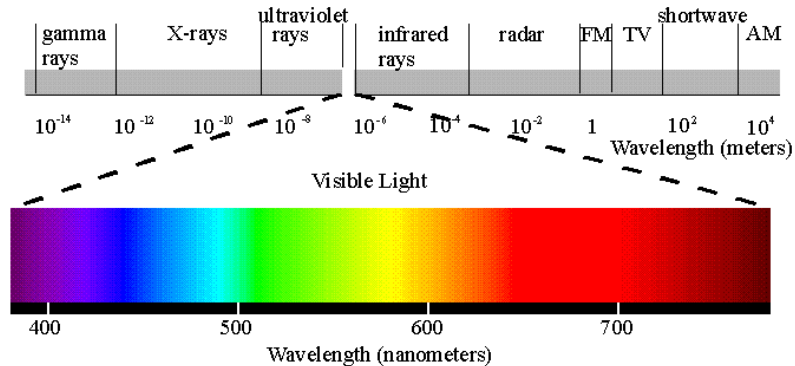


Photosynthesis

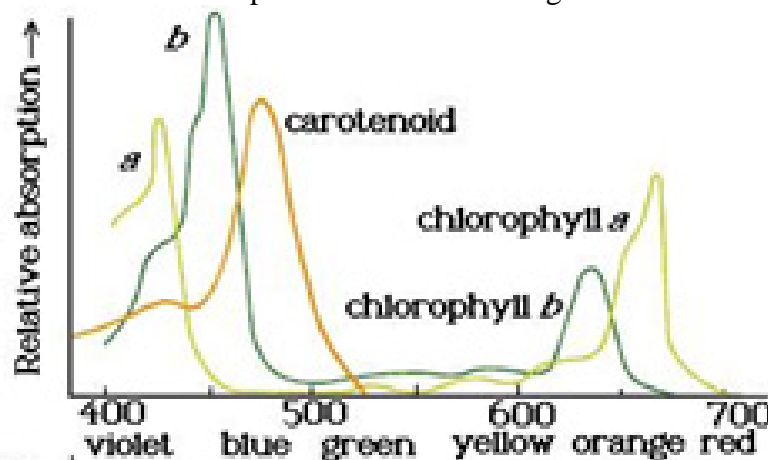
A. Introduction

Plants use the energy from light to make sugars (their food) from CO₂ and H₂O through the process of photosynthesis. Thus, plants are **autotrophs**. This is in contrast to **heterotrophs** such as animals and fungi.

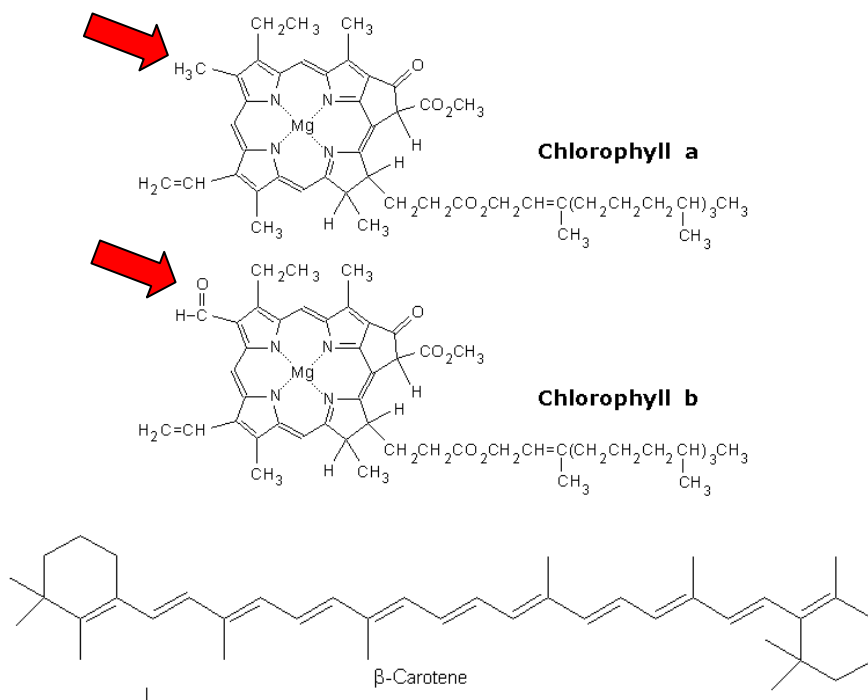


It is known that the light absorbed by plants for photosynthesis falls somewhere within the **visible light** portion of the **electromagnetic (em) spectrum** of radiation that comes from the sun. This visible light portion includes em radiation with **wavelengths (λ)** ranging from just under 400 nm to just over 750 nm. Molecules called **pigments** are, by definition, responsible for the absorption of these wavelengths. We all know that the green pigment chlorophyll is necessary for photosynthesis to occur, but are there are other pigments also involved in photosynthesis?

Autumn in the temperate latitudes brings about changing colors in the leaves of deciduous trees. Before the leaves drop, one can see green fading to yellow, reds, orange, or even purple. Over the years, plant biochemists have identified a variety of different pigments such as **chlorophyll a** (the one molecule absolutely necessary for photosynthesis), **chlorophyll b**, and various carotenoids such as **β -carotene** and **xanthophylls** (see figure on next page). All of these differ in color from one another and so they have different **absorption spectrum** profiles that distinguish them. Today's lab will look for the presence of these through various means.



Above: absorption spectra for chlorophyll a, b, and carotenoids.



B. Pigment Analysis via Paper Chromatography

Spinach leaves are green, so they obviously have chlorophyll. But which chlorophyll, chlorophyll a or b? Furthermore, are the yellow or orange carotenoids that we see during autumn present as well in the green leaves or are they only formed during Autumn? To test your hypothesis, we will make an extract of spinach leaves and subject that extract **to paper chromatography**. Paper chromatography should, if multiple pigments are present, separate them.

Your **INSTRUCTOR** will prepare a pigment mixture for you to analyze by extracting the pigments from spinach leaves. In a Waring Blender, 20g of spinach and 100ml of **ACETONE** are combined and macerated for 3 minutes. This will be followed by vacuum filtration to separate the cellular material from the soluble extract. The pigment extraction will be collected in a 200-400ml beaker and this will be **YOUR SOURCE** for the paper chromatography.

1. Separate individual lipid-soluble pigments via paper chromatography:

- a. Obtain a small amount of the extract in a beaker or vile.
- b. Using a paint brush, place the extract on a pencil line 2.5 cm from the bottom of the sheet of chromatography paper. Add extract to form a dark, thin line. Allow to dry between applications. Label the top in pencil, with your name.
- c. Place 100 ml 9:1 v/v petroleum-ether/acetone solvent into a chromatography tank. The height of this liquid in the tank should be 1.5-2.5 cm. (This may have been done previously by the instructor.)

d. Fold the top of the chromatography paper approximately 1-1.5 cm from the top of the sheet. Hang the folded top edge of the chromatography paper over a string spanning the top of the tank. Make sure the bottom of the chromatography paper is submerged in the chromatography solvent mixture by 1-1.5 cm, but below the line of leaf extract that was applied. **MAKE SURE YOUR PAPER DOES NOT TOUCH THE TANK WALLS OR OTHER PAPERS!!!**

e. After sufficient time to separate any pigments, but before the solvent front reaches the top of the chromatography paper, remove and dry the chromatograms.

f. If multiple pigments are present, then they will have separated according to their different polarities.

How many pigments can you visualize?

What color(s) do you detect?

Can you see the chlorophylls and/or carotenoids? Which?

The chromatography solvent mixture was strongly nonpolar (hydrophobic). With that in mind, if you do see multiple pigments, are the pigments at the top of the chromatogram highly polar or highly nonpolar?

Explain.

Below, List the color bands identified (in order from top to bottom) and PREDICT what pigment they represent:

1. (top)

2.

3.

4.

5. (bottom)

2. Analysis and identification of pigments isolated from Spinach leaves:

Objective: To identify the pigment molecules separated by paper chromatography by determining the absorption spectrum of each and comparing it to the absorption spectrum of known pigments.

1. Turn on Spectrophotometer so it warms up (~15 minutes). Refer to **Appendix A** for the operating procedure of the Spectro Master 415.
2. Isolate an individual pigment by cutting out a single colored band and then cutting the chromatography paper into strips approximately 1 inch long. Be careful not to combine different colored pigments on your cut paper. Then place the pieces into a tape-labeled test tube (use separate test tubes for each pigment being purified).
3. Add 5 ml of petroleum ether/acetone (9:1 v/v) and elute the pigments into solution. **USE THE MINIMUM SOLUTION NECESSARY** to cover the paper! Elute the pigments for 5 minutes or until all color is removed from the paper.
4. Do this for all of your pigment bands. Keep each band separate so that if you have a minimum of four bands, you should have four test tubes in the next step.
5. Add the petroleum ether/acetone pigment extract (minus the paper) to a new glass test tube **OR** simply remove the filter paper with forceps. BE sure that your label does not cover the area of the tube that will fit into the spectrophotometer slot. *Do not mix up your samples.*
6. Adjust the spectrophotometer wavelength to **400nm** and insert a test tube containing 5 ml of petroleum ether/acetone with **NO PIGMENT**. This tube is your **BLANK**. Push the "Set Ref" button to calibrate the % absorbance to zero. Remove the BLANK.
7. Then insert each tube unknown pigment successively and record absorbance for all pigments before moving to the next wavelength.

****IMPORTANT: DO ALL OF YOUR SAMPLES BEFORE MOVING**
TO NEXT WAVELENGTH!**

8. Remove your last sample, increase the wavelength by **20nm** and re-insert the BLANK. Push to the calibrate button and then record the % absorbance for each of your pigments.
9. Repeat step 8 until you have finished with your measurements at **700nm**. Record all values in the provided table.

Table 1. Pigment % absorbance in relation to wavelength.

Pigment #:		Pigment #:		Pigment #:		Pigment #:	
λ (nm)	Absorbance (%)	λ (nm)	Absorbance (%)	λ (nm)	Absorbance (%)	λ (nm)	Absorbance (%)
400		400		400		400	
420		420		420		420	
440		440		440		440	
460		460		460		460	
480		480		480		480	
500		500		500		500	
520		520		520		520	
540		540		540		540	
560		560		560		560	
580		580		580		580	
600		600		600		600	
620		620		620		620	
640		640		640		640	
660		660		660		660	
680		680		680		680	
700		700		700		700	

- 10. Plot** the % absorbance as a function of wavelength for EACH UNKNOWN PIGMENT. Graph paper is attached to the back of this manual. If using one graph, use different colors or data symbols or line patterns to clearly distinguish one line from the next. If using one graph per pigment, then different colors and symbols, etc. is not necessary. Use Figure 7.5 on page 119 of your textbook or the figure on page 1 of this lab manual to IDENTIFY the pigments. Clearly label each pigment band as the number it was on the paper AND as the type of pigment it represents.

Questions:

How many types of pigments could you identify from your green spinach leaves and what are their identities?

Thus, are the autumn colors you see in leaves the result of pigments that were present all summer or of a succession of pigments produced in the fall only?

Do the pigments absorb all wavelengths equally or do they exhibit preferred absorption?

Do the absorption patterns from the different bands differ from one another?

Do any of the bands being analyzed seem to have similar patterns?

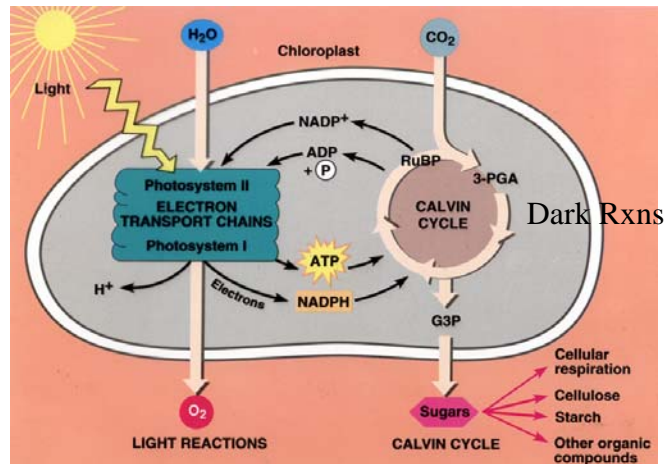
What pigment(s) have you identified?

Relate the pigment identified to the color of the leaf and explain why it was or was not originally seen in the leaf.

C. Photosynthesis produces measurable products

Photosynthesis can be divided into two phases referred to as the **light reactions** (also known as the thylakoid reactions) and the **dark reactions** (more appropriately called the light-independent reactions). Both stages produce biological molecules that can be detected and measured and represent

photosynthesis. The dark reactions produce the **carbohydrate** molecules that are the final energy storing forms of the energy originally harvested from visible light. The light reactions produce the energy needed for the dark reactions (i.e. ATP and NADPH) but also produce **O₂** as a waste product. The dark reactions consume **CO₂**. We can measure the rate of CO₂ consumption as a measure of photosynthesis.



The figure to the right

demonstrates the overall process:

The reaction of photosynthesis can be written:



2. The effect of different light wavelengths on the rate of photosynthesis:

Objective: To measure the rate of photosynthesis through the rate of carbon dioxide consumption and to determine the effect of light quality (wavelength) on photosynthesis.

Notes: To do this, we will use a Vernier CO₂ gas probe connected to a computer and, using the Logger-Pro data analysis computer program, record fluxes in CO₂ concentrations in the atmosphere of a small plastic bottle containing living plant material as it is exposed to several different light regimes. You will be responsible for setting up the experimental conditions, initiating the data collection, recording the data generated from the Vernier CO₂ gas probe, graphing and interpreting your data.

Procedure: (Each group of 4 should perform one group experiment as follows:

1. Under the Windows operating system, OPEN the software for the photosynthesis experiments as follows: **Logger Pro 3.8**

Two windows will be present on the right side of screen, one representing CO₂ and one representing O₂. Live readings will be reported in the lower left corner of the screen for both types of gas.

2. **IMPORTANT:** Confirm that the units for both O₂ and CO₂ are in **ppm** (parts per million). If the units are not ppm, change them as follows (ex. O₂):

Experiment
Change Units
Oxygen Gas
Select "ppm"

3. **IMPORTANT:** Set time and collection rate for the experiment:
Experiment
Data Collection
Length = 90 minutes
Sampling Rate = 4 per minute
4. Set up the CONDITIONS for the experiments as described below (step ?):
5. Once the first condition is set-up, CONFIRM with the instructor before “COLLECTING” the data. Following confirmation, click on COLLECT.
6. **EXPERIMENTAL CONDITIONS:**
- Place a water-filled heat shield between the light and the plant specimen.
 - Use a light meter to measure the QUANTITY of light (lux) coming from the light source (through the water). Mark, with tape, the positions on the bench that represent
 - 20,000 lux = High Light
 - 15,000 lux = Intermediate Light
 - 10,000 lux = Low Light
 - Weigh leaf material. Use either 2 leaves of ivy or four leaves of hibiscus. Place the leaves in the chamber that has two access sites. Curl the leaves so that the adaxial (dark/top side) of the leaf faces outward.

Weight = _____
 - Insert the CO₂ and the O₂ probes into the chamber. Do not let the probes crush/push the leaf material to one end of the chamber.
 - Wrap the chamber in foil and begin the collection = “dark” = 0 lux.
 - Record the data for both O₂ and CO₂ for every minute in each light environment (including dark).
 - After 10 minutes in the “dark” = 0 lux treatment, remove the foil and place the plastic chamber so that it is on the 2,000 lux mark (facing the light source). Record the data every minute for 10 minutes. DO NOT RESTART COLLECTION!
 - Move the chamber to the 10,000 lux position and repeat the data collection (every minute for 10 minutes). DO NOT RESTART COLLECTION!
 - Move the chamber to the 20,000 lux position and repeat the data collection (every minute for 10 minutes). DO NOT RESTART COLLECTION!
 - IMPORTANT:** If the CO₂ level plateaus during a treatment, turn the light off, cover the chamber with aluminum foil, and allow the leaf tissue to rest for 10-15 minutes.

7. Plot the data record above. Prepare two graphs, one comparing the change in CO₂ over time for each light condition and one comparing the change in O₂ over time for each light condition.
8. From the graphs, calculate the rates of oxygen production/consumption and carbon dioxide production/consumption for each light treatment. Use a linear trendline to calculate the slope (slope = rate as “gas” ppm/min)

Condition	Rate (O ₂ ppm/min)	Rate (CO ₂ ppm/min)
0 lux		
2000 lux		
10,000 lux		
20,000 lux		

a) *How does increasing light affect the rate of change of Oxygen?*

Explain your answer:

What specific stage of photosynthesis is involved?

b) *How does increasing light affect the rate of change of Carbon Dioxide?*

Explain your answer:

What specific stage of photosynthesis is involved?

c) *How did O₂ and CO₂ change during the dark treatment?*

What process occurs at 0 lux (dark) that influences gas use/production:

Explain your answer:

d) *At 20,000 lux, which gas responds first to the light exposure? Justify your answer:*

e) *You may have observed a plateau effect during your measurements. Give a possible reason for why this may have happened.*

9. **Independent Investigation**: Use the setup described above to investigate the impact on gas exchange for one of the following scenarios:

a) Normal Hibiscus leaf vs. Frozen Hibiscus leaf

- weight four hibiscus leaves and then place them in the freezer (-20C) for 60 minutes
- remove from the freezer, thaw for 5 minutes and then use as described
- compare the rates of gas exchange (O₂ and CO₂)

-Explain your results:

b) Normal leaf vs. desiccated leaves

- weigh the leaf material provided (use same species as used previously)
- measure the rates of gas exchange as previously described
- compare the rates of gas exchange (O₂ and CO₂)

-Explain your results:

c) Young ivy leaf (light green) vs. Mature ivy leaf (dark green)

- collect fresh young leaves (light green) from the greenhouse
- weigh the material
- measure the rates of gas exchange as previously described
- compare the rates of gas exchange (O₂ and CO₂)

-Explain your results:

*****IMPORTANT** – for comparisons, you will need to calculate as **gas ppm/min/g*****

Appendix A: Operation Instructions for the Spectro Master Model 415

Basic Operation:

1. Turn on and allow it to warm up for 10-15 minutes.
2. Switch the display to absorbance (abs) rather than transmittance.
3. Adjust with dial on right to the desired wavelength.
4. Open chamber lid and adjust black filter knob to the correct filter number that is appropriate for the wavelength.
5. Place a reagent blank in the chamber and push “set ref” to calibrate the machine with zero absorbance for a blank.
6. Place sample(s) to be read into chamber and record absorbance.
7. To read at a different wavelength, repeat steps 2-6.

