

Absorption Spectra of Leaf Pigments

Light is a form of electromagnetic radiation. Visible light is a combination of many wavelengths in the range of 380 - 750 nm that we see as different colors. Each wavelength is associated with a specific photon, or particle of energy. In general, shorter wavelengths have more energy than longer ones.

The light absorbing photosynthetic pigments do not absorb all wavelengths of light equally. The absorption of different wavelengths, or absorption spectrum, of light by the photosynthetic pigments can be demonstrated using a spectrophotometer.

A spectrophotometer is an instrument that can measure the absorption of light by a substance at specific light waves. Instructions for the use of the spectrophotometer are in the laboratory room. You will determine the absorption spectra for 4 leaf pigments: carotene, chlorophyll a, chlorophyll b and xanthophyll.

Paper chromatography is used to separate the component light-absorbing pigments from a leaf extract. Once the pigments are separated from each other, the paper strips will be cut apart and re-suspended in a solvent for use in the spectrophotometer.

Separation of the Leaf Pigments using Paper Chromatography

Materials Needed *

- 28 Wide-top quart jars with lids
- 28 7-inch Square sheets of chromatography paper
- 28 Petri dish bottoms
- 1 Flask of leaf extract (Sufficient to fill 28 petri dish bottoms)
- 1 Waste jar for used chromatography solvent
- 1 Covered stock container of organic chromatography solvent
(184 ml petroleum ether + 16 ml acetone)
- Several staplers

*Each lab group will need 4 jars, 4 pieces of paper and 4 petri dishes.

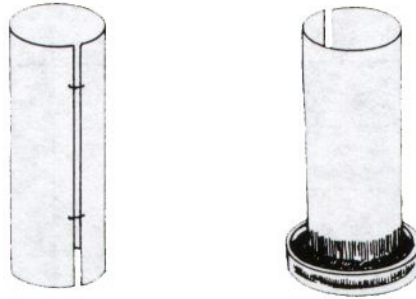
The leaf extract has been prepared by macerating a variety of leaves in a small volume of acetone and then filtering the macerated leaves through several layers of cheesecloth.

Each lab group will make four (4) chromatograms that will be run in the chromatography solvent.

Procedure

1. Roll the chromatography paper into a cylinder as shown by your instructor 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 enough leaf extract into a petri dish to cover the bottom of the dish (0.5 – 2 mm deep).

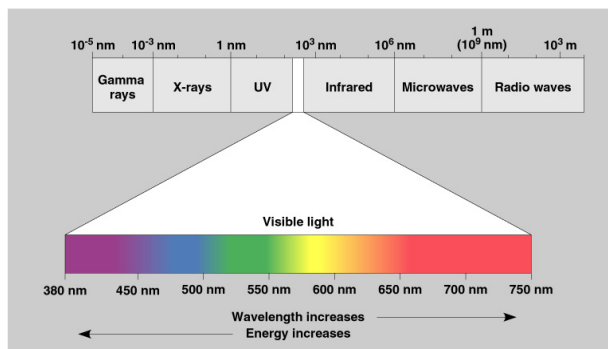
- Place the base of your chromatography cylinder into the leaf extract and leave it sitting in the petri dish until it has migrated about 1cm up the paper. Remove the cylinder and let it dry. If possible, dry outside or under the hood.



- Repeat step 3 at least 12 – 15 times. After the last "dip", let the paper cylinder dry for about 5 minutes.
- While your cylinder is drying, obtain your wide-top quart jar with lid and pour just enough chromatography solvent into the jar to just cover the bottom of it. Keep the lid on the jar at all times. Do not inhale the solvent!
- Put your chromatography cylinder into the quart jar. Put the jar on the top of your lab table, and periodically watch it while you do other lab exercises.
- When the pigments are nicely separated, remove your completed chromatogram from the jar and let it dry.
- Put the chromatography solvent in the designated waste container.
- Unstaple your chromatogram and identify the pigments.

The golden pigments near the top of the chromatogram are the carotenes. You will probably have two bands of lighter yellow xanthophyll pigments in the middle of the chromatogram with the bright green (grass green) chlorophyll a immediately below the lower xanthophyll layer. Chlorophyll b is a more olive green layer immediately below the chlorophyll a layer. You may also see some grayish leaf breakdown products in the xanthophyll regions of the chromatogram. You can ignore those. Depending on the leaves used, you may also find a band of anthocyanin pigments at the bottom of the chromatogram. Why did the anthocyanin pigments not migrate?

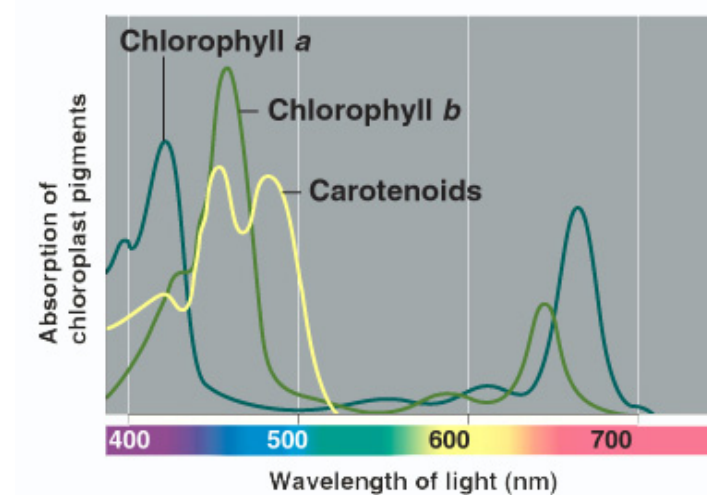
Absorption Spectra of Photosynthetic Pigments



Electromagnetic and Light Spectrum

The pigments that you separated by paper chromatography will be separated from each other and suspended in alcohol to provide pigment extracts to establish an absorption spectrum. These pigments have the property of absorbing and transmitting certain light waves, while reflecting others. To provide energy for the process of photosynthesis (the transformation of light to chemical energy), light must be absorbed by the pigments. The **absorption spectrum** will help you determine which light waves are absorbed by the leaf pigments.

This will be a class exercise. All of the chromatograms will be pooled to concentrate the pigment extract. We will be using as many spectrophotometers as we have available. It's always best to have more than one set of data when doing science. Volunteers will be required to participate in all stages of the exercise: preparing extracts, operating the spectrophotometers and recording the data on the board for the other students. Do not hesitate to volunteer for at least one activity.



Materials Needed

- Spectrophotometers (Turned on at Beginning of Lab)
- Spectrophotometer cuvettes
- 4 250-ml Beakers
- 500 ml Alcohol
- 4 Small labels
- 4 Wood stirrers
- 1 China marker (grease pencil)
- 16 Pairs of sharp scissors

Procedure

Note: While several students are completing steps 1 and 2, other students can be doing the following:

- The students who plan to collect the spectrophotometer data should review the procedure for operating the spectrophotometer from the *Biology 211 Laboratory Exercises* and be getting the spectrophotometer ready for operation. (See below.)
- One student group can reproduce the Absorbance chart on the board.
- One student group can label the 4 beakers for each of the pigments ("A" for chlorophyll a, "B" for chlorophyll b, "C" for carotene and "X" for xanthophyll) and put a small amount of alcohol in each beaker, along with a wooden stirrer. This group will also be in charge of preparation steps 3 - 5 below.

Preparation of the pigment extracts

1. Cut each of the four pigment bands (carotenes, xanthophylls, chlorophyll a and chlorophyll b) from your two chromatograms. Be sure to keep track of which strip is which pigment. Yes, you must cut along the "zigzag" lines. Each strip must have just one pigment.
2. Crumple up the pigment strips and place each strip in its appropriate beaker located at the side table. The beakers will be labeled "A" for chlorophyll a, "B" for chlorophyll b, "C" for carotene and "X" for xanthophyll.

- When all 28 chromatogram strips have been placed in the beakers, stir the paper-alcohol mixtures with the wooden stirrers provided. The pigments will be transferred from the paper to the alcohol. If necessary, add more alcohol.
- You will need 5 cuvettes for each spectrophotometer. Pour the four pigment extracts into 4 cuvettes for each spectrophotometer available. Be sure that you know which cuvette has which pigment. Avoid getting paper fragments in the extract. Pour alcohol into a fifth cuvette. This will be the blank.
- Take one set of the four pigment extracts and a "blank" to each of the spectrophotometers available.

Collecting the absorption data

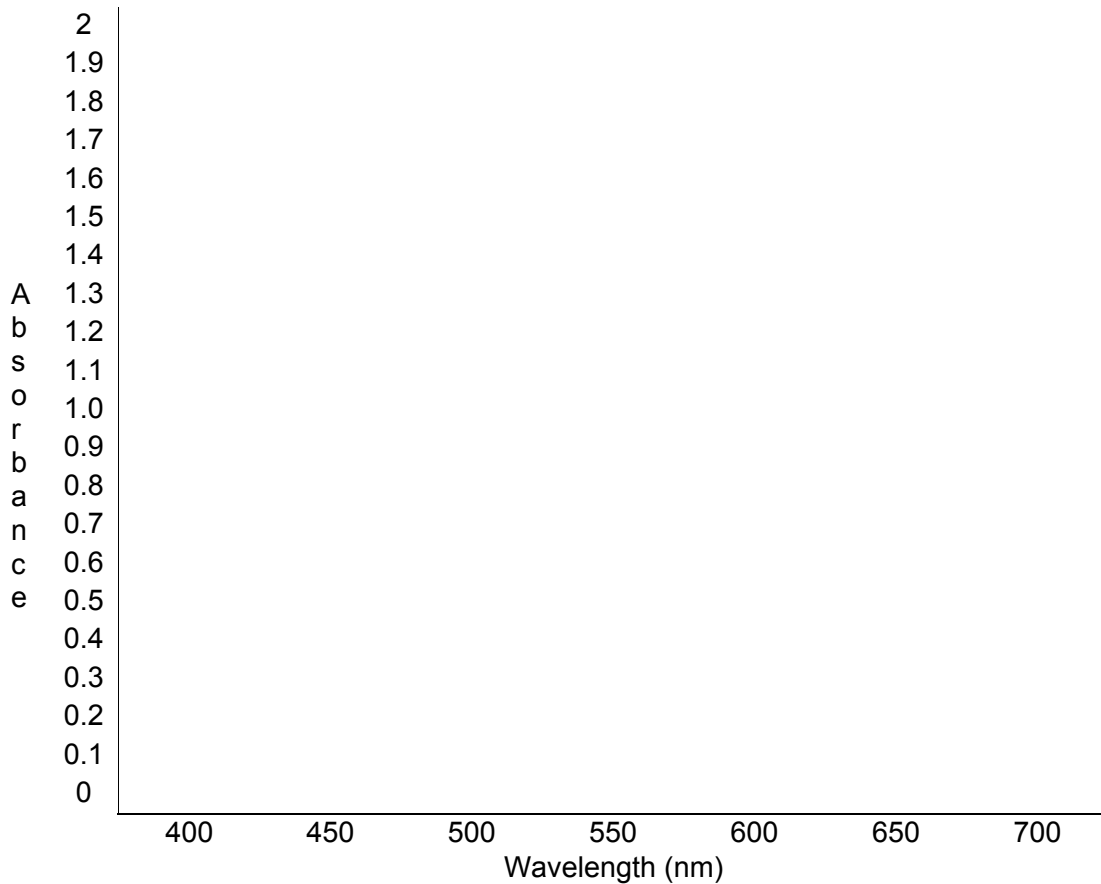
You will measure light absorption at 50 nm intervals from 400 nm → 700 nm for the absorption spectra of the leaf pigments, but you will start at 400 nm.

- Set the wavelength to 400nm and make any other instrument adjustments that are mentioned in the spectrophotometer manual.
- Standardize the absorbance to "0" using the blank cuvette containing the alcohol, following the instructions available in the manual. Each time you change the wavelength selection, you must repeat this step. Remove the blank cuvette. The spectrophotometers should have spaces for four cuvettes.
- Place each of your pigment samples in one of the chambers. Keep track of which chambers the pigments are in.
- Close the cover and read (and record) the **absorbance** on the meter for your first pigment. Repeat for each of the pigments by shifting the cuvette chambers into the correct position for reading the absorbance.
- Record the absorbance numbers for 400 nm for each of the four pigments on the chart on board for the class data.
- Change the wavelength to 450 nm and repeat steps 1 – 5 for 450 nm wavelength.
- Change the wavelength to 500 nm and repeat steps 1 – 5 for 500 nm wavelength.
- Change the wavelength to 550 nm and repeat steps 1 – 5 for 550 nm wavelength.
- Change the wavelength to 600 nm and repeat steps 1 – 5 for 600 nm wavelength.
- Change the wavelength to 650 nm and repeat steps 1 – 5 for 650 nm wavelength.
- Change the wavelength to 700 nm and repeat steps 1 – 5 for 700 nm wavelength.

Absorbance of Leaf Pigments

Wavelength(nm)	Absorbance			
	Chlorophyll a	Chlorophyll b	Carotene	Xanthophyll
400				
450				
500				
550				
600				
650				
700				

Graph the data for each pigment.
Absorbance should be on the y axis and wavelength on the x axis.



Discussion Questions

1. What is the best wavelength for each of the pigments? Do any of the pigments have more than one absorbance peak?
2. Would you expect a plant to be able to photosynthesize if exposed to only the green wavelengths of light?
3. Of what advantage are the accessory pigments to the plant?

One can also measure rates of photosynthesis in different wavelengths to generate an **action spectrum**. This is done by growing plants in light boxes that have just one wavelength of light.