Department of Biochemistry & Microbiology
Chemistry of Biomolecules Lab (CHE 203 L)

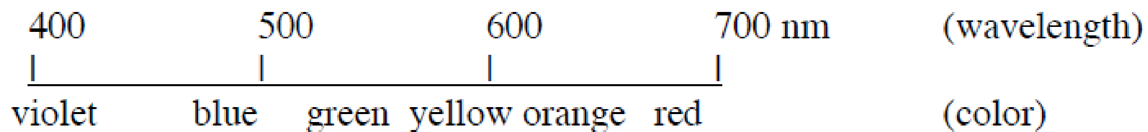
Experiment 2.

Identification of Leaf Pigments by Thin Layer Chromatography

Background:

Plant leaves contain a number of important pigments including chlorophylls, carotenes, and xanthophylls. During the summer when leaves contain large amounts of chlorophyll (and are thus green), the presence of the other pigments is not obvious to the eye. During the fall, however, after most of the chlorophyll has been degraded, these other pigments can be more readily observed, and the leaves of many plants take on the variety of beautiful colors that are typical of fall foliage. During this lab you will have a chance to collect leaves of various colors and analyze the different pigments that are present.

General information about leaf pigments-
Visible Light Spectrum



CHLOROPHYLL
- absorbs red and blue light
- reflects green
- membrane-bound

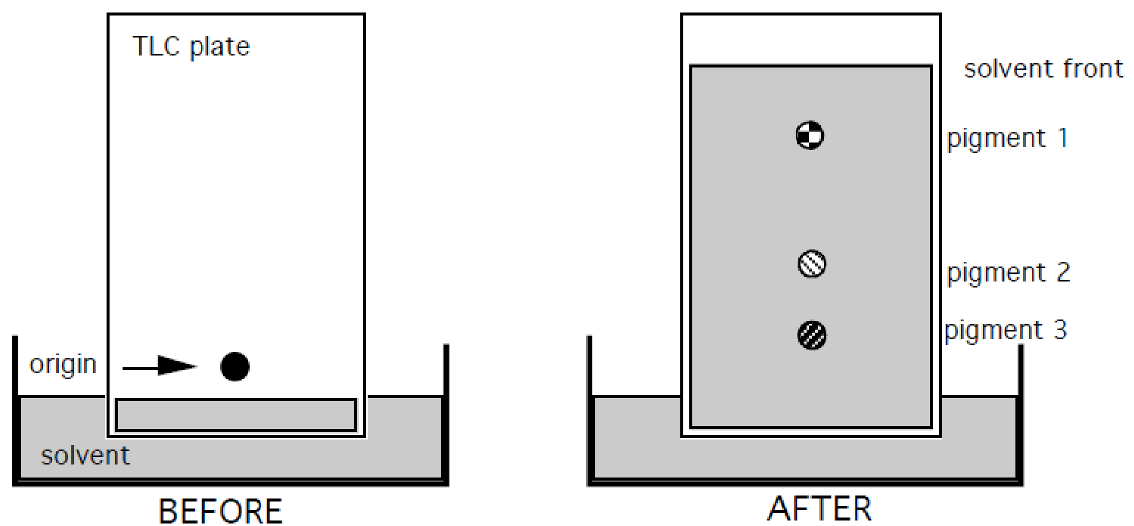
CAROTENES
- absorb violet, blue, and green light
- reflect yellow, orange, and red
- membrane-bound

XANTHOPHYLLS
- absorb violet, blue, and green light
- reflect yellow, orange, and red
- membrane-bound

ANTHOCYANINS
- absorption variable (green, yellow, orange, red)
- reflected colors variable (violet, blue, red)
- water-soluble

Principles of TLC:

Thin-layer chromatography (TLC) is a convenient technique for separating and analyzing the different pigments present in a leaf. A leaf extract containing a mixture of many compounds is spotted onto a TLC plate and an organic solvent is allowed to move up the plate, potentially carrying with it the various compounds in the leaf extract. The different components of a leaf extract are separated based on their affinities for the stationary phase (the silica on the TLC plate) and for the mobile phase (the solvent that is moving up the plate). Compounds with more affinity for the silica (i.e. hydrophilic compounds) will not move very far, while compounds with a high affinity for the organic solvent (i.e. hydrophobic compounds) will move much farther.



In this example, a leaf extract containing three pigments was spotted onto a TLC plate at the "origin". The TLC plate was then placed into a container of solvent. As the solvent front moved up the plate, the three pigments moved at different rates. Pigment 3 is apparently quite hydrophilic and therefore moved much more slowly that the solvent front while pigment 1 is apparently more hydrophobic since it moved almost as fast as the solvent front. The mobility of a compound in a particular TLC system is the R_f value.

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent front}}$$

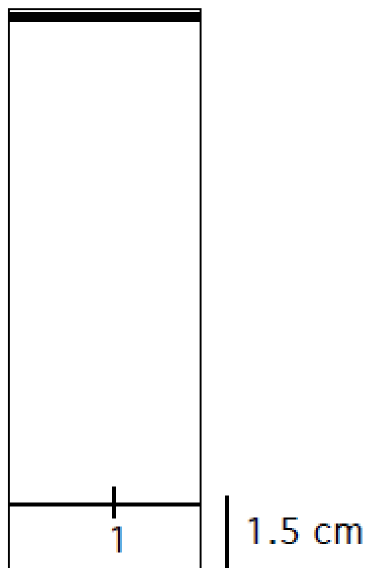
Procedure:

Pigment extraction-

1. Label 1.5 ml tubes for the samples collected. Place 250 mg of leaf tissue into each tube and add 400 µl of 90% acetone (can solubilize the components).
2. Grind each sample thoroughly with a small plastic pestle or hand homogenizer. Add an additional 600 µl of 90% acetone to each tube and mix them thoroughly using a vortex mixer.
3. Centrifuge your tubes for 10,000 rpm for 3 minutes in a microcentrifuge. Carefully transfer the supernatants to fresh tubes and dispose of the pellets.
4. Add 150 µl of hexane to each tube. Add 200 µl of water to each tube. Mix thoroughly using a vortex mixer so that the leaf pigments will partition into the hexane phase.
5. Centrifuge for 1 minute at 12,000 rpm in a microcentrifuge. *Observe what colors have partitioned into the aqueous and hexane phases. What does this mean?* Carefully transfer the hexane fractions into new tubes. Dispose of the aqueous phases.
6. Add 200 µl of water to each of your pigment extracts. Mix them thoroughly using a vortex mixer.
7. Centrifuge for 12,000 rpm for 1 minute in a microcentrifuge. Carefully transfer the hexane fractions into new tubes. These will be the final pigment extracts that you will use. Each one should contain a concentrated mixture of whatever pigments were present in the leaf from which it was derived.

Separation of pigments by TLC plates-

1. Draw a faint pencil line (without damaging the silica surface) 1.5 cm from the bottom edge of a TLC plate. Mark one spot along this line (one for each of your pigment extracts), leaving 2 cm of space between each spot.



2. Measure out 10 µl aliquots of each pigment extract and place them into fresh tubes. Place a glass capillary into each tube. Using the capillary, apply a small amount of each pigment extract to the appropriate spot. Allow the hexane to evaporate. Apply a little bit more of each extract to the TLC plate

and allow the hexane to dry. Keep repeating this process until the entire 10 µl of each pigment extract has been applied to the plate. Allow the TLC plate to dry completely before proceeding to the next step.
3. Place the TLC plate into a **pre-saturated** chromatography chamber containing petroleum ether acetone- chloroform (3:1:1). Since these organic solvents are quite volatile, smell bad, and are not good for your health if you breathe them, do this step completely inside a fume hood.
4. Observe as the solvent front moves up the TLC plate. You should also be able to see the pigments separating as the TLC plate "develops". Allow the solvent front to travel about 3.0 cm past the origin. Once the development is complete (about 2.0 minutes) remove the plate from the chromatography chamber and quickly mark the location of the solvent front with a pencil. Allow the plate to dry completely before removing it from the fume hood. Photograph your TLC plate with a digital camera as soon as it dries (the colors will fade within a few hours) and then print out a copy (on a color printer) for your notebook.

<u>Observation-</u>

Record number of pigments that were present in each of your samples. Identify what each of these pigments is based on your knowledge of the structure and light absorption properties of plant pigments. Calculate the Rf values for each of the pigments you observed. Previously obtained R_f values for several spinach pigments in a similar TLC plate/solvent system to the one you used are listed below.

carotene 0.98
chlorophyll a 0.59
chlorophyll b 0.42
xanthophyll 1 0.28
xanthophyll 2 0.15

How did the values you obtained compare with these values?