LAB 07 – Chromatography Techniques

Objectives:

- Explain how a chromatograph of pigments is formed from both paper and thin layer chromatography.
- Isolate and identify some of the various pigments in spinach.
- Calculate R_f values of pigments and compare them to "Book Value".

Introduction:

Chromatography is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the substance to be measured from other molecules in the mixture and allows it to be isolated.

Chlorophyll *a* is the primary photosynthetic pigment in all plants. A molecule of chlorophyll *a* is located at the reaction center of photosystems. Other chlorophyll *a* molecules, chlorophyll *b*, and the carotenoids (such as carotenes and xanthophylls) capture light energy and transfer it to the chlorophyll *a* at the reaction center. Carotenoids also protect the photosynthetic system from the damaging effects of ultraviolet light. You can measure the relationship of the distance moved by a pigment to the distance moved by the solvent. This constant is called **R_f** and can be calculated for each separate pigment using the following formula.

$$R_f = \frac{\text{distance pigment migrated (mm)}}{\text{distance solvent front migrated (mm)}}$$

Paper Chromatography

Paper chromatography is a useful technique for separating and identifying pigments and other molecules from cell extracts that contain a complex mixture of molecules. The solvent moves up the paper by capillary action, which occurs as a result of the attraction of solvent molecules to the paper and the attraction of solvent molecules to one another. As the solvent moves up the paper, it carries along any substances dissolved in it. The pigments are carried along the different rates because they are not equally soluble in the solvent and because they are attracted, to different degrees, to the fibers in the paper through the formation of intermolecular bonds, such as hydrogen bonds.

Beta carotene, the most abundant carotene in plants, is carried along near the solvent front because it is very soluble in the solvent being used and because it forms no hydrogen bonds with cellulose. Another pigment, xanthophylls, differs from carotene in that it contains oxygen. Xanthophyll is found further from the solvent front because it is less soluble in the solvent and has been slowed down by hydrogen bonding to the cellulose. Chlorophylls contain oxygen and nitrogen and are bound more tightly to the paper than are the other pigments.

Thin Layer Chromatography

Thin layer chromatography (TLC) is a widely used chromatography technique used to separate chemical compounds. It involves a stationary phase consisting of a thin layer of adsorbent material, in this case, silica gel (shown under magnification in **Figure 1**), immobilized onto a flat, inert carrier sheet. A liquid phase consisting of the solution to be separated dissolved in an appropriate solvent is drawn through the plate via capillary action, separating the experimental solution. It can be used to determine the pigments a plant contains, to detect pesticides or

insecticides in food, in forensics to analyze the dye composition of fibers, or to identify compounds present in a given substance, among other uses.

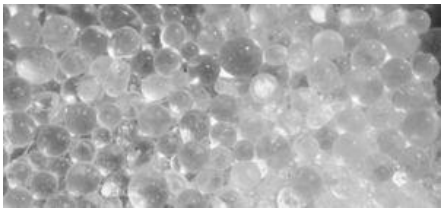

Figure 1

The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products.

The solvent will move up the plate by capillary action, which occurs as a result of the attraction of solvent molecules to the silica gel and the attraction of solvent molecules to one another. As the solvent moves up the plate, it carries along any substances dissolved in it, in this case pigments. The pigments are carried along at different rates because they are not equally soluble in the solvent and because they are attracted to different degrees, to the silica gel.

The order of pigments top to bottom on the silica gel slide following chromatography is different than in paper chromatography due to both different media and solvents used. The colors are the same – carotenes (yellow to yellow-orange to brown), chlorophyll *a* (bright green to blue-green), chlorophyll *b* (yellow-green to olive green), and xanthophylls (yellow).

Procedure:

Before the lab begins, you need to 'story board' both parts of the procedure on the final page of the lab. Once we start, you will not have time to be unsure what you are doing!

PART I—Paper Chromatography

The following suggested procedure is illustrated in **Figure 2**, shown below:

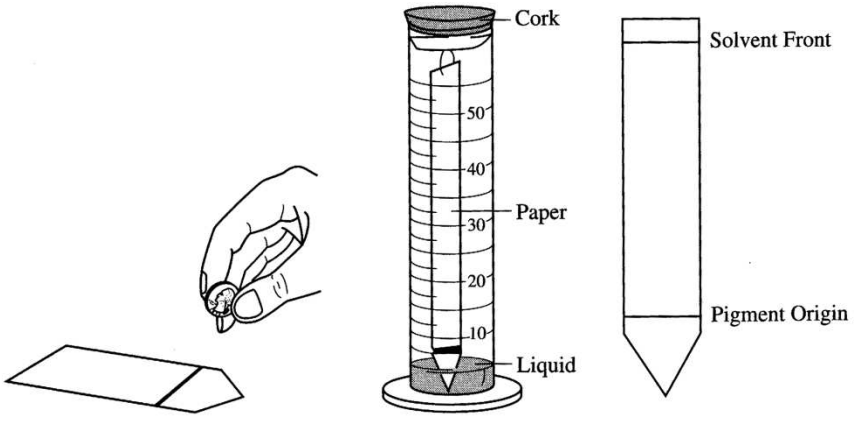

Figure 2

1. Obtain a 250 mL Erlenmeyer flask (or other suitable container) and pour about 1 cm solvent that is 9 parts petroleum ether: 1 part acetone.
2. Cut a piece of filter paper that will be long enough to reach the solvent. Cut one end of this filter paper into a point. Draw a pencil line 1.5 cm above the point.
3. Use a coin to extract the pigments from spinach leaf cells. Place a small section of leaf of the top of the pencil line. Use the ribbed edge of the coin to crush the cells. Be sure that the pigment line is on top of the pencil line. You should repeat this procedure 8 to 10 times, being sure to use a new portion of the leaf each time.
4. Place the chromatography paper in the cylinder so that the pointed end is barely immersed in the solvent. *Do not allow the pigment to be in the solvent*.
5. Stopper the cylinder. When the solvent is about 1 cm from the top of the paper, remove the paper and *immediately* mark the location of the solvent front before it evaporates.
6. Mark the bottom of each pigment band. Measure the distance each pigment migrated from the bottom of the pigment origin to the bottom of the separated pigment band. In **Table 1**, record the distance that each front, including the solvent front, moved. Depending on the species of plant used, you may be able to observe 4 or 5 pigment bands.
7. Calculate the R_f values for the above pigments in **Table 2**.

PART II—Thin Layer Chromatography

1. Homogenize 50 g fresh spinach with 50 mL 100% acetone using a mortar and pestle.
2. Filter to remove debris using layers of folded over cheesecloth.
3. Readjust volume of filtrate to 40 mL (if necessary).
4. Pour filtrate into a separatory funnel.
5. Carefully, pour 60 mL petroleum ether down the sides of the separatory funnel. Gently rotate the separatory funnel. You should now have two layers, petroleum ether (with the dissolved pigments) on top, and the acetone on the bottom.
6. Drain off the lower layer of acetone into a designated waste beaker. Stop the separation at about the layer line.
7. Add 50 mL of distilled water to the pigment mixture (in the remaining petroleum ether) by carefully pouring the water down the sides of the funnel.
8. Rotate slowly until the upper layer contains nearly all of the chlorophyll. Gas pressure will rise in the funnel—vent carefully.
9. After the layers separate, drain off the lower layer of water (with traces of acetone) and discard into the waste beaker. The upper layer of petroleum ether should contain all of the pigments.
10. Add 50 mL distilled water to wash the petroleum either and remove any traces of acetone. Repeat this wash a second time. During the final wash, when removing the acetone, also remove a few milliliters **above** the layer line. We do not want an "oil and water" layer in our extract.
11. Remove 5 mL of the petroleum ether-chlorophyll layer and put it in a small test tube. Allow to evaporate down to get a very concentrated extract. This is the chlorophyll extract to be used for silica gel chromatography.
12. Pour about 1 cm of solvent (9:3:3—petroleum ether: acetone: chloroform) in a 100 mL beaker. Cover the 100 mL beaker with a 400 mL beaker to create a chromatography chamber. Let stand for a few minutes to saturate the plate with the solvent.

13. Using a capillary tube, place about 25 drops of the extract in the center of the silica gel plate about 2 cm from the bottom. *We do not want this pigment mixture to come in direct contact with the solvent mixture!*
14. Slowly place the silica gel plate in the solvent and immediately cover the 100 mL beaker with a 400 mL beaker to create a chromatography chamber.
15. After about 10- minutes, remove the silica gel plate, take a picture of the plate with the digital camera (don't forget your scale!), and measure distances of solvent and pigment from the initial placement point to determine the R_f values for each pigment. Complete **Data Tables 3** and **4**. You might see more bands than 4 representing the other forms of those pigments. (If you need more than the 9 rows given, add an extra row to Data Table 3.) The colors of the main pigments are the same as in paper chromatography, however, as you will find out, the R_f values should be different.

Results:

Part I—Paper Chromatography

Table 1 – Distance Moved by Pigment Band (mm)

Band Number	Band Color	Distance of Pigment (mm)	Distance of Solvent (mm)
1			
2			
3			
4			

Table 2 – R_f Values of Pigments

R_f	Pigment
	carotene
	xanthophylls
	chlorophyll *a*
	chrolophyll *b*

Part II—Thin Layer Chromatography

Table 3 – Distance Moved by Pigment Band (mm)

Band Number	Band Color	Distance of Pigment (mm)	Distance of Solvent (mm)
1			
2			
3			
4			
5			
6			
7			
8			
9			

Table 4 – R_f Values of Pigments

R_f	Pigment (identified by Band Number)
	1
	2
	3
	4
	5
	6
	7
	8
	9

Questions:

1. What factors are involved in the separation of the pigments?

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2. Would you expect the **R_f** value of a pigment to be the same if a different solvent were used? Explain.

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Story Board for Paper Chromatography:

Story Board for Thin Layer Chromatography: