EXERCISE 7 - Lab Procedures

I. Separate photosynthetic pigments by thin layer chromatography (TLC)

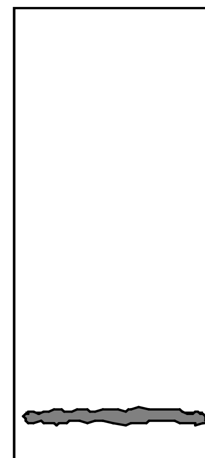
1. An extract of lipids from spinach leaves can be found at your workstation. This dark green liquid contains a mixture of several pigments that spinach plants use to collect light energy for photosynthesis. During this exercise you will use TLC to separate this mixture of pigments into its components.

2. Before you begin the experiment, you will practice applying the extract to a **sample** chromatography plate in order to develop your technique for making thin applications of extract. The chromatography plate consists of a thin layer of silica gel bonded to a plastic sheet. These sheets should only be handled by the edges; never touch the silica gel with your fingers.

   Obtain some **practice** extract and a small **sample piece** of the chromatography plate. Dip a capillary tube into the sample extract, and then make a thin line of extract along the chromatography plate. Do this by touching the tip of the tube to the surface of the silica gel *briefly*–just long enough to make a very small dot of pigment on the plate. Then make another small dot next to it and then another, and so on.

   Note: rather than making individual dots, you can also try dragging the capillary tip very lightly on the surface of the silica gel to make a thin, smooth line of pigment. Use whatever technique works best for you. The goal is to produce a line of pigment that is as thin as possible. **However, make sure you do not scratch the silica gel off the plate with the tip of the tube!** Once you have developed a "feel" for how to apply the extract so as to make a thin, straight line of pigment, you are ready to streak the actual plate.

3. Obtain the chromatography plate for the experiment from your instructor. Make a thin line of pigment, approximately 1.5 cm above the bottom of the plate. This is called "streaking the plate." The streak should remain about 2 mm away from either side edge of the plate. (See the diagram on the right.)



4. When you have made one thin line of pigment extract, allow the streak to dry thoroughly, then apply another line on top of the first–making your line darker and more concentrated. Repeat this process until you have transfered ALL 0.3 mL of the extract, in several layers, onto the plate.

5. Allow the solvent in the extract to evaporate completely after you are finished streaking the plate. This may take several minutes. While you are waiting, examine the glass jar, called a chromatography tank, located in the fume hood.

   ⇒ **CAUTION:** The developing solution inside the chromatography tank is highly volatile and will rapidly evaporate when the jar is open. Volatile organic solvents tend to be unhealthful, and breathing these vapors should be avoided. For this reason, containers with developing solution should ALWAYS remain inside the fume hood and should never be kept open any longer than absolutely necessary.

   The solution in the chromatography tank should rise to a height of about 1 cm above the bottom of the jar. If it does not, add enough of the "chromatography developing solution" so that it rises to the required height. The developing solution consists of a mixture of organic solvents. Your developing solution may be 58% petroleum ether, 30% ethyl acetate, and 12% diethylamine, or it may be a 7:3 mixture of petroleum ether and acetone. With the cover firmly screwed on, swirl the jar so that the developing solution completely saturates the air inside the chamber.

6. When your streak is completely dry, carefully place the chromatography plate vertically into the developing tank with the streak near the bottom of the jar. Make sure the streak itself does not dip into the solution. If it looks like it might, pour some of the developing solution out of the jar and into the container labeled “chromatography developing solution” to prevent this from happening. The lid should remain on the jar, except for the second or so needed to place the plate inside.

7. As the developing solvent moves up the plate, it will pull the pigments along with it. Pigments that are more strongly attracted to the developing solvent (the **mobile phase**) will move up the plate faster than those more strongly attracted to the silica gel (the **immobile or stationary phase**). The leading edge of the solvent is called the **solvent front** and the original site of the streak is called the **origin**. Allow the chromatogram to develop until the solvent front is about 1 cm below the top edge of the sheet, then remove the plate from the developing tank.

8. Place your plate on a paper towel in the fume hood. Quickly (while you can still see the level of the solvent) mark the level of the solvent front by placing a ruler across the top of the plate at the level of the solvent front and scratching a straight line through the silica gel. This should create a thin line that clearly marks how far the solvent traveled from the origin.

9. Leave the developed plate in the fume hood until all of the solvent evaporates. This may take 5 minutes or longer. Then make a diagram of the chromatogram, showing the location and color of all the pigment bands.

10. Measure and record the final height of the solvent front above the origin (the distance from the middle of the origin to the scored line at the top of the plate.) Also measure and record the final height of each pigment band above the origin. Heights of the bands should be measured from the middle of the origin to the middle of each band.

II. Remove your pigments from the silica gel and suspend each in ethanol

1. With label tape, label three centrifuge tubes with the colors of the three major bands found on your chromatography plate (the 2 green bands and the orange-yellow band nearest the solvent front.) Ask your instructor if you are not sure which are the major bands.

2. With a spatula, carefully scratch off the silica gel containing the orange-yellow band nearest the solvent front. Be careful to scratch off ONLY this band. Transfer the loosened silica gel containing the pigment onto a piece of waxed paper, and then pour it into the correspondingly labeled centrifuge tube.

3. With a 5 mL pipette, transfer 3.0 mL of ethanol into the centrifuge tube containing the silica gel. Mix the contents with a glass rod to dissolve the pigment in the ethanol.

4. Remove each of the two major green bands from the chromatogram and place them in the other labeled centrifuge tubes using the same procedure you followed for the orange-yellow band. Add 3.0 mL of ethanol to each of these tubes and mix as was done with the orange-yellow band.

5. Place a 150 mL beaker on each of two separate electronic balances and tare the balances. Place one of the conical centrifuge tubes in each beaker and balance the two tubes by adding ethanol to the lighter tube. Place the balanced tubes directly opposite each other in the rotor of a clinical centrifuge, and then tare the balances again with the empty beakers on them.

6. Add 3.0 mL dH_2O to a clean centrifuge tube and place the tube in one of the two beakers. Place the third tube of pigment in the other beaker and balance the two tubes by adding ethanol to the pigment tube or more dH_2O to the other tube. Place the balanced tubes directly opposite each other in the clinical centrifuge.

7. Centrifuge for 3 minutes. Once the centrifuge has come to a complete stop, remove the tubes and pour each supernatant into a separate, labeled test tube without disturbing the pellet.

8. You should now have 3 test tubes of pigment, each containing approximately 3 mL of solution. Discard the pellets of silica gel as directed by your instructor.

III. Plot an absorption spectrum for each pigment using the scanning spectrophotometer

1. Pour each of the 3 tubes of pigment into a separate plastic cuvette. (NOTE: when handling the cuvettes, touch the frosted or ridged sides only, not the smooth sides!) Prepare a reagent blank by filling a fourth cuvette with ethanol.

2. Take the 4 cuvettes to the **Genesys 2 Scanning Spectrophotometer**. Familiarize yourself with the parts and operation of the spectrophotometer before you begin. Check with your instructor to see if any supplemental instructions are available.

3. From the Main Menu, select “4” (Advanced Scanning) by pressing “4” on the keypad.

4. Press “Setup Tests” on the keypad.

5. Select “1”, Test Name

6. Enter a test name of your group’s choosing, and press “Exit” on the keypad.

7. Select “4”, Start Wavelength, and set to 400nm. Press “Enter”.

8. Select “5”, Stop Wavelength, and set to 720 nm. Press “Enter”.

9. Select “6”, Scan Speed, and set to Medium (1pt/1.0nm) by repeatedly pressing “6” on the keypad until display reads “Medium”.

10. Select “7”, Measurement Mode, and set to ABS (absorbance) by repeatedly pressing “7” on the keypad until display reads “ABS”.

11. Select “8”, Number of Samples. Set to desired number of samples (NOT counting the blank) by entering the number “3” on the keypad. Press “Enter”.

12. Select “Next Page”. This is done by pressing the yellow arrow on the keypad under the “Next Page” icon on the display.

13. Select “1”, Overlay Scans, by pressing, “1” on the keypad until display reads “ON”.

14. Press “Exit”

15. Open the sample compartment on the spectrophotometer, and examine the sample holder. It has 7 cells (cell # 1 is closest to the front of the spectrophotometer and cell # 7 is closest to the back). The cuvettes are placed in the cells with the smooth sides facing left and right, and the ridged or frosted sides facing front and back. Wipe off the smooth sides of each cuvette with a Kimwipe, and place them in the sample holder as follows:

    Place the reagent blank (ethanol) in cell # 1
    Place the orange-yellow pigment in cell # 2
    Place the first green pigment band in cell # 3
    Place the second green pigment band in cell # 4

16. Select “Collect Baseline” by pressing the yellow arrow on the keypad under the “Collect Baseline” icon on the display. Wait until machine is finished scanning.

17. Select “Scan” by pressing the yellow arrow on the keypad under the “Scan” icon.

18. Machine will scan all of the samples, and a graphic representation will be made for each sample. The scale of the graph will automatically be corrected at the end of all scans.

19. Print out the graph of the scans by pressing the "Printed Icon" on the bottom left of the keypad. If a "Printer Error" message is displayed, select "Retry" by pressing the yellow arrow on the keypad below the "Retry" icon on the display.

20. Ask your instructor for help if you have any difficulties. When you are finished, remove the graph from the printer and label the three curves that were plotted so you know which one corresponds to each pigment band.

21. Remove the cuvettes from the machine, and press "Exit" to return to the Main Menu.

Clean up

Remove all labels and clean your glassware.

All disposable glassware goes into the special glass disposal receptacle.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Ask your instructor to check your work area before you leave.