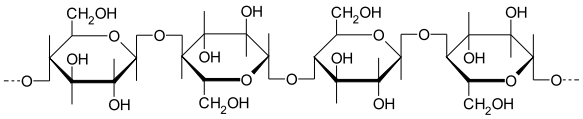
The Separation and Spectral Identification of Leaf Pigments

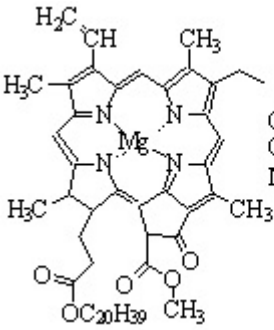
The separation and identification of the components of leaf pigments involves two important techniques in chemistry. The first is a separation procedure called chromatography. There are a number of different types of chromatography such as HPLC (high performance liquid chromatography), GC (gas chromatography), TLC (thin layer chromatography), and paper chromatography. While they have different names and different mobile or stationary phases, they all involve the same principles. When the mobile phase, a liquid or gas, passes over a solid or stationary phase, the substances in the mixture are separated by their attraction to the stationary phase or solubility in the mobile phase. In our experiment, the mobile phase will be a mixture of C5-C6 hydrocarbons called petroleum ether (PE), while the stationary phase will be filter paper composed of pure cellulose. Note the adjacent cellulose structure with its large number of polar -OH groups.



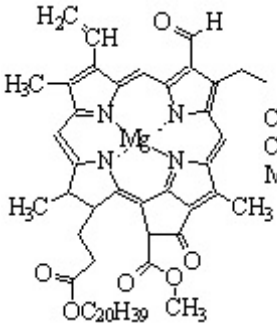
We will prepare our chromatogram by placing a small amount of petroleum ether containing a small amount of acetone in a test tube. The acetone is used to slightly increase the polarity of the solvent. Pieces of the leaf under study will then be ground in a mortar with a pestle to crush the leaf structure and release the pigment. The pigment:acetone solution is drawn into a piece of capillary tubing and transferred onto a filter paper strip. The paper is then placed into the test tube where the separation process will occur. At first, the petroleum ether/acetone mobile phase will rise quickly on the paper by capillary action as it has only a slight attraction for the cellulose dissolving pigment as it moves along. Here is where the separation begins. Some of the pigments have little or no attraction for the paper and will be carried along with the solvent. The rule, "likes dissolve likes," applies here. Others have a number of non-bonded electrons on highly electronegative elements such as oxygen or nitrogen. They are attracted to the polar -OH groups on the paper, moving slowly. We must also consider the molar mass of the molecules involved. High molecular mass molecules will have greater London Dispersion or van der Waals forces which will retard their movement. This process of dissolving and dragging will continue up the filter paper chromatogram. Substances with a lower attraction for the paper and, hence, greater solubility in the petroleum ether, will travel faster and farther than the rest of the pack. Those with the greatest attraction will travel the slowest and, therefore, will remain at the bottom of the paper. In the end we will have a paper strip with separated bands of colored pigments similar to the adjacent chromatogram.

A typical chromatogram

Let's look at the chemical structures of some of the substances we will expect to see on our completed chromatogram. The two obvious compounds are the two chlorophylls. Their only difference is that chlorophyll A has a methyl group, $-CH_3$, in the upper right hand corner of the molecule while chlorophyll B has an aldehyde group, -CHO, in the same position. The chlorophylls will be conspicuous on our chromatogram as they are the only green leaf pigments we will observe.

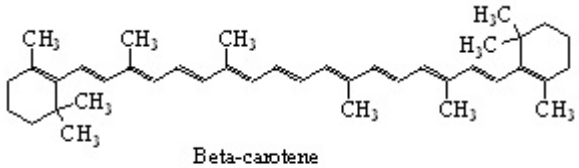


Chlorophyll a
$C_{55}H_{72}O_5N_4Mg$
Mole wt = 893 g/mol



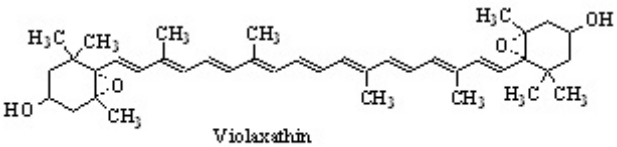
Chlorophyll b
$C_{55}H_{70}O_6N_4Mg$
Mole wt = 907 g/mol

The second group of substances we should expect to find are the carotenes. The most well known is beta-carotene; but, in our experiment we will not have one, but a mixture of very similar isomers which can not be resolved by this technique. Note that the beta-carotene is a hydrocarbon.

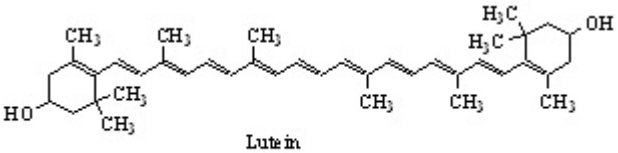


Beta-carotene
$C_{40}H_{56}$
mole wt = 537 g/mol

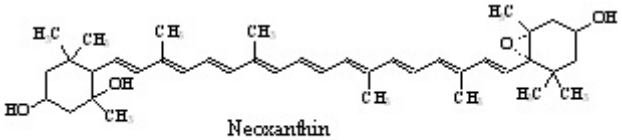
Another group of substances which might be found are the xanthophylls: lutein, violaxathin, and neoxanthin. They are almost identical to the carotenes except that they have -OH groups at opposite ends of the molecule or an epoxide oxygen bonded across two carbons on one or both terminal rings. We may not observe neoxanthin as it is usually found in a low concentration or is too similar chemically to either lutein or violaxathin to separate well. However, there is a test which we will perform to distinguish violaxathin and neoxanthin from the other pigments. When placed in contact HCl(vapor), violaxathin turns blue and neoxanthin turns green. Lutein, the carotenes, and the chlorophylls are not effected.



Violaxathin
$C_{40}H_{56}O_4$
mole wt = 601g/mol

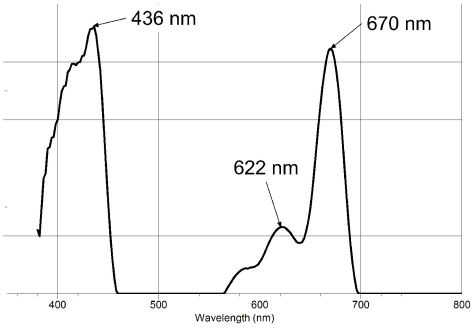


Lutein
$C_{40}H_{56}O_2$
mole wt = 568 g/mol

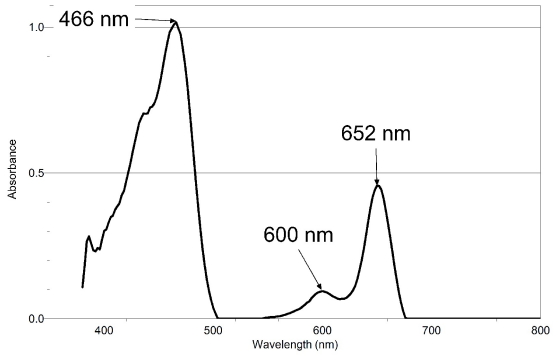


Neoxanthin
$C_{40}H_{56}O_3$
mole wt = 584 g/mol

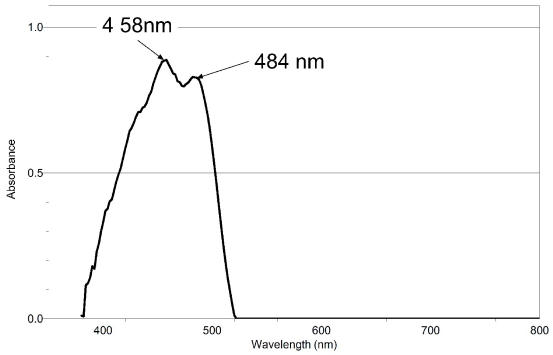
The second part of this experiment is the identification of the separated leaf pigment components by means of an instrument called a spectrophotometer. Spectrophotometers measure the intensity of light passing through a substance or solution at each wavelength producing a graph such as the adjacent chlorophyll spectrum. These spectra are a chemical fingerprint of the substance allowing us to identify it. Note that the identifying peaks in the chlorophyll A spectrum have been labeled. However, the actual values for the identifying peaks will vary somewhat according to the resolution of the spectrophotometer and the environment of the pigment when measured. For example, the identifying peaks will have a slightly different value when measured in a hydrocarbon than in an alcohol. Likewise, a spectrophotometer with a 0.2 nm resolution will give a different value than one with a 3-nm resolution. That is, the 670 nm value measure with a high resolution spectrophotometer could vary from 667 nm to 673 nm when measured with a spectrophotometer with a 3-nm resolution. However, the patterns of both the spectrum and the trend of identifying peaks will be same. Let's look at some other leaf pigment spectra.



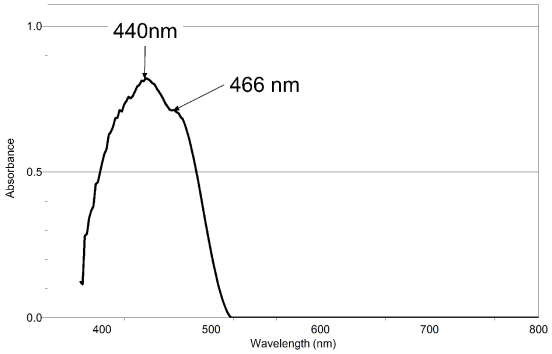
The absorption spectrum of chlorophyll a



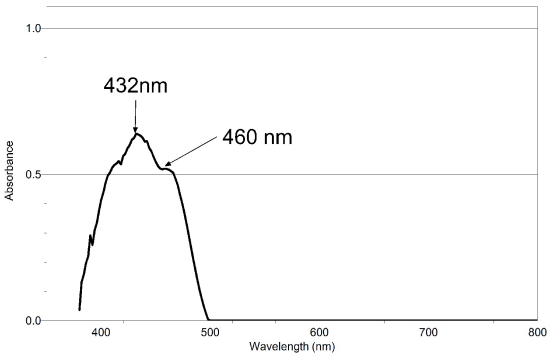
The absorption spectrum of chlorophyll b



The absorption spectrum of the carotenes

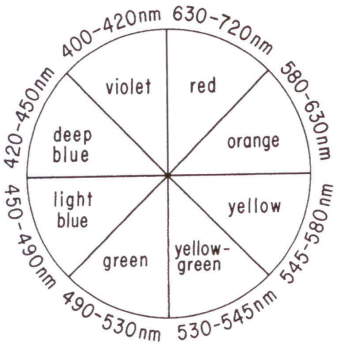


The absorption spectrum of lutein

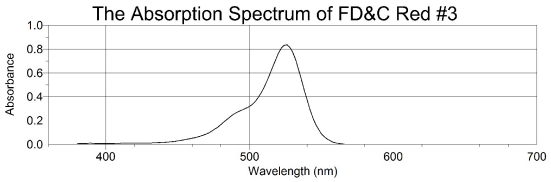


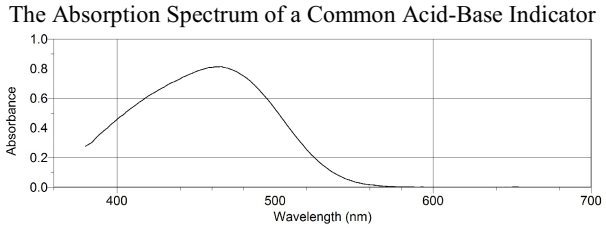
The absorption spectrum on violaxathin

An interesting application of absorption spectra is predicting the color of a substance as its color is determined by the color(s) it absorbs. If it absorbs many colors, then it will be that color or mixture of colors, that is transmitted through the solution. However, what if it only absorbs one color, what would be its color? Note the adjacent color rosette. It consists of various colors and their complimentary colors. If a solution absorbs only one color, then its color will be that of its complimentary color. Note the red food dye spectrum below. We would predict it would have a red color as it absorbs light in the green or 490 nm to 530 region. What about the second spectrum of a common acid-base indicator? What would you predict its color to be?



A color rosette [1]





If you guessed orange, you would be right. Note that its maximum absorbance is about 465 nm. According to the color rosette, it absorbs in the light blue and therefore its complimentary color would be orange. In fact, the indicator is called methyl orange.

Pre-Laboratory Exercise:
Study the chemical structures of the common leaf pigment components given above. Predict the order you would expect the separated pigments to occur from the top of the chromatogram to the bottom. Consider their relative polarity, symmetry, molar mass, the polarity of both the mobile phase and stationary phases, and any other unique feature you feel might effect the rate of migration up the paper. Place your predictions on the adjacent table in order from top to bottom. Be prepared to discuss the rationale behind you predictions.

Position	Pigment
1	
2	
3	
4	
5	
6	

Materials:
a green leaf or pine needles
petroleum ether: acetone solution
test tube, 20 mm x 150 mm
cork or rubber stopper to fit test tube
test tube rack
1-ml calibrated pipet
acetone
filter paper strips
scissors
mortar & pestle
sand
capillary tube or microtip pipet
computer w/Logger Pro software
spectrophotometer
USB cable
fiber optic cable
ruler
pencil
overhead projector
stoppered test tube containing HCl

Procedures:

1. With the aid of a 1-ml calibrated pipet, add 2 ml of the petroleum ether:acetone solution to a 20 mm x 150 mm test tube, stopper, and place in a test tube rack to allow the vapor to saturate the air in the test tube.

Caution: Both petroleum ether and acetone are quite volatile and highly flammable. Do not perform this experiment near flames or faulty motors.

2. Cut a 25 mm x 50 mm or so (2 in^2) portion of your leaf or pine needles into small pieces with a scissors. If you are using pine needles, cut enough to give a thick layer on the bottom of the mortar. Sprinkle sand over the material, add 1-ml of acetone, and grind with the pestle. If the materials dries, add another ½ ml of acetone and grind some more. Continue until a small amount of the green pigment solution is evident in the mortar. If not, add another ½ ml of acetone and again grind the material releasing more pigment.

3. Touch the pigment solution with a piece of fine capillary tubing or microtip pipet allowing the solution to be drawn into the tube. Practice streaking the solution onto a scrap piece of the filter paper strips. This is best accomplished by touching one edge of the paper with the capillary tubing filled with solution allowing the capillary action to draw some of the solution onto the paper. Once the solution starts to flow, drag the tube across the paper at a constant rate. The best streaks must be uniform across the paper and as narrow as possible. Allow the streak to dry thoroughly between applications. Repeat two more times or until a dark green band appears across the paper. If the solution dries in the mortar, add more acetone and again grind the material. Once you are satisfied you have mastered the technique, prepare your chromatogram as described below.

4. Place a light pencil mark approximately two centimeters from the bottom of a strip of filter paper. Streak the solution across the paper at the mark 2-3 times, allowing the paper to dry between applications as with your test strip.

5. Place the streaked paper strip into the test tube containing the petroleum:acetone solution and stopper loosely. Let the paper touch the bottom of the test tube but not the sides. The paper can be moved from side to side or raised easily if the stopper is not fitted tightly. Once centered in the tube, allow the chromatogram to proceed until the solvent front is within a few millimeters from the bottom of the stopper.

6. While the chromatogram is developing, clean the mortar and pestle, discarding the contents of the mortar in the trash. Return all unnecessary materials to their designated area.

7. Attach the fiber optic cable to your spectrophotometer and connect it a UBS port on your computer. After powering your computer, click on the Logger Pro software icon. Logger Pro should automatically identify the spectrophotometer after a few seconds.

8. Next, calibrate the spectrophotometer. This is completed by clicking on the "Experiment" drop down menu, scroll down to "Calibrate," then "Spectrophotometer." Wait until the spectrophotometer lamp has warmed sufficiently, usually 60 seconds, and turn on the overhead projector. Place a piece of blank chromatography paper on the bright area of the overhead stage. Touch the end of the fiber optic cable on the paper, click on "Finish Calibration," then "OK." This procedure subtracts the absorbance of the paper from your spectrum. Do not close Logger Pro, turn off your computer, or disconnect the USB cable from either the spectrophotometer or your computer. Keep everything in a ready state until your chromatogram is finished.

9. Once your chromatogram is complete, measure the absorption spectra for the separated pigments on the paper strip. Remove the paper from the test tube and allow it to dry. When measuring the absorption spectra of the separated pigments, choose the most intense area in the center of the color band. Avoid an area where consecutive bands overlap. To measure the spectra, place the paper on the same bright area of the overhead, touch the top most color band at the "best" spot, and either click on "Collect," or press the space bar. Logger Pro will record the absorption spectra for that material. When you are satisfied with your spectrum, click on "Stop" or again press the space bar. Save this trial by clicking on the "Experiment" drop down menu, followed by "Store Latest Run." Repeat the process of recording the spectra and saving each trial in order from top to bottom of the paper strip until the spectrum of all of the separated pigments has been measured.

10. Using a pencil, trace around each colored band as the pigments fade quickly. Record the colors of each band in order from top to bottom in the space provided on the table below.

11. Remove the stopper from the test tube containing a few milliliters of concentrated hydrochloric acid. Hold your chromatogram in the HCl vapor for five to ten seconds but do not let it touch the solution. If either violaxathin or neoxanthin is present, it will turn blue or green respectively. The color intensity of the other pigments may increase but will not change color.

12. When finished, pour the spent petroleum ether:acetone mixture into the waste solvent container and return all other materials except the computer to their designated area.

Questions:

Q1. If the hydrochloric acid test has verified the presence of either violaxathin or neoxanthin, write its name in the proper position on the summary table below.

Q2. Identify the remaining bands by comparing your spectra with the known spectra above recording your results on the summary table below. Consider both the patterns and the relative peak positions in the process. Unfortunately, your graph is a collection of overlapping spectra. To ease the identification process, you may want to isolate individual

components and view the legion. First, click on the graph anywhere away from the plots. A "Graph Option" menu will appear. Uncheck the "Draw Visible Spectrum" box and check the "Legion" box. Click "Done" and the spectrum will be removed and the legion will appear.

Summary Table of Your Results

Position	Color	Name
1		
2		
3		
4		
5		
6		

The next step will be to view either individual spectrum or a group with similar colors simultaneously. Click on the word "Absorbance" on the vertical or "y" axis, then "More." The "y-axis options" box will appear. By checking or unchecking the various trials, you can select those you wish to study. It may be better to view several spectra together with a similar color to compare the patterns. The peak heights are not as important as the wavelength at the maximum absorbance. The peak heights are related to the intensity of the spot on the chromatogram where you measured the spectrum or the amount of pigment in the sample. Remember the run number reflects the order in which you recorded the data where Run 1 is the top most band and the latest is the last band. Moreover, the color of the band may be helpful in resolving its identity. When you have resolved the identity of each of your colored bands and have completed the table above, save your spectrum by clicking on the "Save" icon or "Save" in the file drop down menu.

3. How does your predicted order the pigments separated on the paper compare with the actual order determined from their spectra? If your predicted order was wrong, explain why you think you were wrong.

4. If prior to this experiment, you had copies of the absorption spectra for various leaf pigments, what would you have predicted for the color of chlorophyll A, the carotenes, violaxathin and lutein? Explain your reasoning.

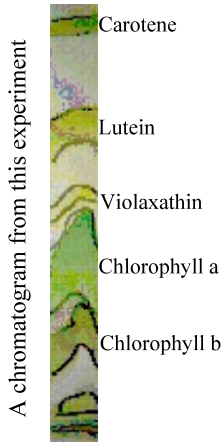
5. The relationship between the wavelength the light and its energy, ΔE, is described by two equations: c = frequency x wavelength, and ΔE = h(Planck's constant) x frequency.
   (a) What is the relationship between the wavelength of light and its energy?

   (b) Where in the visible spectrum do you find light with the greatest energy? The least?

   (c) What is the relationship between the color of the leaf pigments and the energy they absorb? According to your observation, which color(s) of light are the most useful for photosynthesis? Why?

6. Why is do you think a pencil is used to mark your chromatogram and not a ball point or felt tipped pen?

NOTES FOR TEACHERS



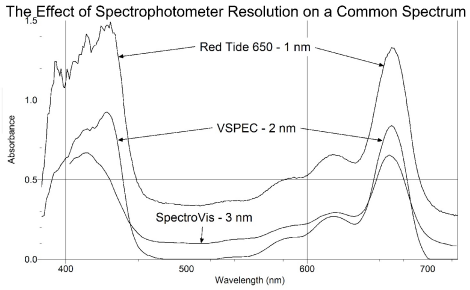
1. A mixture composed of 90% petroleum ether and 10% acetone seems to give the best separation in this experiment.

2. The chromatography paper was prepared from 24 cm diameter, Whatman #1 filter paper, by cutting it into ½ inch strips with a paper cutter. Save the pieces which are too short for the test tube, less than 16 cm, for the students to practice streaking the paper.

3. A large test tube, 150 mm or more longer, is needed to obtain the best separation of the chlorophyll pigments. A 20 mm wide or larger test tube reduces the chance that the paper will touch the sides.

4. Commercial 5 µl glass capillary micropipets work best for streaking the paper but they can be prepared by drawing out a small diameter glass tube. A microtip polyethylene transfer pipet or even a Pasteur pipet can also be used to streak the paper but gives a much wider band. If the students put too much pigment on the paper, the bands tend to run together.

5. It doesn't seem to matter which spectrophotometer is used in this experiment. The spectra from Vernier's relatively inexpensive SpectroVis is just as useful as the high end Ocean Optics models. Note the adjacent chlorophyll A spectrum measured with three different spectrophotometers.



6. The overhead projector used in this experiment must have an incandescent bulb. Fluorescent bulbs are too noisy or may not emit the full spectrum. Alternately, the chromatogram may be taped to a sunny window to back light the chromatogram when calibrating the spectrophotometer and measuring its spectrum.

7. An extension of this activity is to have the students determine the optimum petroleum ether - acetone mixture which will give the best separation of all the pigments. Have them start by testing the pure solvents and then vary the mixture until they are satisfied with their results.

8. While this experiment is written for a computer, Vernier's LabQuest works equally as well. Not only can it be used with all the spectrophotometers sold by Vernier but it has a smaller foot print minimizing the clutter around the overhead projector or at the windows. All of the spectra displayed in this experimental write-up were measured with the spectrophotometer connected to Vernier's LabQuest and later transferred to a computer.

[1] Shakhashiri, Bassam, *Chemical Demonstrations: Volume 1*, 1983, Univ. Of Wisconsin Press, p.262