Allow the layers to separate then use the same Pasteur pipet to aspirate and discard the lower layer.

6. To the washed upper layer remaining in tube # 2, add 10 ml of 92% methanol. Mix well. Let stand until the layers separate, then use a clean Pasteur pipet to aspirate the upper (pet. ether) layer to tube # 3.

You now have two solutions: 1) a methanolic layer in tube # 2, and 2) a pet. ether layer in tube # 3. Two students will proceed with the methanol layer in steps 7 and 8, and two students will proceed with the pet. ether layer in step 9. At this time, turn on the Spectronic 20.

7. **To the methanolic layer in tube # 2:**

a. Add 10 ml of ethyl ether and mix.
b. Add water 1 ml at a time. Add 1 ml, Vortex. Add a second 1 ml, Vortex.
c. Repeat 1-ml additions of water until the lower layer is colorless. (Note: separate layers become apparent only upon addition of the fourth or fifth ml of water. Just keep going, adding 1 ml at a time.)
d. When the lower layer is colorless, aspirate the upper layer to tube # 4 and proceed to step 8. (You are now finished with tube # 2. Place it in the discard rack in the fume hood.)

8. **To the ethyl ether layer in tube # 4:**

a. Add 3 ml of 30% KOH in methanol, mix on the Vortex; add 6 ml water, and mix again. During the next 10 min., repeat the vigorous mixing four or five times, allowing the mixture to stand during each interval between mixing.
b. Aspirate the upper layer and transfer it to tube # 6. This is solution C, in ethyl ether. This solution should be yellow. If it is not, wash it with 10 ml distilled water.
c. If there is still some upper layer afloat on the lower layer, aspirate and discard it. The layer remaining in tube # 4 is solution D, in methanol (and KOH).

9. **To the pet. ether layer in tube # 3:**

a. Add 3 ml of 30 % KOH in methanol, mix on the Vortex; add 6 ml water, and mix again. During the next 10 min., repeat the vigorous mixing four or five times, allowing the mixture to stand during each interval between mixing.
b. Aspirate the upper layer and transfer it to tube # 5. This is solution A, in pet. ether.
This solution should be yellow. If it is not, wash it with 10 ml distilled water.
c. If there is still some upper layer afloat on the lower layer, aspirate and discard it. The layer remaining in tube # 3 is solution B, in methanol (and KOH).

10. Your group now has four pigment extracts (solutions A, B, C and D), one for each student to use for spectral analysis:

Solution A in tube # 5, the pet. ether upper layer.
Solution B in tube # 3, the pet. ether lower layer, now in methanol.
Solution C in tube # 6, the ethyl ether upper layer.
Solution D in tube # 4, the ethyl ether lower layer, now in methanol.

The closed tubes may now be removed from the fume hood.

Dilute the extracts according to the following table. To dilute any extract, the solvent already present in the extract must be used, but because all the solvents are colorless, a single reference cuvette containing distilled water can be used for all the spectra. To dilute an extract, first measure the required volume of

solvent into the cuvette, then add the measured volume of extract and mix well. Close the cuvette tightly with Parafilm to prevent evaporation of the solvent.

Extract	Extract volume	Solvent volume
Solution A	2.5 ml	2.5 ml (pet. ether)
Solution B	0.5 ml	4.5 ml (methanol)
Solution C	1.0 ml	4.0 ml (ethyl ether)
Solution D	5 ml	none; use undiluted

11. Determine the absorption spectrum of each extract or dilution at 10-nm intervals from just below 400 nm, up to 700 nm, recording the Absorbance readings in your notebook. If the Absorbance of your pigment solution is greater than 1.2, add 3 ml of the appropriate solvent to your sample, mix it well, cover it again, and determine the spectrum of this more dilute sample. (Don't forget to account for any extra dilutions in your calculations.)

12. When you have completed determination of the spectra of your solutions, return your samples and reference cuvette to the fume hood, where a rack will be available for discards.

Calculations

Your group will have obtained five sets of data, and each student should have a record the spectra of all five extracts.

C1. Plot each set of absorbance readings (on the Y-axis) as a function of wavelength (on the X-axis) on two pages of graph paper--one page for Solutions A and B (the pet. ether fractions), and one for Solutions C and D (the ethyl ether fractions). Plot the absorption spectrum of the chloroplast extract from experiment 9 on each graph.

C2. Normalize the Absorbance reading for the major peak of each spectrum to an Absorbance value calculated for the undiluted solution. (To normalize them, divide each one by the largest value. The resulting values will all range from 0 to 1.) Then, assuming that the E value (see Laboratory 1) is the same for the absorption maximum of each pigment, rank the four types of pigments in order of relative abundance in spinach chloroplasts.

C3. Use the following information to infer the identify of the principal pigment in each of your solutions:
   a. Absorption peaks for the chlorophyllides in methanol are at 4l0 and at 640-660 nm.
   b. Absorption peaks for carotenoids and xanthophylls in ethers are broad, from 420-460 nm.
   c. The more abundant chlorophyll (a) is more hydrophobic than the minor chlorophyll (b).
   d. Carotenoids are more hydrophobic than xanthophylls.

Lab Report

You are required to write a lab report on experiments 9 and 10 together. Following the standard format report your conclusions concerning which extract contains which pigment and the relative amount of each.