

LABORATORY EXPLORATION

Physical Properties of Photosynthetic Pigments

In this week's lab, you will extract and examine some of the physical properties of the photosynthetic pigments plants use to capture photons for photosynthesis. Next week, you should be able to apply what you learn today and devise an interesting experiment in which you will manipulate the light-capturing reactions of photosynthesis by using isolated, live chloroplasts. So be very careful in your work today, and don't let the thinking stop here. Before you leave lab today, you should meet with your lab team members and start to think about a problem you'd like to address about the light reactions and chloroplast function for next week.

I. Examination of Photosynthetic Pigments.

A **pigment** is any substance that absorbs and reflects light in a characteristic fashion. Light is energy, and when absorbed by a pigment, that energy changes in form. Sometimes the energy is lost as entropy, as when the hood of your black car absorbs sunlight and heats up (increased random molecular motion). In one of the most amazing biological systems of all, **photosynthesis**, light absorbed by plant pigments is not lost. Rather, it is converted by a series of electron transfers into electrical energy (the light-dependent reactions of photosynthesis), and then by a complex series of chemical reactions into the chemical energy of covalent bonds (the light-independent reactions of photosynthesis).

Consider that without plants and their light-capturing pigments, life on earth as we know it would not exist. Before you begin today's laboratory exercise, be sure to read and understand the appropriate sections in your textbook on photosynthesis. Recall the roles of chlorophyll a and the antenna pigments, chlorophyll b and carotenoids. In today's laboratory exercise you will extract photosynthetic pigments and examine some of their physical properties.

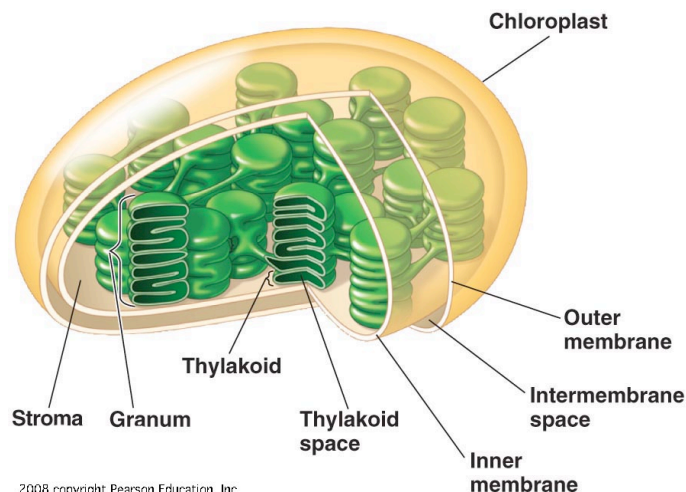


Figure 1. The chloroplast.

Chlorophylls and carotenoids are embedded in the thylakoid membranes inside the chloroplasts (Figure 1). In this exercise, you will extract these pigment molecules from

a species of plant you have probably eaten (sometimes under protest), but probably never considered as anything other than something to get caught between your teeth at embarrassing moments: spinach (*Tetragonia tetragonioides*, Family Aizoaceae). Spinach has dark green leaves, indicating a high concentration of photosynthetic pigments. You will isolate the pigments from the living cells, separate the various types of pigments from one another, and examine their absorbance spectra and other physical properties.

As you do today's extractions and exercises, consider the implications of the pigments' physical properties and what this might imply about the process of photosynthesis. Near the end of the laboratory, you and your team will engage in a bit of hypothesis formulation and experimental design. Consider the physical appearance of the plant you are using. How does it compare to other plants with which you are familiar? What is the significance of pigment concentrations in a plant? Do you think all plants have the same pigment concentrations and in the same ratios? These are only a few of the many possible avenues of research you may wish to consider.

A. Extraction of pigments from spinach

Follow this step-by-step guide for a lovely extract of chlorophylls and carotenoids. Don't forget that the techniques you use here might be useful to you in the future.

1. In a 400 ml beaker, start a boiling water bath on your small hotplate.
2. Place 40 ml of 85% ethyl alcohol (EtOH) in a clean 250 ml beaker and heat it on the large electric hot plate in the center of your lab table (you will be sharing this hotplate with the other groups at your table). Heat the solution well, but *do not allow it to boil*. (CAUTION: Do not allow open flames near the highly flammable ethanol!)
3. Select five (large to medium sized) spinach leaves and remove their petioles (leaf stems). Bunch the leaves, hold them loosely in a **test tube clamp** (Figure 2d) and immerse them for 10-15 seconds in the beaker of boiling water. This process breaks down the internal and external cell membranes, facilitating pigment extraction.
4. Thoroughly blot dry the scalded leaves between paper towels. If the leaves are too wet, they will dilute the ethanol, and you will be unable to extract enough pigment to do the following exercises.
5. Our non-polar pigments will readily dissolve in alcohol. Place the blotted leaves in the hot EtOH in your beaker. This is a somewhat delicate step: the EtOH must be hot enough to extract pigments, but not so hot as to boil over. To prevent overheating, set your *hot* EtOH beaker on the lab table and steep the leaves for about 3 to 5 minutes. When ready, the liquid should be a very deep, somewhat opaque green. *Voila!* You have extracted the pigments responsible for photosynthesis.
6. Draw up four droppersful of this alcohol pigment extract and place in a **shell vial** (Figure 2a). Set this aside for use in parts B and D.
7. Fill an 18 x 150 mm **test tube** (Figure 2b) half full of the extract; set aside for use in part C.

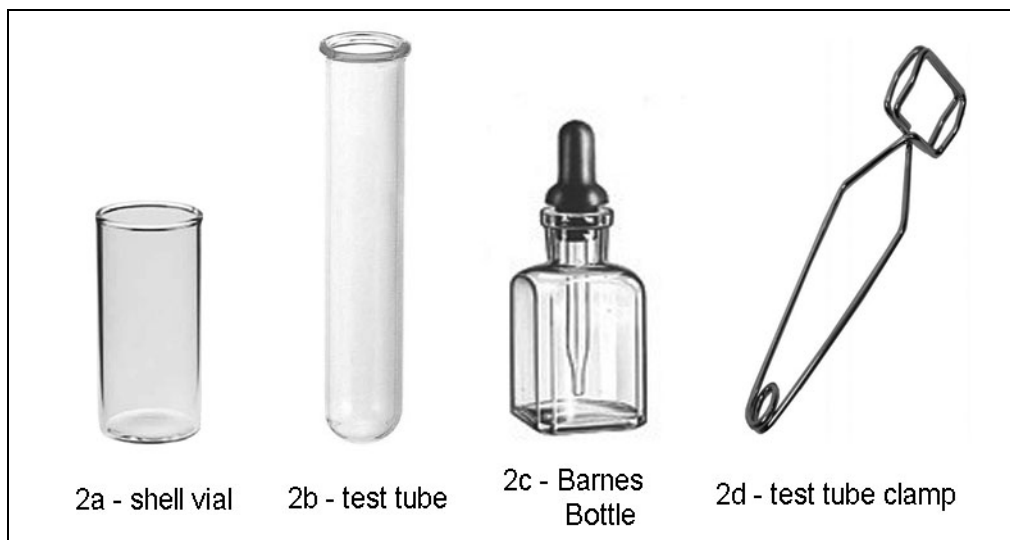


Figure 2. Common tools of the trade: 2a. shell vial; 2b. standard test tube; 2c. Barnes Bottle (with stopper) ; 2d. test tube clamp

B. Separation of chlorophylls and carotenoids via paper chromatography

Chlorophylls and carotenoids have different molecular structures and hence, different physical and chemical properties. Before the next laboratory, you and your team should do further research on the molecular structure of these types of molecules, to better understand their physical properties. Use what you learn to consider possible problems to explore in the next lab.

Each pigment has its own affinity for solid matter and its own degree of solubility in polar and non-polar solvents. Because of these differences, pigments can be separated via a process known as **paper chromatography**.

The term chromatography (from the Greek *chrom*, "color" and *graph*, "to write") was first introduced in 1906 by the Russian botanist Michae Tsweet. Paper chromatography separates a mixture of compounds based on their different affinities for a stationary phase (paper, gel or other material) and their differential solubility in a moving phase (a suitable liquid solvent). In our case, you will see how the particular affinities and solubilities of the various photosynthetic pigments from a spinach chloroplast cause them to migrate up a piece of chromatography paper at different rates. The migration rates depend upon

- 1) The composition of the solvent flowing up (or down) through the paper and,
- 2) The degree to which migrating molecules are absorbed by the type of paper used.

The finished product of paper chromatography, a sheet of paper with separate, identifiable components of a previously homogeneous mixture, is called a **chromatogram**.

1. Chromatography Procedure I.

We will use 1mm Whatman chromatography paper and petroleum ether as a non-polar solvent.

1. Obtain a strip of chromatography paper (9" x 1") from the front desk. Handle it only by its edges! Oil from your fingers will affect the migration of your pigments.
2. Lay the strip across the top of a beaker, horizontal to the tabletop (Figure 3)
3. At your station is a tall chromatography jar capped with a cork or rubber stopper. Note the level of the clear chromatography solvent in the jar. You will be preparing your paper to be placed on its end into this solvent, and the line of pigment you are about to draw must not drop into the solvent; it should be above the solvent level.
4. Use a capillary tube to draw up small amounts of pigment extract from the shell vial (from step A-5 above). Deposit a thin line of pigments *parallel to the narrow end* of the paper, about 2.5 cm from the end. Draw the end of the capillary tube quickly and lightly across the strip. (It is wise to practice using the capillary tube on a paper towel before starting your chromatogram, if you're not sure how to do this.)
4. Allow the alcohol to evaporate for a few minutes, then repeat the procedure several times until you have built up a narrow, dark green line across the strip. Allow pigment to dry thoroughly. Set the shell vial aside for use in Part D.

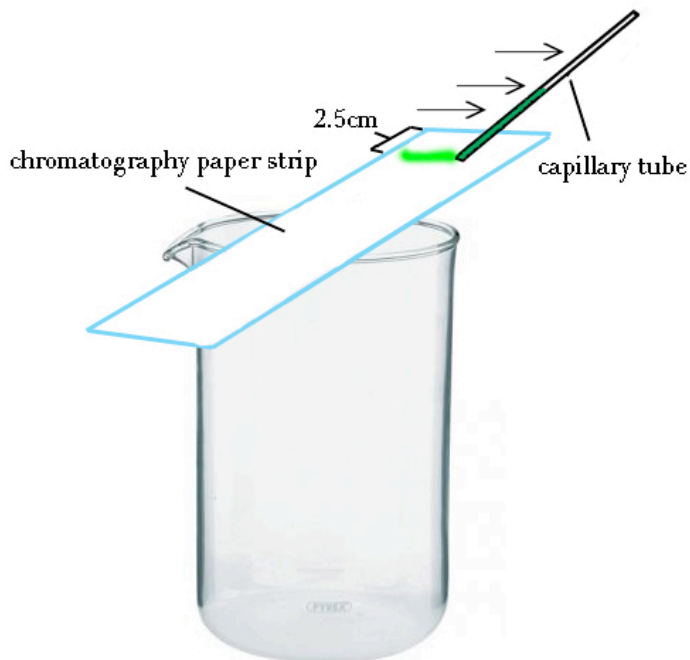


Figure 3. Chromatography Paper Loading. Position a strip of chromatography paper across the top of a beaker, for stability. Carefully touch and pull a capillary tube (full of pigment extract) across the width of the strip, approximately 2.5cm from the bottom of the paper. The pigment line should be high enough on the paper so that it is NOT immersed in solvent when you place it in the chromatography jar.

5. Remove the stopper from your chromatography jar and gently lower the chromatography paper into the jar--pigment line closest to the solvent--but making

sure not to immerse the solvent. Move the jar and solvent as little as possible during this procedure, to avoid splashing the paper.

- Loosely re-stopper the jar with the strip held in position by the stopper. *Once the jar is set up, DO NOT MOVE OR JOSTLE IT.* The chromatography solvent is a combination of petroleum ether and acetone, both of which are very volatile. Observe movement of the pigments up the paper.
- While you wait for your chromatogram to develop, you may proceed to Part C. However, be sure to monitor the chromatogram's progress closely so that the pigments do not migrate all the way to the top of the strip.
- Once you have obtained sufficient pigment separation, remove the strip and allow it to air dry.

Some carotenoids ride the solvent front, and should form a pale yellow wash across the top of your chromatogram. Farther down the paper, a darker yellow-orange pigment band is formed by the xanthophylls, a class of yellow carotenoids. Chlorophyll a should form a dark green band below the xanthophylls, and chlorophyll b a lighter green band near the bottom of the chromatogram.

Do all of these pigments function in Photosystems I and II? _____

What is the function of each pigment?

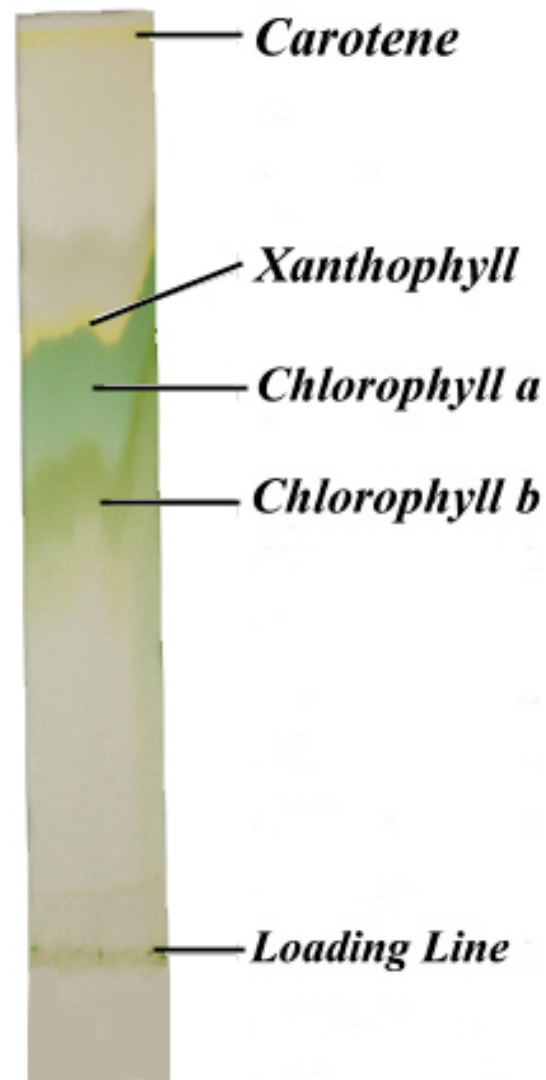


Figure 4. A sample chromatograph

Compare your results with those of other teams. Before leaving lab, cut the strip lengthwise, label each pigment and give one strip to each team member as a sample for his or her lab notebook. Alternatively, photograph the chromatogram with your cell phone or other camera, and be sure each team member gets a copy.

2. Calculating an R_f value

The relative position and color of the bands on a chromatograph may not be sufficient for positive identification. To more accurately identify what is in the sample, one can quantify the movement of the substances by comparing the migration rate of the solute (in this case, photosynthetic pigments) to that of the solvent itself.

For each separated substance, you will calculate an **R_f value**: the ratio of the distance moved by the pigment to the distance traveled by the solvent. The R_f of a substance can be useful in identifying it on chromatograms. By comparing R_f values of substances present in an unknown sample to the R_f values of known standards, the investigator can more confidently identify his/her unknowns.

$$R_f = \frac{\text{Distance the substance moved from the base line}}{\text{Distance the solvent moved from the base line}}$$

Calculate an R_f value for each of your photosynthetic pigment bands. You will notice that the bands are not completely straight. Which area of the pigment band do you think is the most useful in comparing migration rates of pigments with one another? The top of the pigment line? The bottom? An average? Make a decision and write your rationale here:

What pigments are present in your chromatogram?

What is the R_f value for each pigment?

Why do you think the R_f values are different for each pigment type?

C. Separation of Pigments by Differential Solubility

As you know from the previous exercise, chlorophylls, and carotenoids have different solubilities in various non-polar solvents. In this exercise, you will be able to separate chlorophylls from carotenoids because one is more soluble in ethanol, and the other is more soluble in petroleum ether.

1. To the large test tube containing cooled ethanol-based pigment extract (Part A, Step 6), add enough petroleum ether to nearly fill it.
2. Cover the test tube opening with a small piece of Parafilm, and place your thumb over the covered opening. Gently tilt the tube 10 to 20 times until the contents are thoroughly mixed. As you do so, *gradually* lift your thumb to release pressure build-up caused by the highly volatile ether (CAUTION: release with care to avoid spraying ether all over yourself and your partners or rivals!)
3. Place the test tube in your test tube rack and allow the contents to settle for about five minutes. If two distinct layers do not form, try adding a drop or two of water and repeat the mixing procedure. Do not proceed until separation is complete (Ask your lab instructor for help, if necessary.)

Ether, less dense than ethanol, floats on top in your test tube. What color is the ether layer? _____

Which pigments are dissolved in it? _____

What color is the ethanol layer at the bottom of your test tube? _____

Which pigments are dissolved in it? _____

Of what significance might this be? _____

Retain this test tube for use in Parts D and E.

D. Fluorescence: capture & release of solar energy

Before proceeding, review the electromagnetic spectrum in Figure 5. What color light (photons) have the highest energy (shortest wavelength)? Which have the lowest energy (longest wavelength)? Which photons (colors) do you predict are most likely to be captured by plants, and their energy packaged for later use? Why? _____

When a molecule of chlorophyll *a* absorbs a photon, one of its orbiting electrons is boosted to a higher energy level. In a living thylakoid membrane, this electron would be accepted by a protein called the **primary electron acceptor**, and sent through the cyclic or non-cyclic photophosphorylation chain, ultimately to deliver the energy of the absorbed photon to the cellular machinery that packages it as ATP or NADPH. (You will learn more about this in the next laboratory.)

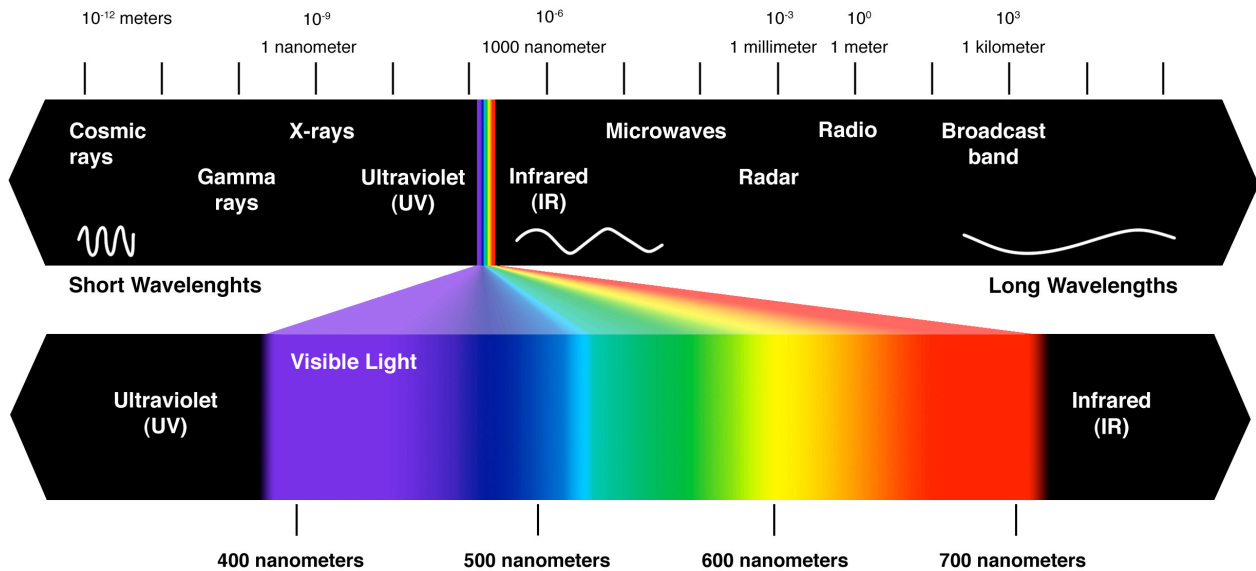


Figure 5. The electromagnetic spectrum. Visible light ranges from approximately 380nm (violet) to 750nm (red).

Since you have pulled them out of their cozy membrane homes, your extracted chlorophyll molecules are suspended in the *absence* of primary electron acceptor proteins. The electrons of the pigments can still be boosted to a higher orbital when they absorb a photon, but there's no primary electron acceptor to carry them on their photosynthetic journey.

Unable to enter the photophosphorylation cycle, the chlorophyll's excited electron falls from its unstable, high-energy orbital back into its original orbital. But what happens to the photon energy that was originally absorbed? As you know from Newton's First Law of Thermodynamics, energy can be changed in form, but it cannot be created or destroyed. So where has the photon energy gone?

When the photon was first absorbed, its energy was momentarily changed from *light* energy to *electrical* energy (the excited electron). As the excited electron falls back to its original, stable orbital, it essentially "spits out" the extra energy of the absorbed photon. That energy is re-emitted as *light* energy--a photon once more!

Because energy transductions like this are never 100% efficient, the photon emitted is lower in energy than the one that was originally absorbed. Some of the original photon energy is lost as **entropy** (randomized energy unavailable to do work). When the remaining energy comes back out of the system, it appears as long wavelength, low energy *red* photons.

You can observe this phenomenon, **fluorescence**, in your chlorophyll extract.

1. With a Pasteur pipet, extract the ether/chlorophyll layer from your test tube from Part C. Place it in a clean Barnes bottle. Your pigment solution must be clear, with no turbidity: TAKE CARE NOT TO EXTRACT ANY OF THE "SLUDGY" INTERFACE LAYER BETWEEN THE ETHER AND THE ETHANOL!
2. Hold the bottle close to the 100 Watt desk lamp at your table. If possible, view the

bottle from a direction such that the background is your black lab tabletop. Under such bright irradiation, the solution in the bottle should appear to change from clear green to blood red. The intensity of the color will depend upon the chlorophyll concentration of your solution (and, hence, on your original extraction technique!).

3. Isn't that the *coolest* thing? Well, we think so. But we don't get out much.

What is the significance of fluorescence? _____

Do you think it occurs in living plants? _____

Why or why not? _____

E. Absorbance spectra of photosynthetic pigments

Review the light-dependent reactions of photosynthesis in your textbook. Understand the role of the photosynthetic pigments before you begin this next section.

The range of wavelengths absorbed by a pigment (such as a chlorophyll) is known as that pigment's **absorbance spectrum**. The range of wavelengths that drive a particular photochemical reaction (e.g., photosynthesis) is known as that reaction's **action spectrum**.

When the action spectrum of a particular reaction corresponds to the absorbance spectrum of the pigments present in the vicinity of the reaction, the investigator may hypothesize that the pigments are in some way involved in that chemical reaction. Further studies can then be undertaken to test predictions based on this hypothesis.

Study the absorbance spectra of chlorophyll *a*, chlorophyll *b* and typical carotenoids (Figure 6). Compare these with the action spectrum of photosynthesis (Figure 7).

What is the significance of the overlap (or lack thereof) between the absorbance spectra and the action spectrum? _____

Why might it be advantageous to have several photosynthetic pigments instead of just one? _____

In this part of the laboratory, you will perform a qualitative analysis of the absorbance spectra of the pigments you extracted in Part C. To do this, you will use a device called a **spectroscope**. Your lab instructor will demonstrate its use.

1. Before you begin, look out the lab window through the spectroscope to familiarize yourself with the unfiltered spectrum of typical daylight. What colors, and in what order, do you see reflected on the inside of the spectroscope?

NOTE: If you are in a night lab, use the 100-Watt desk lamps provided. These tend to be slightly more rich in red wavelengths than sunlight, but will work well for your comparison.

2. To the Barnes bottle of chlorophyll extract you used in Part D, add the same solvent (ether) until the contents come up to the “shoulder” of the bottle.
3. Hold the spectroscope up horizontally and look through it. You should be able to see reflected visible spectra shining against the dark walls of the spectroscope.
4. While still looking through the spectroscope, press the Barnes bottle flat against the aperture opposite your eye. Compare the full daylight spectrum to the pigment-filtered spectrum by moving the bottle away from the aperture, then back again.
5. Examine each color individually, noting its **presence**, **absence** and **intensity** as you move your chlorophyll sample into and out of the spectroscope window. Use the colored pens/pencils provided to record your observations in Figure 8.
6. Retain your chlorophyll solution for use in Part F.

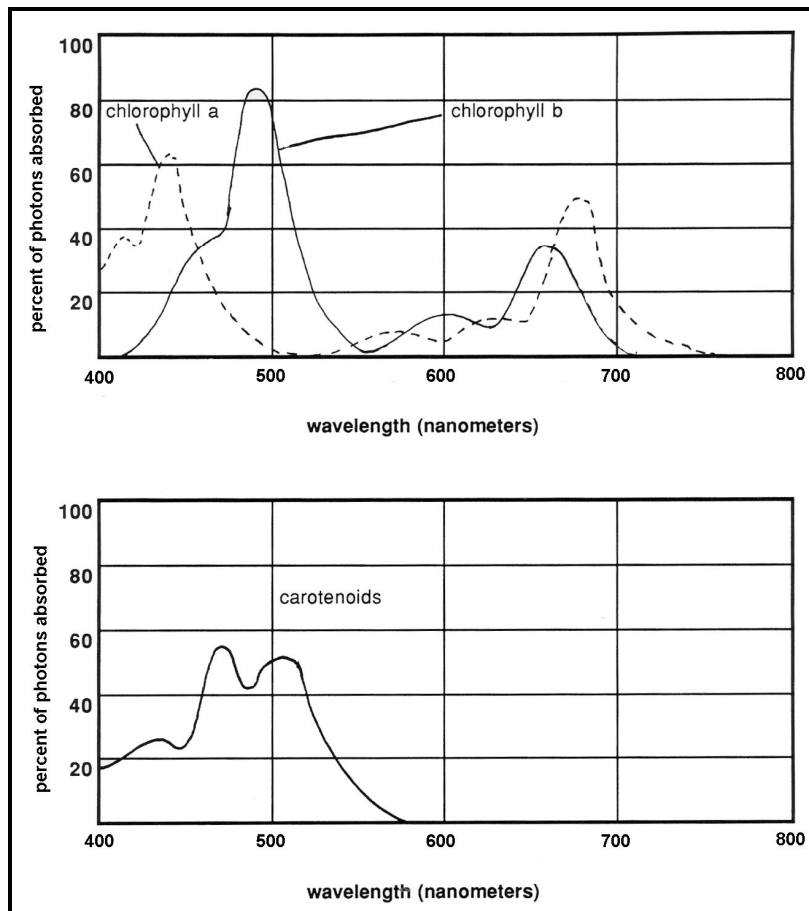


Figure 6. The absorbance spectra of chlorophylls *a* and *b* (above) combined carotenoids (below).

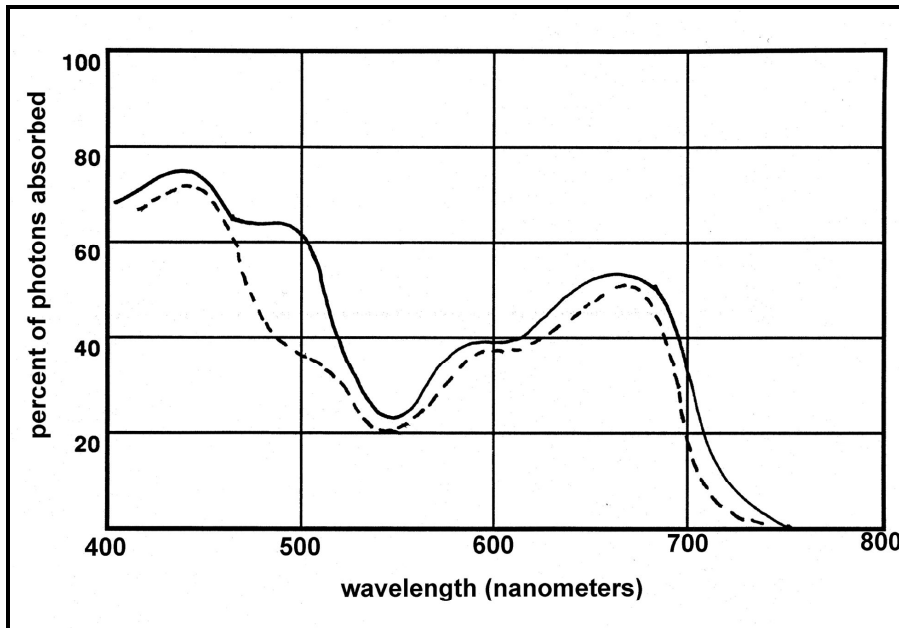


Figure 7. The approximate absorption spectrum of the combined photosynthetic pigments of a typical plant, (solid line) superimposed on the action spectrum of photosynthesis.

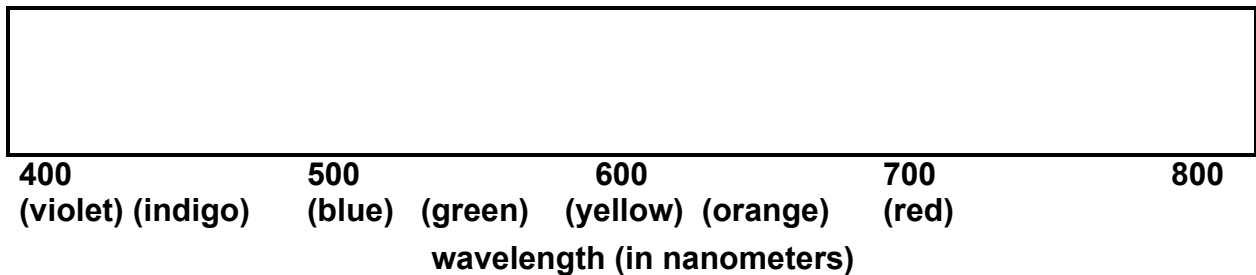


Figure 8. Qualitative absorbance spectrum of chlorophyll solution, with colors (as perceived by the human eye with normal color vision) listed at their approximate wavelengths.

6. With a Pasteur pipet, extract the cloudy ether/ethanol interface layer in your large test tube from part C.
7. With a *clean* Pasteur pipet, extract the carotenoid/ethanol solution from the test tube and place it in a clean, empty Barnes Bottle. Add additional ethanol until the bottle is full enough to cover the spectroscope aperture.
8. As you did for the chlorophyll/ether mixture, study and record the qualitative absorbance spectrum of the carotenoid solution. Use Figure 9 to record your results. When you have finished, you may discard the carotenoid solution.

Which colors are absorbed by the chlorophyll solution? _____

Which colors are transmitted or reflected? _____

Which colors are absorbed by the carotenoid solution? _____

Which colors are transmitted or reflected? _____

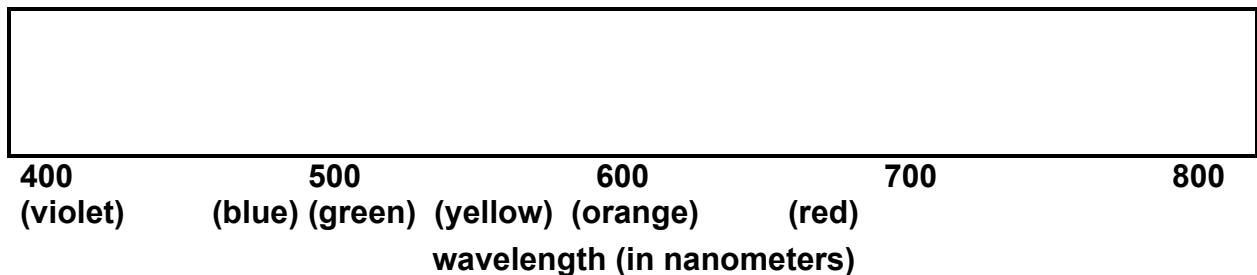


Figure 9. Qualitative absorbance spectrum of carotenoid solution, with colors (as perceived by a trichromatic human) listed at their approximate wavelengths.

What is the relationship between light absorbed and the energy available for photosynthesis? _____

Review Figure 7, showing the relative effectiveness with which various wavelengths of light drive the light dependent reactions of photosynthesis. The two spectra diverge in the blue region of the visible spectrum, which corresponds to the absorbance of carotenoid pigments. This is because carotenoids are far less efficient at driving photosynthesis than are chlorophylls. If this is so, why do plants use carotenoids at all? Consider this in an evolutionary context.

F. Separation of Chlorophylls a and b by Paper Chromatography

In the following exercise you'll learn yet another paper chromatography technique, and use it to separate chlorophyll a from chlorophyll b.

1. From the front desk, obtain an 6 x 6 cm piece of chromatography paper. Take care to keep the paper free of dirt and finger prints.
2. Pour the contents of the Barnes bottle in part E, step 5 into a 250 ml beaker. Take this beaker and your paper to the hood at the back of the room.
3. Place the paper into the jar marked "chromatography dip" (the "dip" is 100% petroleum ether), screw on the cap, and shake it gently until the paper square is completely wet.
4. Drain the surplus and set the paper upright in the beaker of pigment-ether mixture, with its bottom edge immersed in the solution.
5. Allow the pigments to travel up the paper for 5-10 minutes. When the pigments have formed separate layers, remove the paper and air dry it on a paper towel.

When the chromatogram is dry, label the layers and cut strips for your notebooks. (Or photograph and share, as before.)

Which band of pigment is chlorophyll a? _____
 Which band is chlorophyll b? _____
 Are any other pigments present? _____

II. Photosynthesis and the Scientific Process.

Now that you and your partners understand something about the nature of photosynthetic pigments, reconsider their source: living plants. The techniques you used in laboratory today are useful for demonstrations and observations, but you might also put them to good use in experimental design.

For the next ten minutes, you and your partners should sit at your station (or even sit outside on the steps and observe the myriad diversity and fluffiness of the plant life all around you) and do what you do best as a budding scientist: Consider what you do *not* know about the photosynthetic process. Make observations. Observations, as you should know by now, are the triggers of the scientific process.

Once you have observed something interesting, consider what are the possible explanations for this observation. List as many possibilities as you can, trying to consider every option. This is the basis of strong inference: working from a point of knowledge and known theories, pose hypotheses to explain an observation.

Finally, consider how you would test each of your hypotheses by making mutually exclusive predictions about the results of an experiment you design to test a particular hypothesis. By now, you should be getting better at doing this without too much agony.

Observation:

Hypotheses about cause(s) of your observation:

1. _____

Prediction: _____

2. _____

Prediction: _____

3. _____

Prediction: _____

4. _____

Prediction: _____

Choose ONE of the above hypotheses and design a simple experiment to test it. What are the possible outcomes of the experiment, and the explanations for each possible outcome?

Materials and Methods:

Type of data collected:

POSSIBLE OUTCOMES:

Outcome 1:

Explanation:

Outcome 2:

Explanation:

Outcome 3:

Explanation:

Outcome 4:

Explanation:

You may wish to use this week's thoughts about photosynthesis as a springboard for designing an experiment next week using live, extracted chloroplasts from spinach. So start team-planning now for the big finish!