Lab 6: Photosynthetic Pigments

Objectives: To determine the pigments responsible for leaf color.

Background

During autumn in Vermont, tree leaves "paint the landscape" with awe-inspiring colors. While some plants exhibit a single shade of color in the fall, such as birch and aspen that have yellow leaves and sumacs that have deep red leaves, other species can have multiple color signatures, such as maples that dazzle with red, orange, and gold, and ashes that show maroon and yellow. The colors of maples and ash, among others, can vary considerably from one locality to another or even from one leaf to another, depending on the combination of pigments present in the fall leaves.

In this lab, you will separate out the pigments in leaves using **paper chromatography**, and then measure the quantity of each pigment present using **spectrophotometry**. Through chromatography, individual pigments are isolated from the many other substances found in living tissues. Once separated, the amount of pigments present can be determined with spectrophotometry, which measures the light absorbed by a given substance.

KEY WORDS

Chromatography: a process that separates the components of a gaseous or liquid mixture that involves passing the mixture over or through a medium that absorbs the components at different rates.

Paper chromatography: a process that separates pigments (or other substances) using special paper. Similar to the process that causes ink to run when it gets wet.

Spectrophotometry: a process that uses a spectrophotometer to measure how much light at different wavelengths is absorbed by a substance.

Photosynthesis: the process by which carbon dioxide and water are converted into sugar, water, and oxygen using light energy.

Pigment: a substance that absorbs light and gives a tissue its color.

Water insoluble: does not dissolve in water. Typically this means that the molecules are not especially polar.

Water soluble: dissolves in water. Typically this means that the molecules are polar, and therefore interact with the polar water molecules.

Photosynthesis is the process by which green plants and other organisms produce simple carbohydrates from carbon dioxide and water, using energy that chlorophyll or other organic cellular pigments absorb from radiant sources. **Photosynthesis is the most important series of chemical reactions on earth; without it, life as we know it would not exist.** It is a complex chemical process that converts radiant energy (light) to chemical energy (sugar):

Light + 6 CO_2 + 12 H_2O → $C_6H_{12}O_6$ (sugar) + 6 H_2O + 6 O_2

Pigments are natural substances in plant and animal tissues that absorb light and give the tissue its color. Note that the color that is NOT absorbed by the pigment is reflected, and that's the color we see. The chloroplasts (those photosynthesizing organelles) of mature leaves contain several groups of pigments:

Chlorophylls – water insoluble
chlorophyll a – grass green *chlorophyll b* – yellow-green

Carotenoids – water insoluble
carotenes – orange/yellow *xanthophylls* – pale yellow

Cyanins – water soluble
anthocyanins – red/violet/blue *betacyanins* – red/violet betaxanthins – orange/yellow

Chlorophyll and **carotenoid** pigments play a role in photosynthesis. Research suggests that the large amounts of **chlorophylls** and their intense green color usually hide the presence of the **carotenes** and **xanthophylls.**

Most plants regularly destroy and re-synthesize their **chlorophyll** during their growing season, but as the fall progresses, the rate of **chlorophyll** synthesis lags behind its breakdown. Eventually it no longer masks the other pigments, and the fall color change begins.

While plant physiologists are unsure of the role that the **cyanin** pigments play in plants, research suggests that they may help in plant defense, perhaps as a fungicide. These water-soluble pigments are non-photosynthetic and are not present in the chloroplasts, but instead are localized in vacuoles, especially in epidermal cells (like your onion epidermal peel).

Many pigments have an affinity for paper and are also easily dissolved in solvents. This technique takes advantage of two facts. First, paper is made up of cellulose, which has many -OH (hydroxyl) groups present. These groups form hydrogen bonds to other hydrophilic groups, and as a result many substances (like chlorophyll) form hydrogen bonds to cellulose. Second, pigments can be dislodged from their cozy hydrogen bonds if a solvent is present.

If we extract pigments from plant tissue, apply them to paper, and then wet the paper gradually with a solvent, a pigment molecule can be displaced slightly from its original position. It will migrate over the paper as the solvent flows over it. The other pigments present in the extract also bind to the cellulose, but with different affinities since different types of pigment molecules have different chemical structures, sizes, polarities and solubilities. Therefore, the substances in the mixture separate; some are slightly soluble in solvent and don't migrate very far on the paper, while others are more soluble and migrate farther, separating from each other.

Forming Hypotheses:
Observation: Many lettuce leaves are green.

Question: What makes leaves green?

Vague Hypothesis: Plant leaves are green because they contain pigments that absorb some wavelengths of light and not others.

Testable Hypotheses:
Based on the above information, which pigment(s) will have the greatest concentration in green lettuce? Which pigments will have the least concentration in these two types of lettuce? We will have data only on chlorophyll a, chlorophyll b, xanthophyll and carotene.

Write your hypotheses here.

The Experiment: You will use the techniques of paper chromatography and spectrophotometry to test these hypotheses as they relate to lettuce leaves.

Your Results: Record your results in the data table and graph them. Make inferences from your results and from the results of others.

Lab Procedure
You and your lab team will harvest 5 grams of green lettuce. Then you will extract the pigments, separate them, and measure how much light they absorb at various wavelengths.

<u>Pigment Extraction</u>
Routine laboratory procedures including paper chromatography and spectrophotometry can be used to **(E)** extract, **(S)** separate, **(I)** identify, and **(Q)** quantify leaf pigments - **ESIQ.**

1. Each group of 4-5 students will collect 5 grams of lettuce leaves to analyze for pigments. Weigh your fresh leaves to make sure you have 5 grams.

2. Place half of the leaves in a chilled mortar, add ~10 mL of cold acetone, and then grind the leaves with a pestle. When they have squished down enough to fit the rest of the leaves in the mortar, add them. Grind until all color is in the liquid phase, and the pulp is white or colorless (Why is this important?). **Caution: acetone is flammable! (Hint: make sure there is always a little puddle of acetone in your mortar.)**

3. Pour off the liquid extract from the mortar into a 50 mL graduated cylinder being careful to get as little pulp in the cylinder as possible. Rinse the pulp in the mortar with a small amount of acetone, and add this to the graduated cylinder. Place the extract in an ice bath, allowing any solids to settle for 5 minutes.

Chromatography (Pigment Separation & Identification)

µL: microliter, or 1/1000 of a milliliter (mL).

4. Draw a light pencil line across a piece of chromatography paper 2 cm from the bottom edge. Use a micropipetter to withdraw 100 µL from the extract solution and apply it evenly and continuously along the pencil line. Do NOT touch the pipette tip to the paper. Allow the paper to air-dry completely before applying another 100 µL. Repeat this process until you have applied 1000 µL (1 mL) of extract to the paper and let it dry. The line of green extract is called the **origin**.

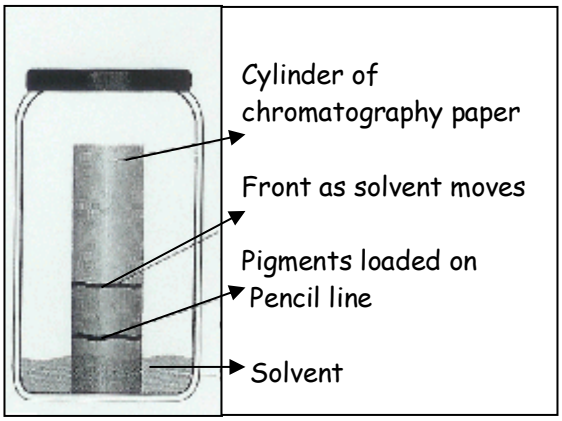

Figure 3

5. Roll the paper into a tube so that the pigment line forms a ring around the bottom (Fig 3). Try to avoid creasing or otherwise damaging the paper.

6. Under the ventilation hood, swirl the chromatography tank (a.k.a., jar) to coat the inside surfaces with the liquid (90% acetone/10% petroleum ether = a solvent that will carry the extract up the paper). Place the paper tube in the chromatography tank and quickly place the cap on the jar. The tank contains 50 mL of 10% acetone/90% petroleum ether solvent, which is 1 cm deep. (The solvent must not cover the origin). *Leave the tank under the hood and remember the number written on the lid.* You should not open it unless it is under the ventilation hood because acetone and petroleum ether are both toxic to breathe.

7. The solvent will travel upward, wetting the paper. This process is called irrigation. Allow the solvent to irrigate until the solvent front is 1-2 cm from the top of the paper (this should take ~15 minutes). Remove the chromatogram from the tank and let the paper dry (do all this under the hood!).

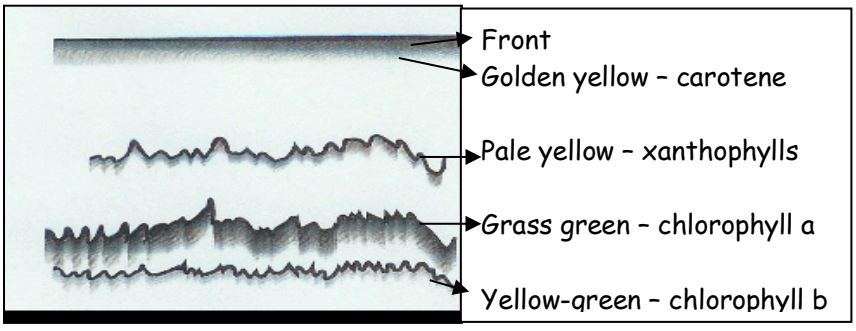

Figure 4. Chromatography paper, unrolled, showing the movement fronts of various pigments. Some of your pigments may be difficult to see and some varieties may contain more than four pigment bands. Do your best to identify these four pigments.

8. Locate the *chlorophyll a and b* bands and the *xanthophyll* and *carotene* bands. (Note: in this procedure, you have extracted only non-polar photosynthetic

pigments. For example, the water-soluble *anthocyanin* pigments were not extracted and cannot be measured.)

9. Cut out each band separately and place each band in a small marked vial to which 10 mL of acetone has been added. The acetone will dissolve the pigment in about 5 minutes. Remove the strips with forceps and dispose of them in a designated waste container. If there are four groups extracting pigments in the lab, the four groups should swap samples after pigment extraction, so that two groups will measure absorbance for chlorophyll a and b for both groups and two groups will measure absorbance of xanthophyll and carotene for both groups. The readings for the four pigments can then be shared among the groups at the end of the experiment.

10. Using one disposable plastic pipette for each pigment, fill four cuvettes, each **halfway**, with the pigment solutions from your vials (one cuvette per pigment). Add acetone to a fifth cuvette. **Do not dump out your pigment solutions yet; if you spill a cuvette, you'll need to refill it.**

Spectrophotometry (Pigment Quantification)
Allow the spectrophotometer to warm up for 30 minutes before use. Please be careful with these. They are expensive and fragile. NEVER pour the acetone directly into the spectrophotometer. Always use a cuvette (a small, rectangular container).

The TA will demonstrate the use of the spectrophotometer, based on the instructions below.

If you are using a spectrophotometer other than the Spec 200, obtain instructions from your TA. Otherwise, follow these user instructions:

Spectronic 200: User Instructions: Measuring absorbance at a specific wavelength:

1. The spectrophotometer will be turned on 30 minutes prior to use.
2. From the Home screen, press **Enter** on SPEC200 Modern Interface screen.
3. Press the (>) key once to get to the **Multi wavelength page**, and then press Enter.
4. Turn the **Wavelength knob** to the desired wavelength.
5. Put in the blank (acetone) cuvette to "Blank" the spectrophotometer. The absorbance (ABS) should read zero or a negative number.
6. Press Enter to **HOLD.**
7. Put in cuvette containing the sample and press **Enter** again when **GO** is highlighted.
8. Watch real time results of absorbance values at specified wavelength.
9. Press the **down arrow** to go to next wavelength and repeat steps 5-8.

Measuring absorbance over a wavelength range (Wavelength scan):

1. From the Home screen, press **Enter** on SPEC200 Modern Interface screen.
2. Press the (>) key three times to get to the **Scan page.**
3. Press the down arrows to set the Low and High wavelengths using the **Wavelength knob.**
4. On the screen, arrow down to **Next** and press **Enter**.

5. Put the blank (acetone) cuvette in the spectrophotometer and **press the 0.00 button on the screen.** Wait for it to finish auto-zeroing.
6. Remove the blank cuvette and place the cuvette containing the sample in the spectrophotometer.
7. Press **Enter** (it should say "**Scanning**…")
8. Use the right and left arrows on the screen to scan absorbances at various points across wavelength range.

Record the absorbance of each of your samples at the indicated wavelengths on the chart below.

nm	Chlorophyll a	Chlorophyll b	Xanthophyll	Carotene
400				
425				
450				
475				
500				
600				
650				
675				
700				
800				

9. Share data between the groups that measured chlorophyll a and b and the groups that measured absorbance for xanthophyll and carotene.
10. **Plot your data on the graph paper on the last page of this manual, using colored pencils to represent the different pigments.** Each line on the graph is called an absorption spectrum (plural = spectra).

Pigment Concentrations

Important: answer question 1 before reading this section!

Calculating the concentrations of each pigment in the leaves that you crushed is possible, but it is time-consuming, so we aren't doing it today. Instead, we are going to use typical pigment concentrations for lettuce to answer the questions below.

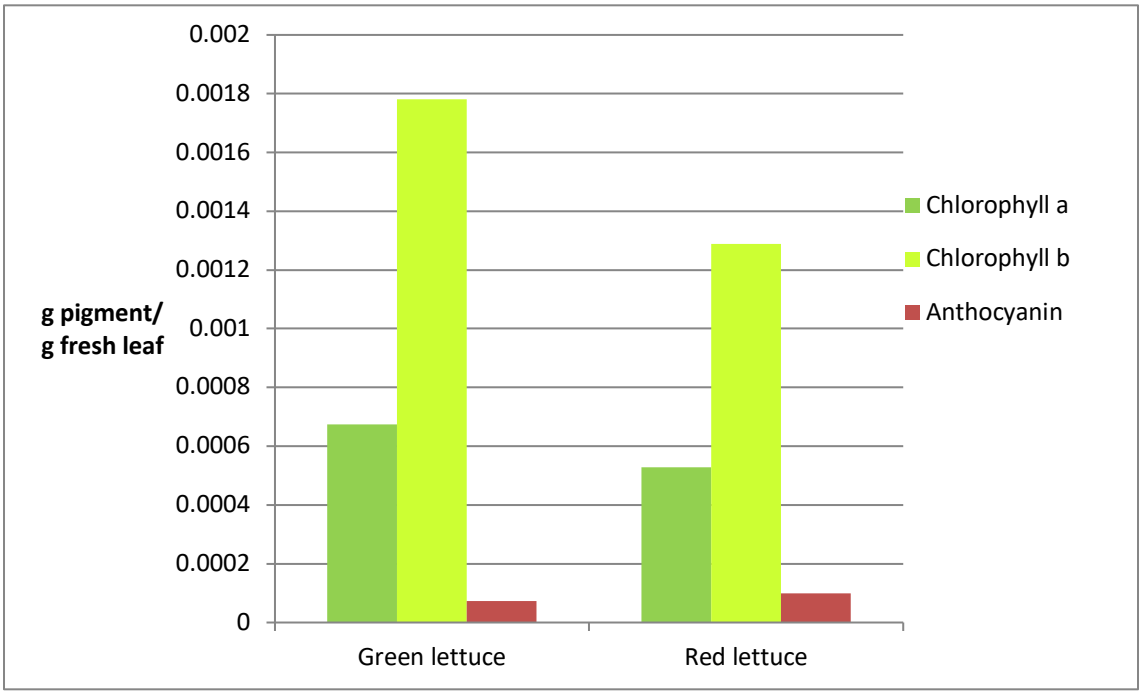

Figure 5. Chlorophyll a, chlorophyll b, and anthocyanin concentrations in green and red lettuce varieties. Anthocyanin is a red, purple, or blue pigment that is common in many lettuces. Data from Kleinhenz, M.D., D.G. French, A. Gazula, and J.C. Scheerens. 2003. Variety, Shading, and Growth Stage Effects on Pigment Concentrations in Lettuce Grown under Contrasting Temperature Regimens. HortTechnology 13(4):677-683.

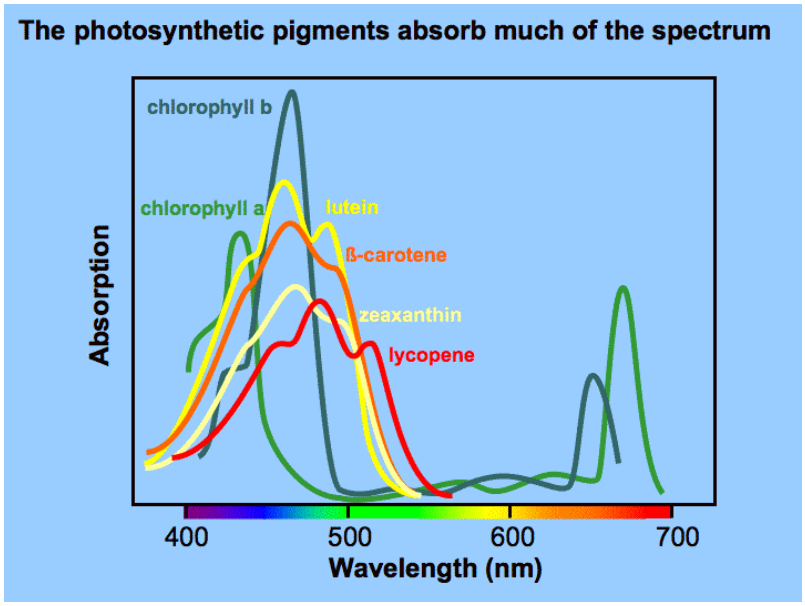

Figure 6. Absorption spectrum of photosynthetic pigments in plants

Questions

1. List the peak absorbances of each of the four pigments. Speculate on why you see the patterns that you see in the absorption spectra that you drew. For example, why did the chlorophylls peak in the violet end of the spectrum? Why did they have secondary, smaller peaks in the red end of the spectrum? Did your absorption spectrum look similar to the spectrum shown in Figure 6?

2. Did the results from the Pigment Concentrations section on p. 6 support or fail to support your hypotheses? Why do you think you see the patterns that you see in the graph?

3. What might be an advantage to having an unusual leaf color/combination of leaf pigments? In what situations might this be beneficial? Think creatively and put yourself in the plant's place. What are its threats? What does it need?

Before leaving lab...

Turn in your complete lab assignment (all questions answered) to your TA. Points will be docked if you do not turn in your assignment before leaving lab.

Your graded assignment will be returned to you next week.

TOTAL: _____/10