LAB: SEPARATION OF PLANT PIGMENTS USING PAPER CHROMATOGRAPHY

INTRODUCTION

In this activity, you will separate the different photosynthetic pigments found in a spinach leaf using the technique of paper chromatography. A mixture of plant pigments is placed near the bottom of a strip of chromatography paper. The bottom edge of the paper is then placed into a nonaqueous (i.e. nonpolar) mixture of acetone and petroleum ether. As the solvents migrate up the paper by capillary action, those pigments in the mixture that are nonpolar will dissolve in the solvents and travel upwards. By contrast, those pigments in the mixture that are polar have a high affinity for the polar chromatography paper and thus do not travel far. At the end of a measured time interval, the various pigments in the original mixture have come to rest at different places along the chromatography paper. The separation might look something like this.

Pigment molecules are attracted to the solvent and/or paper by intermolecular forces (hydrophobic attractions, dipole-dipole interactions, and hydrogen bonds) which vary in strength. Which type of intermolecular force is formed depends on the types and locations of the polar and nonpolar functional groups on the pigment molecule. The collective effect of these functional groups will determine whether the pigment molecule is strongly polar, mildly polar, mildly nonpolar, or strongly nonpolar. This in turn influences whether the pigment is more likely to bind, in this lab, to the polar paper or to the nonpolar solvent, and whether this binding is relatively weak or strong. In addition, the size and shape of the pigment molecule will affect how many of these intermolecular forces are bonding the pigment to the paper/solvent at any given moment. Pigment molecules whose size and shape create a larger surface area of contact with the paper/solvent tend to bind more strongly because they can make a greater number of intermolecular attractions. Size and shape may also be important because traveling pigment molecules must slip between the narrow spacing of the closely compacted cellulose fibers of the paper as they migrate upwards. Imagine two people, one fat and the other skinny, both trying to move quickly and efficiently through a crowded airport terminal in order to get the last seat on a flight bound for Hawaii.

The pigments that are commonly extracted from spinach include chlorophyll a (blue-green in color), chlorophyll b (yellow-green), and a collection of carotenoids including beta carotene (bright yellow), and one or more xanthophylls (bright yellow). Structural formulae for these pigments are shown below.

The Chlorophylls are large molecules with a central atom of magnesium held in a polar porphyrin ring. Attached to the ring is a long, hydrophobic carbon-hydrogen chain that may help to anchor the molecule in the thylakoid membranes of the chloroplast. Chlorophyll b differs from chlorophyll a in that it has a polar aldehyde functional group attached to the porphyrin ring in place of a nonpolar methyl functional group (see diagram). This makes chlorophyll b a bit more polar, and so it tends to “stick” to the paper a bit more than chlorophyll a.

In addition to their differences in color and polar properties, the carotenoids are also much smaller molecules than the chlorophylls. A subtle difference between beta-carotene and xanthophylls is the presence, at each end of the molecule, of polar hydroxyl groups, instead of a nonpolar hydrogen atom. Again, this means that this molecule will “stick” very slightly more to the paper than beta-carotene.
Once the pigments have been separated from one another, a dimensionless value known as the Rf value is calculated. This is a ratio comparison of the distance traveled by the pigment compared to the distance traveled by the solvent front. The following example illustrates how Rf values are determined.

**Example Of Calculating Rf Values**

\[
R_f \text{ value molecule } A = \frac{2.0 \text{ cm}}{12.0 \text{ cm}} = 0.17
\]

\[
R_f \text{ value molecule } B = \frac{6.0 \text{ cm}}{12.0 \text{ cm}} = 0.50
\]

**PROCEDURE**

**Part 1: Separating the Pigments**

1. From a strip of chromatography paper provided by the instructor, cut one end of the paper strip so that a 1 cm point is formed. (See diagram above).

2. At a position 2.5 cm from the tip of pointed end, make a light pencil mark at the edge of the paper strip.

3. Gently lay a plant leaf on top of the paper strip, making sure the underside of the leaf is in contact with the paper.

4. Using the serrated edge of a coin (a dime is preferable but a quarter will work) or washer, carefully roll the coin back and forth on the upper side of the spinach leaf. This should transfer a solid green line of pigments onto the chromatography paper. It is important that your line be straight and dark.

5. Flip the chromatography paper over and repeat step 4...making sure that the second line of pigments you lay down is directly opposite the pigment line on the reverse side of the strip. Let the paper dry and repeat until a dark green line is visible.

6. Obtain a chromatography tube containing the chromatography solvent mixture from your instructor; place the tube in a test-tube rack and DO NOT move the tube again. Remove the cork lid and place the paper strip into the tube, point-side down. The green band of pigments you laid down on the chromatography paper must not touch the solvent mixture at the bottom of the tube. Quickly replace the foil lid to prevent vapors from escaping.

7. Remove the strip when the wet solvent front (line) moves to within 1 cm of the top of the paper.

8. Quickly mark and label the edge of this solvent front with a pencil. Gently air dry the strip by waving it back and forth for several seconds.

9. Mark and label the origin.

10. Using a pencil and an appropriate identifying color abbreviation, lightly circle each of these pigment bands. You should see 4-5 separate pigment bands, though one or two of these may be quite faint. Make sure you distinguish between yellow-green and blue-green pigments.

11. Carefully measure the distance the solvent moved as well as how far each pigment moved. **Measure to the front of the pigment band.** Take a picture of your chromatogram and print a copy on a color printer. This will be the Data for your report.

12. Make a Results table that shows the distances you measured and the Rf you calculated for each pigment.

**Part 2: Eluting (extracting) Pigments to Separate them from Chromatography Paper**

13. Once you have measured and recorded the distance traveled by all four individual pigments, cut the chromatography paper into four pieces so that there is only one pigment type on each fragment of paper (you should have 4 or 5 fragments). Be careful to keep track of which pigment is which. Trim away any excess paper lacking pigment.

14. Place each colored fragment in the appropriately labeled beaker, along with those from the rest of the class. These will sit overnight so that the pigment molecules can diffuse out of the paper into the isopropanol solution.
Part 3: Conducting a Spectral Analysis of Pigments

15. Start the computer and open the APAbsorptionSpectrum Template with Logger Pro. Pour a sample of pure isopropanol into a clean spectrophotometer cuvette (the little rectangular test tube). This cuvette is the “blank” that will be used to calibrate the spectrophotometer. Place the blank in the receptacle in of the spectrophotometer (only touch the ribbed part sides of the cuvette, which should NOT face the light source when inserting the cuvette.)

16. Go to the Experiment toolbar and open Calibrate ….then follow directions indicated on the computer. This will blank the spectrophotometer, meaning that it will set the spectrophotometer to cancel out the absorbance of the isopropanol and plastic molecules in the blank; thus the spectrophotometer will only measure the absorbance of the pigment molecules when a pigment sample is inserted into the spectrophotometer. At the end of the blanking the transmittance should be 100%.

17. You will be provided with samples of each of the individual pigment solutions eluted from the class chromatograms. Put the tube, ribs facing you, into the receptacle and push the green COLLECT button in the top menu. Push STOP once the graph is complete. Remove the sample and place your next pigment into the receptacle. After you press COLLECT , select STORE the previous run from the menu and collect the new pigment run. Logger-pro will continue to show all curves, but you will correct for that later.

18. Your last sample will be a vial of plant pigments that has been directly extracted from the plant. Determine the absorbance spectrum of this complete extract, which will be superimposed with the absorption spectra of the individual pigments you measured. Use Snip-it to make a copy of the graph and send it to yourself.

19. To make graphs of individual pigments, select GRAPH OPTIONS from the OPTIONS menu, then open AXIS OPTIONS and look for the Y-Axis column. In it you will find a listing for each run. Presently they are all selected, so unselect all of them except for run 1, then push DONE. Snip-it the resulting graph and send yourself a copy. Repeat this for each of your pigments, sending yourself a copy of each. Print these on a color printer and include in your lab report. When you are finished, save your Logger-Pro file to a flash drive and keep as a backup.

ANALYSIS

1. In the RESULTS section of your lab, securely attach your chromatogram photograph to a clean sheet of paper. Measure and record the exact distances traveled from the origin by the solvent front and each of the various pigments and the calculated Rf in a results table as indicated in step 12. Next to each pigment band on the chromatography strip, write the identity of the pigment and its corresponding Rf value.

2. Why were some bands of pigment more difficult to see than others?

3. In this exercise, you separated four pigments. For each of the following pigment pairings, identify which pigment(s) moved the furthest and then describe the chemical basis for this difference:
   a. chlorophylls from carotenoids.
   b. beta carotene from r xanthophyll(s)
   c. chlorophyll a from chlorophyll b.

4. In general terms, how does a calculated Rf value correspond to the position of the pigment on the paper? For any paper chromatography separation, what is the largest Rf value that could be possible? The lowest Rf value possible?

5. Your pigment separation demonstrated the presence of several accessory pigments in the plant (i.e. pigments other than chlorophyll a). Collectively, what is their likely role in the plant?

6. On your printed pigment absorption spectra curves mark the range(s) of wavelengths that each pigment absorbed and did not absorb, and the peak(s) wavelengths of absorption. Enter these into a table that should also include the perceived color of the pigment.

7. Describe the difference between an absorption spectrum and an action spectrum. Which of these did you determine?

8. Given the information on your graphs, does any single pigment account for all the light absorbed at a particular color (wavelength)? With which pigment(s) does the action spectrum’s peak resemble most?