Plant Pigment Chromatography

- Students will isolate and identify photosynthetic pigments in spinach leaves.
- Students will calculate Rf values of photosynthetic pigments and graph the absorption spectrum for each pigment.

Introduction

As primary producers in the food chain with some bacteria and algae, plants produce their own food by using the sun's energy to transform carbon dioxide and water into glucose. In this process of photosynthesis, plants convert the sun's energy into chemical energy that is stored in the bonds of the glucose molecule. This energy fuels the metabolic processes of cells and is essential for life on earth. Glucose is a simple carbohydrate that provides immediate fuel to cells but it is also a building block for more complex carbohydrates stored by living organisms for future use.

For photosynthesis to transform light energy from the sun into chemical energy (bond energy) in plants, the pigment molecules absorb light to power the chemical reactions. Plant pigments are macromolecules produced by the plant, and these pigments absorb specified wavelengths of visible light to provide the energy required for photosynthesis. (Appendix A) Chlorophyll is necessary for photosynthesis, but accessory pigments collect and transfer energy to chlorophyll. Although pigments absorb light, the wavelengths of light that are not absorbed by the plant pigments are reflected back to the eye. The reflected wavelengths are the colors we see in observing the plant. (Example: green pigments reflect green light) Plants contain different pigments, and some of the pigments observed include:

- chlorophylls (greens)
- carotenoids (yellow, orange red)
- anthocyanins (red to blue, depending on pH)
- betalains (red or yellow)

The process of chromatography separates molecules because of the different solubilities of the molecules in a selected solvent. In paper chromatography, paper marked with an unknown, such as plant extract, is placed in a developing chamber with a specified solvent. The solvent carries the dissolved pigments as it moves up the paper. The pigments are carried at different rates because they are not equally soluble. A pigment that is the most soluble will travel the greatest distance and a pigment that is less soluble will move a shorter distance.

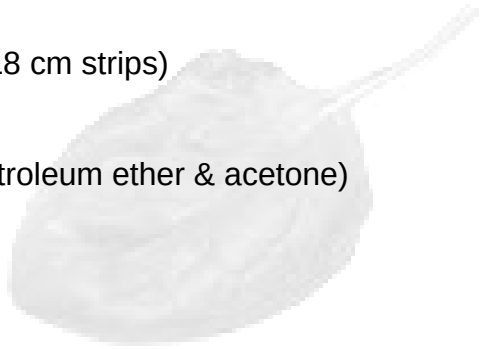
The distance the pigment travels is unique for that pigment in set conditions and is used to identify the pigment. The ratio is the Rf (retention factor) value. Standards are available for comparison. (Appendix B)

$$Rf = \frac{\text{distance pigment travels (cm)}}{\text{distance solvent travels (cm)}}$$

The bands derived in paper chromatography contain the pigments found in the plant. The bands can be cut apart, and placed in alcohol to elute the pigment in an extract. Each pigment can be tested to derive the wavelength absorption spectrum for that pigment. A spectrophotometer measures the absorption of light by an extract containing the pigment and provides information that is plotted in a graph to illustrate the absorption spectrum for the isolated pigment.

EQUIPMENT AND MATERIALS (per group)

2 or 3 fresh spinach leaves
Ruler
Large test tube
Cork with push pin
Chromatography paper (precut 18 cm strips)
Pencil
Copper penny coin
Chromatography solvent (9:1 petroleum ether & acetone)
6 ml syringe
Colored pencils
Calculator
Scissors
Plastic wrap
70 % Isopropyl alcohol
Plastic pipettes
5 test tubes (20-30 mL)
Test tube rack
Sharpie markers or tape (for labeling test tubes)
4- 6 spectrophotometer cuvettes
Test tube rack for cuvettes
Kimwipes
Genesys 20 Spectrophotometer



SAFETY
Wear goggles and aprons when working with chemicals.
Petroleum ether, acetone and alcohol are volatile and flammable.
Avoid breathing vapors of the reagents.

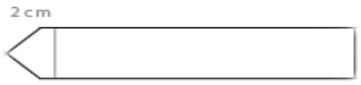
Day One
Work in teams of two for this activity. Make sure the work area is clean and dry.

Preparation of the Sample:

(Important! Oil from the skin affects the separation, so handle paper as little as possible and only by the edges.)

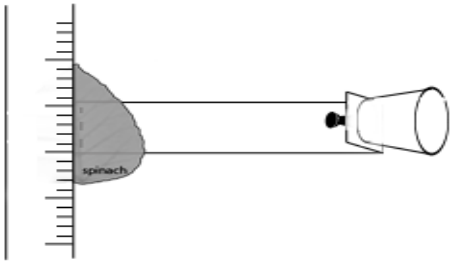
1. Take a strip of chromatography paper approximately 18 cm long. One end is blunt and the other is pointed.

2. With a pencil lightly make a line 2 cm from the pointed end of the paper.



3. Bend the strip of paper at the blunt end and attach it to the small end of the cork with the push pin. Adjust the length of the paper so that when it is inserted into the test tube, it will touch the bottom without curling.

4. Place a ruler over the leaf so that is covers the pencil line on either end.



5. Using a penny coin, press down firmly and roll along the ruler edge several times to form a definite green line.
6. Allow the green line to dry thoroughly.
7. Use a fresh area of the leaf and repeat several times until the pencil line is covered completely with a narrow green band. Be careful not to smear this green line.

Separation of Pigments:

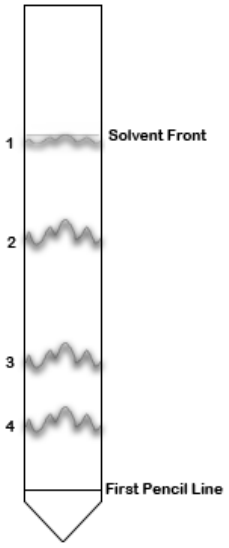
1. Place the test tube in the test tube rack. Using the 6mL syringe, dispense 5 mL of chromatography solvent in the test tube.
2. Carefully lower the paper strip into the test tube and secure the cork in the top. The solvent must touch the pointed end of the paper but should not touch the green line.
3. Be careful not to slosh the solvent. Allow the tube to stand undisturbed.
4. Observe the solvent movement and the band separation.
5. When the pigments have separated into distinct bands (the solvent has moved approximately half the distance of the paper), lift the cork with paper attached from the test tube. Mark the edge of the solvent front with a pencil. Remove the push pin and detach the paper from the cork.
6. Place the push pin back in the cork and place the cork back on the test tube to minimize fumes. Follow safe disposal instructions in Appendix C.
7. Allow the paper to dry completely.

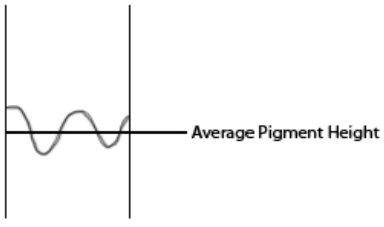
Extraction of Pigments:

1. On the **Student Data Sheet**, color the diagram to illustrate the color bands on the chromatogram. Label the band that traveled the greatest distance **1**, the next **2**, the next **3.** Continue until all bands are labeled.

2. Describe the color of each band in **Data Table 1**, column B.

3. Measure the distance from the first pencil line to the solvent front. Record this value in **Data Table 1** (column C) for each pigment.



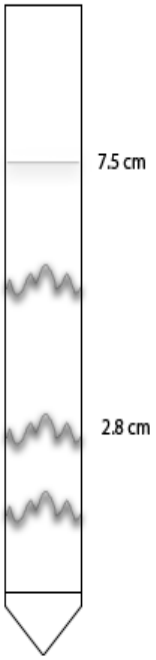


4. Now measure the distance from the first pencil line to the average peak of each color band.

5. Record these values in **Data Table 1** (column D). (Depending on the results, groups may have differing numbers of pigments.)

6. Cut the different colored bands apart carefully and trim off excess paper being careful to include all the pigment for each band.
7. Label each test tube, one for each pigment in **Data Table 1**.
8. Cut each band of color into pieces small enough to fit into a 20-30 mL test tube. Insert the paper pieces in the appropriate test tubes.
9. Add 5 ml of isopropyl alcohol to each test tube and seal with a small piece of plastic wrap. Allow samples to stand overnight until the color is completely eluted from the paper. These solutions will be used in the next activity.
10. Calculate the Rf values for each pigment and record the values in **Data Table 1** (column E) using the following formula

    $$Rf = \frac{\text{distance pigment travels}}{\text{distance the solvent front travels}}$$



Rf = 2.8 = .37
7.5

11. Use Appendix B to determine the name of each pigment and record the name in **Data Table 1** (column F).

Day Two

Upon entering the lab, turn on the Genesys 20 Spectrophotometer to allow warm-up time.

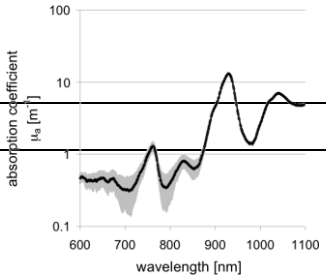
Preparation of samples for spectrophotometeric analysis

1. Using a clean plastic pipette, fill a cuvette about half full with isopropyl alcohol. Label it **bl**. This is the blank used to standardize the spectrophotometer.
2. Using another clean plastic pipette, transfer enough of the solution from the test tube containing Pigment 1 to a second cuvette until it is about half full. Label this cuvette **1.**
3. Using another clean plastic pipette, transfer enough of the solution from the test tube containing Pigment 2 to a third cuvette until it is about half full. Label this cuvette **2.**

4. Using another clean plastic pipette, transfer enough of the solution from the test tube containing Pigment 3 to a fourth cuvette until it is about half full. Label this cuvette **3.**
5. More pigments will require additional cuvettes.
6. Wipe the sides of the cuvette with a Kimwipe and handle by the top edge to avoid fingerprints. Be sure that the label does not interfere with the path of the light beam.

Measuring Absorbance of Pigments

1. Set the wavelength on the spectrophotometer to 360 nm.
2. Set the mode to **Absorbance** by pressing the **A/T/C** button until an **A** appears at the right of the digital display.
3. Insert the blank into the cell holder and close the door.
4. Press the **0 ABS/100%T** key to set the blank to **0 absorbance**.
5. Remove the blank.
6. Insert the clean cuvette **1**. Close the door and read the **Absorbance** shown. Record the results in **Data Table 2.** Remove the cuvette.
7. Repeat this procedure for all pigments in Data Table 1.
8. Once all readings are recorded for 360 nm, increase the wavelength to 380 nm.
9. Follow steps #3- #7 for the 380 nm wavelength.
10. Continue this same process to complete **Data Table 2**.



DATA ANALYSIS

Instructions for Excel 2007 Version

1. Enter the data in an Excel Spreadsheet
   - Column A: Wavelength
   - Column B, C, D,(and if necessary E and F) : Absorbance
2. Click on any cell in the data
3. Next, click on Insert tab. Then choose Scatter.
4. Choose the wavy line graph without data points. A graph will appear using your data.

To change increments on the X axis a. Make sure to click on the graph b. Under "chart tools" click on Layout→ Axes→ Primary Horizontal axis→ More Primary Horizontal Axis Options c. In order to change the values, click the "**Fixed** option. Choose Minimum to be 350; Maximum to be 750; Major unit to 50; then click **close**	**To change increments on the Y axis** a. Make sure you are clicked on the graph b. Under "chart tools" click on Layout→ Axes→ Primary Vertical axis→ More Primary Vertical Axis Options Choose Minimum to be 0; leave the other options as "Auto"; then click **close**
To label your X axis a. Go to Layout→ Axis Titles→Primary Horizontal Axis b. Choose "Title below Axis" and then type "Wavelength (nm)" in the text box	**To label your Y axis** a. Go to Layout→ Axis Titles→Primary Vertical axis b. Choose "rotated title" and then type "Absorbance" in the text box
To edit the legend a. Click on the graph; click on Design; click on select data; click on edit; then type in the series name and click OK; then click **OK** again	
To put a title on your graph a. Go to Layout→ Chart Title b. Choose "above Chart" and then type a name for your graph in the text box	

References

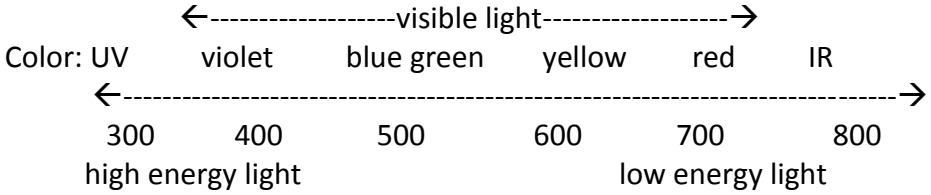
Reiss, Carol 1994. Experiments in Plant Physiology. Englewood Cliffs, NJ: Prentice Hall

http://voh.chem.ucla.edu/vohtar/spring03/classes/14CL/pdf/14clisol.pdf
pubs.acs.org/doi/abs/10.1021/jf00083a011

http://biology.wsc.ma.edu/Biol129Labs/sites/default/files/129PHOTOsp09.pdf

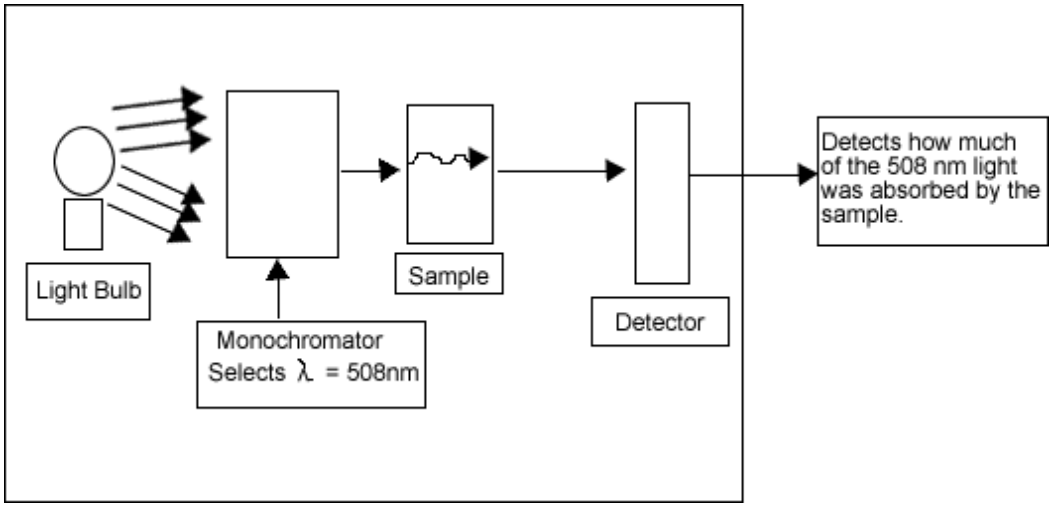
APPENDIX A

Visible light is the portion of the electromagnetic spectrum with wavelengths in the range of approximately 380 nm to 760 nm. If a beam of light shines on a colored solution, the colored component of the solution typically absorbs some of the wavelengths in the light beam and transmits other wavelengths.



A spectrophotometer is an instrument that can determine the wavelengths in the visible region transmitted or absorbed by the colored solution. The instrument also can be used to determine the degree or extent of absorption at any wavelength. The degree of absorption is called the absorbance of the solution at that wavelength. A plot of absorbance versus wavelength for a solution is the absorption spectrum for the colored substance in that solution. A wavelength, or continuous wavelength range, where a maximum in the absorbance value occurs in the absorption spectrum is called a peak.

A spectrophotometer has five basic parts: a light source, a device that separates the light into its constituent wavelengths, a compartment to hold the sample, a detector that analyzes the light which has passed through the sample, and a readout device to provide data to the instrument's user.



References
Spectrophotometry Educational Manual Thermo Electron Corporation, Madison, WI 5371
Spectrophotometer Image: Wellesley.edu Lab 04 Analysis of Ferrous Iron in a Vitamin Pill

APPENDIX B

Rf Values:

- β-carotene - 0.99
- chlorophyll a - 0.30
- chlorophyll b - 0.13
- violaxanthin - .40
- lutein - .68

carotene, violaxanthin, and lutein are carotenoids

APPENDIX C

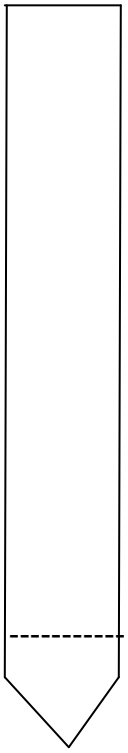
If the chromatography solvent has not become contaminated (as evidenced by it no longer being colorless), it can be reused each class period.

Set up a waste container in the fume hood for disposal of used chromatography solvent. To properly dispose of the solvent, dilute with three volumes of water and then pour the diluted solvent down the drain.

Student Sheet

Plant Pigment Chromatography

Name _____



1. Illustrate the results of the chromatogram on the diagram.
2. Why are two solvents used in the process?
3. Justify the separation of the bands.

4. Why is energy required for life?
5. How does energy enter the living world?
6. What composes visible white light?
7. How do you think the results would differ if you had used spinach leaves which had been stored in a dark room for five days before the experiment? Explain your answer?
8. The spinach leaf looks green, but your chromatogram demonstrated that other pigments are in the leaf as well. Why can you not readily see the other pigments in the leaf?
9. What is a benefit of the pigments in photosynthesis?

10. Would you expect a plant to grow well in only green light? Explain.

11. Why was isopropyl alcohol used to **ZERO** the spectrophotometer? (to get 100% T setting)

12. What is the significance of the R_f values?

13. Why is wavelength information of value in studying plants?

14. In which colors of light would you expect a plant to obtain maximum photosynthetic activity?

15. Why are plants generally green?

16. What does the absorption spectrum convey about each pigment? Be specific with details

Data Table 1

A	B	C	D	E	F
Pigment *	Description of Color	Distance Solvent Front Traveled	Distance Color Traveled	Rf value (column D /column C)	Name of Pigment (Use Appendix B)
1					
2					
3					
4					
5					

* Number of distinct pigments will vary per lab group. The chart accommodates a maximum of 5 pigments.

Data Table 2

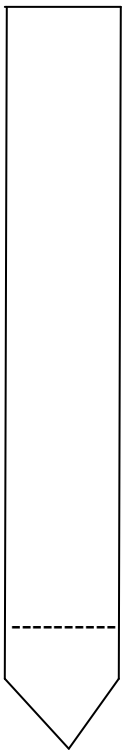
WAVELENGTH (nm)	ABSORBANCE*				
	1	2	3	4	5
360					
380					
400					
420					
440					
460					
480					
500					
520					
540					
560					
580					
600					
620					
640					
660					
680					
700					
720					

* Number of distinct pigments will vary per lab group. The chart accommodates a maximum of 5 pigments.

Teacher Answer Page

Plant Pigment Chromatography

Name _____



1. Illustrate the results of the chromatogram on the diagram. Student's drawings will vary

2. Why are two solvents used in the process? Different pigments will be soluble in one solvent but not another. Better separation of pigment bands will result if a combination of solvents is used.

3. Justify the separation of the bands. Bands that travel the greatest distance are more soluble in the solvent than those that traveled less distance.

4. Why is energy required for life? Energy fuels all the metabolic processes of cells.

5. How does light energy enter the living world? Photosynthetic organisms, or primary producers, take this energy and convert it to chemical energy to be utilized in the food web as a source of energy.

6. What composes visible white light? Visible light waves are the only electromagnetic waves humans can see. We see these waves as the colors of the rainbow. Each color has a different wavelength. Red has the longest wavelength and violet has the shortest wavelength. When all the waves are seen together, they make white light. When white light shines through a prism, the white light is broken apart into the colors of the visible light spectrum.

7. How do you think the results would differ if you had used spinach leaves which had been stored in a dark room for five days before the experiment? Explain your answer? When plants are deprived of light, chlorophyll gradually breaks down and cannot be replenished. If the spinach leaves used for this lab had been stored in a dark room, there would have been less chlorophyll present in the extract and much lower peaks in the absorption spectrum would be expected.

8. The spinach leaf looks green, but your chromatogram demonstrated that other pigments are in the leaf as well. Why can you not readily see the other pigments in the leaf? The other pigments are present in the leaf in smaller quantities. Their presence is covered up by the abundance of chlorophyll.

9. What is a benefit of the pigments in photosynthesis? Pigments absorb the light energy necessary for powering photosynthesis.

10. Would you expect a plant to grow well in only green light? Explain. No, plants do not absorb green light, but rather reflect it. If a plant is reflecting green light it is not using it to power photosynthetic reactions.

11. Why was isopropyl alcohol used to **ZERO** the spectrophotometer? (to get 100% T setting) The pigment bands on the chromatogram were eluted using isopropyl alcohol. The spectrophotometer needs to be calibrated to display the absorbance of the pigments, not the absorbance of the alcohol.

12. What is the significance of the R_f values? An Rf value that is close to 1 indicates that the pigment is very soluble in the solvent. An Rf value that is very small indicates that the pigment is not very soluble in the solvent.

13. Why is wavelength information of value in studying plants? Certain wavelengths of visible light are not absorbed by plants to power photosynthesis. Other wavelengths are readily absorbed. Commercial plant nurseries can take advantage of this knowledge to provide necessary wavelengths and boost growth and overall production.

14. In which colors of light would you expect a plant to obtain maximum photosynthetic activity and why? Blue and red because the graph shows they are pigments with the greatest absorbance peaks. The peaks indicate they are absorbed by the plant pigments.

15. Why are plants generally green? Plants contain chlorophyll, a green pigment that is necessary for the absorption of light energy to power photosynthesis. It reflects green, so it appears green.

16. What does the absorption spectrum convey about each pigment? Be specific with details? Each pigment absorbs distinct wavelengths. By looking at all the absorbed wavelengths cumulatively, it is possible to see the broad range of wavelengths that a plant can use for photosynthesis.

TEACHER ANSWER SHEET

Data Table 1

A	B	C	D	E	F
Pigment *	Description of Color	Distance Solvent Front Traveled	Distance Color Traveled	Rf value (column D /column C)	Name of Pigment (Use Appendix B)
1	yellow/orange				Beta Carotene
2	yellow				Lutein
3	yellow				Violaxanthin
4	grassy green				Chlorophyll a
5	olive green				Chlorophyll b

* Number of distinct pigments will vary per lab group. The chart accommodates a maximum of 5 pigments.

VALUES FOR COLUMNS C, D AND E WILL VARY.

Wavelength	Pigment 1	Pigment 2	Pigment 3	Pigment 4	Pigment 5
360	0.009	0.015	0.006	0.065	0.032
380	0.011	0.017	0.009	0.085	0.033
400	0.018	0.025	0.015	0.091	0.035
420	0.026	0.035	0.020	0.113	0.047
440	0.033	0.042	0.024	0.106	0.064
460	0.037	0.038	0.017	0.021	0.067
480	0.034	0.036	0.016	0.015	0.037
500	0.015	0.007	0.001	0.008	0.006
520	0.004	0.002	0.001	0.008	0.005
540	0.002	0.001	0.001	0.009	0.007
560	0	0.002	0	0.011	0.006
580	0.001	0.003	0.001	0.017	0.009
600	0.001	0.001	0	0.019	0.011
620	0	0	0	0.026	0.010
640	0	0.001	0	0.029	0.018
660	0	0	0	0.083	0.030
680	0	0.001	0.001	0.034	0.009
700	0	0	0	0.003	0.001
720	0.009	0.001	0.001	0.003	0.001

This is an example of data taken from a chromatogram of a spinach leaf. The role of the accessory pigments in absorbing a wider range of wavelengths is obvious.