Leaf Pigment Chromatography

Light is a form of electromagnetic radiation. The different colors of light we see are a combination of many wavelengths that vary from 380 - 750 nm in length. Each wavelength also has a specific amount of energy measured in particles, called photons. In general, shorter wavelengths have more energy than longer ones.

The color that we see when we look at something is the wavelength of light that is not absorbed, but is being reflected instead. Leaves look green because they have green pigments that reflect the green wavelength of light. These pigments are used in the process of photosynthesis, the process by which plants trap light energy and use that energy to make sugars and starches (from CO_2 and H_2O). The sugars made by plants ultimately provide the food supply, not just for plants, but for the world's animals and other organisms, too. When you eat a hamburger, you are really eating photosynthetic products (the plant parts) that have been processed by a cow; the plant nutrients were used by the cow to make its muscles so we can have our hamburger.

Although we see green when we look at leaves, there are many pigments in a leaf. The pigment that reflects green light in leaves is chlorophyll. There are two forms of chlorophyll: chlorophyll A and chlorophyll B. There are also gold and yellow pigments (the carotenes and xanthophylls) in the leaf that are "masked" by the green chlorophyll until fall, when the chlorophyll breaks down before the leaves fall off the trees.

We can separate the different pigments found in leaves by using paper chromatography. Chromatography works because different substances, such as different pigment molecules, when dissolved in a specific solvent, will migrate through paper at different rates. The rate at which each specific substance moves on the chromatography paper is determined by 1) how soluble the substance is in the solvent being used and 2) how well the substance adheres to the chromatography paper.

Once individual types of molecules have been separated by chromatography they can be identified by calculating **"Rf" (Relative front) values.** The experimental Rf values are then compared to standard Rf values in chromatography reference manuals. Two substances that have the same Rf for a given solvent are probably the same molecule. The probability that the tested molecules are the same substance increases if they continue to have the same Rf value in a variety of solvents. Since Rf values can be used to identify unknown substances, it is important to know how to determine Rf values in chromatography experiments.

Separation of the Leaf Pigments using Paper Chromatography
Materials needed for each group*

- 3 Wide-top quart jars with lids
- 3 7-inch Square sheets of chromatography paper
- 3 Petri dish bottoms

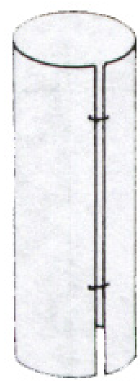
Materials needed for the entire class

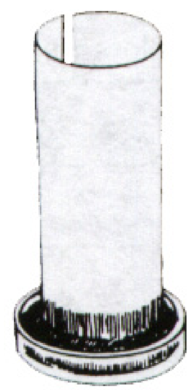
- 1 flask of leaf extract*
  (The leaf extract has been prepared by blending a variety of leaves in a small volume of acetone and then filtering the blended leaves through several layers of cheesecloth.)
- 1 Waste jar for used chromatography solvent
- 1 Covered stock container of organic chromatography solvent
  (184 ml petroleum ether + 16 ml acetone)*
- Several staplers

***CAUTION:** The leaf extract and the chromatography solvent contain aromatic organic solvents. **It is very important that you do not breathe the fumes from these solvents.** Be very careful with this solvent and wash your hands thoroughly after this exercise. The leaf extract will stain clothes. Do not spill either the leaf extract or the chromatography solvent. If there is an accidental spill, notify your instructor immediately.

Procedure
Each group will make three chromatograms. Two of the chromatograms will be run in the organic solvent. The third chromatogram will be run in water.

1. Roll the chromatography paper into a cylinder as shown and staple the edges together at the top and bottom. Ideally, the edges should not overlap when you staple them. Touch the paper as little as possible; the oils on your fingers can affect the chromatogram.
2. Pour just enough leaf extract into a petri dish to cover the bottom of the dish (0.5 – 1 mm deep).
3. Place the base of each chromatography cylinder into the leaf extract and leave it sitting in the petri dish until it has migrated about 1cm up the paper. Remove the cylinder and let it dry. If possible, dry outside or under the hood.





4. Repeat step 3 many, many times (at least 20) for each of your three cylinders. After the last "dip", let the paper cylinders dry for several more minutes.
5. While your cylinders are drying, obtain three wide-top quart jars with lids.
    a. Pour just enough chromotography solvent into two of the jars to just cover the bottom. Keep the lids on the jars at all times. Do not inhale the solvent!
    b. Pour just enough water into the third jar to just cover the bottom. Keep the lid on the jar.
6. Put your three chromatography cylinders into the quart jars. Put the jars on the top of your lab table, and periodically watch them while you do other lab exercises or review text materials.
7. When the pigments are nicely separated, remove your completed chromatograms from the jars.
8. Unstaple your chromatograms and **immediately** measure (in mm or cm) the distance traveled by the solvent and the distance traveled by each of the pigments on each of the three chromatograms. After you identify the pigments on your chromatograms (see below) record your data in **Table 1**.
9. Dispose of the chromatography solvent in the designated waste container under the hood.

Leaf Extract Pigments

The leaf extract contains at least five pigments. Four of the pigments are soluble in the organic solvent and are found in the chloroplasts of the leaf. The fifth is water-soluble and stored in the central plant vacuole.

- The golden pigment near the top of the organic solvent chromatograms is **carotene**.
- You will probably have two bands of lighter yellow **xanthophyll** pigments in the middle of the chromatogram. You may also see some grayish leaf breakdown products in the xanthophyll regions of the chromatogram. You can ignore those.
- The bright green (grass green) **chlorophyll a** pigment is immediately below the lower xanthophyll layer.
- **Chlorophyll b** is a more olive green layer immediately below the chlorophyll a layer.
- At the base of the cylinder you should find the fifth pigment, the purple-blue **anthocyanin**.

Draw and identify the pigments on each of your chromatograms below.

Organic Solvent #1 Organic Solvent #2 Water

Compare the organic solvent chromatograms with the water chromatogram. Are the two organic solvent chromatogram patterns the same?

Which pigment(s) migrated in the organic solvent chromatogram? Which did not?

How does the chromatogram pattern of the water solvent chromatogram differ from those done in the organic solvent? Which pigment(s) migrated in the water chromatogram?

Table 1: Solvent and Leaf Extract Pigment Migration Distances			
	Organic Solvent 1	Organic Solvent 2	Water
Initial Pigment Distance on Paper			
Distance to the Solvent Front			
Anthocyanin Distance Traveled			
Carotene Distance Traveled			
Chlorophyll a Distance Traveled			
Chlorophyll b Distance Traveled			
Xanthophyll Distance Traveled			

Calculating Rf values

The distance traveled by the solvent through the chromatography medium is referred to as the "**solvent front**". Since the distance the unknown molecules (in this case the pigments of the leaf) travel through the chromatography medium is relative to the distance traveled by the solvent front, the distance traveled by the unknown (pigment) is called the "**relative front**", or Rf. To calculate the Rf value:

$$Rf = \frac{\text{Distance traveled by the unknown substance (the pigment)}}{\text{Distance traveled by the solvent}}$$

Note: The Rf value is a ratio; the Rf will always be between 0 and 1.

If you were doing research and needed to isolate and identify one or more unknown substances using chromatography, you could calculate Rf values of the unknowns and then compare the Rf values obtained in your experiment with values in reference manuals to identify the substances. In today's laboratory exercise, we used the pigment color to identify the different leaf pigments.

Calculate and record the Rf values for each of the pigments in the three chromatograms in **Table 2**. The Rf values for the two organic solvent chromatograms should be very similar. Are they?

Table 2: Leaf Pigment Rf Values			
Rf Value	Organic Solvent 1	Organic Solvent 2	Water
Anthocyanin			
Carotene			
Chlorophyll a			
Chlorophyll b			
Xanthophyll			

Save the organic solvent chromatograms for the Leaf Pigment Absorption Spectra Exercise. You make take the water chromatograms home for appropriate display of work accomplished in Biology class.