Photosynthesis:
Pigment Extraction, and Spectral Analysis

**Purpose**

This lab is designed to reinforce the student’s understanding of the process of photosynthesis by observing two of its main components: separation of plant pigments and light absorbance by chlorophyll. The primary objectives of this lab are for the student to:

- Understand how the wavelength of light energy corresponds its position in the electromagnetic spectrum and the color of visible light.
- Understand the light absorption role that plant pigments play in the process of photosynthesis.
- Separate and identify different plant pigments contained in spinach leaves using the technique of thin layer chromatography.
- Learn the basic premise underlying the functioning of a spectrophotometer.
- Use a spectrophotometer to take light absorbance data and generate an absorbance spectrum for chlorophyll.
- Demonstrate proper data presentation techniques learned in previous labs.

**Background**

**I. Introduction.**

Nearly all life on Earth depends on the ability of certain organisms to convert solar (light) energy into chemical energy (molecules with high-energy bonds). This process, called photosynthesis, goes on in all green plants and a few other producers (blue-green algae and some types of bacteria). Today’s lab will introduce you to some of the individual concepts involved in the overall process of photosynthesis. There are many different reactions involved in the overall process, but in its most basic form, photosynthesis can be broken down to the following reaction:

\[ 6\text{CO}_2 + 6\text{H}_2\text{O} + \text{light energy} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 \]

The process of photosynthesis takes place in the chloroplasts of green plant cells. The sun’s light energy is the primary source of energy on the planet (there are some organisms which use heat or other chemicals to produce their chemical energy, but they are rare and highly environment specific) and most of the organisms on Earth are directly or indirectly dependant on photosynthesis. If you observe the reaction closely, you will also notice the other by product of the reaction is oxygen, a requirement for all of us animals. So, as animals we are doubly dependant on photosynthesis for food as well as oxygen. This is something to consider the next time you hear about clear cutting of the rainforests and pollution of the seas.

**II. Light.**

Light energy is contained in discrete pockets of energy called photons. Different photons have differing amounts of energy. It is helpful to think of the behavior of light in terms of a wave form. The higher the energy contained in the photons, the shorter the distance between adjacent “peaks” of the wave (figure 6.2 on p. 116 of your text). The distance between wave peaks, called the wavelength of the light, is used describe the energy content of light photons. The entire continuum of electromagnetic energy is called the electromagnetic spectrum, and visible light (what our eyes can detect) is only a small part it. The visible light spectrum runs from ~400 nanometers (nm) to ~740 nm, and the primary
colors contained within it are Red, Orange, Yellow, Green, Blue, Indigo & Violet (all others are combinations of the primary colors). Sometimes it helps to remember the name Roy G. Biv to remember the colors in order.

III. Plant Pigments.

Plant pigments are molecules that absorb light (photons). The plant pigment that you are probable most familiar with is Chlorophyll. Because chlorophyll pigments (there are actually 2, chlorophyll A & B) absorb photons in the red and yellow end of the visible light spectrum and transmit photons in the green area, it is the reason that we perceive green plants to be green. There are, however, many different plant pigments which absorb light of different wavelengths (though there may be some overlap between similar pigments) that are also used by plants. The energy from these photons is then shuttled to the second which actually builds the sugar molecules using stored CO\textsubscript{2} (carbon dioxide) and H\textsubscript{2}O.

**Laboratory Exercises**

I. Thin Layer Chromatography.

Different plant groups utilize different sets of plant pigments. In this exercise, using the process of thin layer chromatography (TLC), you will be separating out the different individual pigments found in a leaf based upon their molecular weight. By applying the leaf sample to a strip of paper and allowing it to absorb a solvent, it is possible to separate the individual pigments contained in the leaf. The procedure for setting up the TLC experiment is as follows:

1. Put on a pair of gloves and goggles. The goggles are to protect your eyes – you will be working with solvents during this lab activity. The gloves will protect your hands, but they will also prevent oils on your skin from getting on the TLC square, which can interfere with results.
2. Obtain a TLC square and a spinach leaf from the supply area.
3. Using a penny, score the leaf across the square approximately 1.5 cm from the bottom edge (the end opposite the fold). This region may be marked with an “X.” It is best to double the leaf over so you are transferring as much pigment as possible. You may want to go over the line several times so that you have a thick dark band on the TLC square. While one team member is preparing the TLC square, another team member should be preparing a developing chamber at the fume hood.
4. To prepare the chamber, using one of the large plastic pipettes provided to dispense botany chromatography solvent into a chamber to the fill line indicated on the side of the chamber, and promptly replace the lid. Keep the pipette vertical at all times to prevent solvent squirting out across the hood or into someone’s face. The solvent consists primarily of Petroleum Ether with small fractions of Acetone and n-Propanol (1-propanol). It is flammable and volatile. Do not inhale the solvent directly. In order to prevent crowding, there should be no more than two students working at the fume hood at any time.
5. Bring the covered chamber back to your group’s workstation. Score the fold at the top of the TLC square and use the fold to hang your TLC square from the nylon thread at the top of the chamber. Promptly replace the lid on top of the chamber. Observe the movement of the solvent and pigments along your chromatogram.
6. When the solvent is within 2 cm of the nylon thread, remove the TLC square, and cover the chamber. Draw what you see on your TLC Square, and describe the colors. Using a pair of scissors, carefully cut out the chlorophyll A and B band – this should be a blue-green band above the olive green Phaeophytin band located at the point where you applied the pigment. The
chlorophyll A and B pigments do not separate completely during this process, so you will be extracting both. Try to avoid including the yellow Xanthophyll band in your chlorophyll strip.

7. One representative from your group should take the chlorophyll strip to the fume hood. Find a rack holding two corked test tubes, two pipets, and a cuvette (the small test tube with a white line and circle on it). Remove the cork from one of the test tubes. Hold the strip with forceps and suspend it inside the tube. Use the acetone wash bottle to rinse the paper strip with acetone. The pigment is polar and thus highly soluble in the polar acetone. Add enough acetone to the tube to fill the tube to the line indicated. The acetone rinse should have a faint green tint. In the second tube, add pure acetone. Replace the corks on both. These will be your extract and blank. Take the rack back to your table and follow the steps in part II to perform spectral analysis of your extract.

II. Spectrophotometry.

In this exercise, you will demonstrate that not all pigments absorb light of the same wavelength. A spectrophotometer is an apparatus which allows us to determine which wavelengths of light are absorbed by a pigment sample. Recall that white light is composed of all of the colors (wavelengths) of the visible light spectrum. The spectrophotometer has a white light source which is shined through a refracting prism to separate out the different colors. The light rays then strike a metal plate with a small slit in it. By positioning the metal plate, you can control what wavelength of light is allowed to pass through the slit and strike the pigment sample on the other side. After passing through the pigment sample, the light strikes a photoelectric tube which records absorbance.

The spectrophotometer is as follows.
1. Beware, acetone is a solvent and can be hazardous. Please keep your corked tubes closed at all times possible, and use the parafilm squares available on the supply counter to cover the top.
of your cuvette when you change between your extract and your blank solution. Let me know if any of your glassware breaks or if there is any spillage.

2. Turn the spectrophotometer on by turning the power switch/zero control knob clockwise (you should feel it click) and allow the machine to warm up for at least 5 minutes. Check the Filter located at the bottom left of the machine. It should match the wavelength that you are testing. (You will start with it set at 340-599 and then move it to 600-950).

3. Adjust the wavelength to 380nm using the wavelength control knob.

4. Set the red dot on the digital readout to transmittance using the white “mode” button.

5. Using the power switch/zero control knob, adjust the transmittance to zero percent.

6. Adjust the red dot on the digital readout to absorbance using the “mode” button again.

7. Using the appropriate pipet, add pure acetone from the test tube to your cuvette until the liquid reaches the bottom of the circle beneath the vertical line. Cover your cuvette with a parafilm square. Wipe the bottom and sides of the tube (where the liquid is) with a kimwipe. Open the sample compartment and insert the cuvette completely into the well (be sure to line up the white vertical line on the cuvette with the line in front of the well) and close the lid.

8. Adjust the absorbance number on the digital readout to zero using the transmittance/absorbance control knob.

9. Remove the cuvette from the sample compartment and empty the acetone back into its test tube. Now add the same volume of your extract using the appropriate pipet. Re-cover the top of the cuvette with a parafilm square (you can use the same one if it is still in good shape). Wipe the cuvette with a kim wipe, insert it into the sample well, and close the lid.

10. Record the percent absorbance value for 380nm on a table, remove the cuvette from the sample compartment and completely empty it back into the corked tube containing your extract.

11. Adjust the wavelength to 400nm and repeat steps 7 – 10. Continue this procedure, increasing the wavelength by 20nm intervals up to 680nm. For your readings above 600nm, you will need to switch the filter by moving the lever located on the lower front left side of the machine over to the right.

**Troubleshooting tip:** If the Spec 20 flashes 1999 when reading the absorbance value, turn the right knob (100% T / 0 A) to the left until it stops flashing. This may require several revolutions. For some reason the flashing occurs because the knob has been turned too far in one direction.

**Quick Overview for each reading:**
1. New wavelength (check filter)
2. Mode to Transmittance
3. Zero with power switch/zero knob
4. Put in blank (pure acetone)
5. Mode to Absorbance
6. Zero using Transmittance/Absorbance knob
7. Put in sample (extract)
8. Read absorbance
9. Remove sample
Results Sheet  
Photosynthesis

The color of light that is ______________________ is the color that we perceive the leaf to be.

II. Spectrophotometry.
    Record your absorbance data for chlorophyll in the table below.

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<thead>
<tr>
<th>Wavelength</th>
<th>% Absorbance</th>
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Attach your graph of the absorption spectrum for chlorophyll below (be sure it is complete and labeled).
In what range of wavelengths did chlorophyll best absorb light? ________________________

What color of visible light does this range correspond to? _________________________

Why might it be advantageous for plants to use different pigments (which absorb at different wavelengths) rather than just chlorophyll?

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