Separation and Identification of Plant Pigments

Dr. Gergens - SD Mesa College

PURPOSE

In this experiment, the photosynthetic pigments common to all flowering plants will be extracted by liquid-liquid extraction. The four main pigment components of plant leaves are chlorophyll a, chlorophyll b, carotene, and xanthophyll. The solvents needed for the extraction and separation will be petroleum ether, diethyl ether, methanol, and water. Finally, these pigments will be analyzed by measuring the absorption spectra of the plant pigments.

BACKGROUND

The four main pigment components of plant leaves are chlorophyll a, chlorophyll b, carotene, and xanthophyll. While most plant leaves appear green to our eyes, several different photosynthetic pigments of various colors are usually present in the chloroplast of green plants. The colors of these pigments are due to the numerous double bonds in their structure. The green color of leaves is provided by chlorophyll a and b, which absorb most of the light energy needed for photosynthesis to occur. The greatest absorption of light by chlorophyll is in the red, blue and violet wavelengths. Associated with these green pigments are several other pigments known as carotenoids. These are commonly yellow to red in color, and are also involved in harvesting light energy for photosynthesis.

SAFETY PRECAUTIONS

- Wear safety goggles and aprons at all times in the laboratory.
- No open flames in or outside the door of the laboratory. The solvents in this experiment are highly flammable. Diethyl ether is very volatile and is highly flammable. Petroleum ether and methanol are flammable as well.
- Work underneath the hood.
- Keep all Erlenmeyer flasks, beakers, and solvent containers capped and/or covered.
- 30% methanolic KOH is extremely corrosive. Avoid contact with skin, eye, or cloths. If you spill any on yourself, rinse thoroughly with water and contact the instructor.
- Dispose of all reagents in the reclaim container provide in the fume hood.
- The chlorophyll solution is very concentrated. It will stain skin, cloths, etc. Clean up any spill. Wash all glassware to avoid any permanent green stains.
- Keep in mind the safety precautions when using a separatory funnel:
    a. never extract hot solutions.
    b. never forget to vent constantly.
    c. never clamp a separatory funnel to a ring stand; use an iron ring.
    d. never forget to unplug the glass stopper when draining.

EXPERIMENTAL PROCEDURE

PART A. Separation of Photosynthetic Pigments by Liquid-Liquid Extraction.

Take care not to spill this solution. Concentrated chlorophyll solutions will produce a very nasty green stain on anything it comes in contact with. Please was all glassware with soap as soon as possible to avoid permanent green strains.

0. Work in pairs. Only two individuals are to work per group. Begin by sharing one 125 mL separatory funnel placed in an iron ring attached to a ring stand. Take turns extracting as outlined in the flow chart of procedures up to juncture "A" and "B." At the A" and "B" juncture, the mixture is split between partners. One partner will complete the extraction in his/her own 125 mL separatory funnel for the isolation of chlorophyll a and xanthophyll in "A", and the other partner will complete the isolation of chlorophyll b and carotene in "B" in their separatory funnel. Finally, individuals will run one visible absorption analyses for each pigment isolated for a total of two spectra per individual. Partners will be responsible for exchanging and sharing graphical data gathered in the spectroscopic analysis.

If there is an odd number of students in the laboratory, a single individual will complete all work up to the juncture "A" and "B" shown in the flow chart scheme of the procedures. At this juncture, he or she will make a choice; complete the isolation and analysis for part A, or for part B. Finally, the individual will need to acquire the missing graphical data for the part he/she did not complete from someone in the classroom.

1. Weigh a 2 grams of spinach onto a weigh boat.
2. Transfer the 2 grams of spinach to a mortar and add a rice sized portion of sand.
3. Grind the spinach to a pulp.
4. Working underneath a fume hood, add 10 mL of acetone and 2 mL of water to the pulp and grind.
5. Avoid transferring sand contained in the mixture. Using a pipette, place the tip of the pipette flush against the ceramic mortar. Pipette the liquid mixture away from the pulp/sand mixture and transfer the liquid a 125 mL separatory funnel.
6. Rinse the pulp in the mortar with a fresh portion of a mixture of 10 mL of acetone and 2 mL of water and transfer the rinse with a pipette to the same 125 mL separatory funnel.
7. Carefully follow the directions outlined in the flow chart for the separation of chlorophyll a, chlorophyll b, carotene, and xanthophyll.
8. As you perform each step, WRITE YOUR PROCEDURES into your laboratory notebook. Experimental procedures and observations are to be written in such a manner anyone could repeat the experiment. For example, if I were to give your notebook to a group of high school students, could they repeat any given part of the experiment base on what you have written into your laboratory notebook?

   IMPORTANT: <u>It is essential that you record what you actually did and observed while you are performing the experiment. Have your instructor initialize your laboratory notebook before leaving class at the end of the period. Points will be deducted for recording data in pencil or erasable ink, or recording it in a place other than your laboratory notebook. Poorly written experimental procedures, observations, illegible and sloppy data and sample calculations will not be tolerated and will not be graded.</u>

9. At juncture "A" and "B," split the mixture. One partner completes the isolation and runs the absorption spectrum of chlorophyll a and carotene, the other will complete the same for chlorophyll b and xanthophyll.
10. Be sure to record your partners procedures for the part you did not do into your laboratory notebook.

PART B. Analysis of the Four Plant Pigments by Absorption Spectroscopy.

The Carey 50 in the laboratory will be used to analyze the visible light absorption spectrum of each pigment. Two blanks containing methanol and diethyl ether have been prepared for zeroing the instrument in the analysis. Keep your samples capped and parafilmed to prevent evaporation of the solvents.

You will need a diskette. The laboratory technicians and your instructor will help you scan each sample. The data will be saved as an ASCI file to be worked up in Excel.

RESULTS/DISCUSSION

There is no formal report for this experiment, only a data and results summary sheet that can be downloaded and completed. The results sheet will be submitted for grading.

Place 1 mL of pigment mixture in 125 mL sep. funnel;
Add 20 mL of 80% acetone

→ Add 25 mL pet ether; gently swirl in the sep funnel; allow the two liq layers to separate; draw off lower layer

- keep → pigments / pet ether → Add 25 mL H_2O; gently swirl; draw off lower layer
  - keep → pigments / pet ether → *
  - discard → H_2O
- discard → acetone / H_2O

Circle the best answer

pet ether is (more, less) polar than methanol.

chlorophyll a and carotene are (more, less) polar than chlorophyll b and xanthophyll

* repeat washing w/ 25 mL H_2O two more times; draw off lower layer each time

- keep → pigments / pet ether → Add 25mL 95% methanol; gently swirl; draw off lower layer
  - keep → upper pet ether layer contains chlorophyll a and carotene → A.
  - keep → lower methanol layer contains chlorophyll b and xanthophyll → B.
- discard → H_2O

A. Slowly add 10 mL of 30% KOH sol.; gently shake for 10 minutes; allow layers to separate; top layer should be yellow.

- keep → upper yellow pigment / pet ether layer
  - the upper pet ether layer is (green, yellow)?
  - the upper layer contains (chlorophyll a, carotene)
- keep → lower pigment / methanol/H_2O layer
  - the lower methanol/H2O layer is (green, yellow)?
  - the lower layer contains (chlorophyll a, carotene)

diethyl ether is (more, less) polar than methanol.

chlorophyll b and xanthophyl are (more, less) polar than methanol.

B. Place methanol layer back into the sep funnel; add 25 mL diethyl ether; gently swirl; add 5 mL portions of water; swirl after each addition until layers separate (about 15 mL H_2O)

- keep → pigments / diethyl ether → Slowly add 10 mL of 30% KOH solution; gently shake for 10 minutes; allow layers to separate
  - keep → upper pigment / diethyl ether layer
    - the upper diethyl ether layer is (green, yellow)?
    - the upper layer contains (chlorophyll b, xanthophyl)
  - keep → lower pigment / methanol/H_2O layer
    - the lower methanolic KOH layer is (green, yellow)?
    - the lower layer contains (chlorophyll b, xanthophyl)
- discard → methanol / H_2O

PROCEDURES for running your sample on the Cary 50 UV-visible spectrophotometer.

1) Open Varian Scan on the desktop

2) Select Cary 50 <OK>

3) Then WAIT until you hear the Cary 50 make a buzzing noise. After buzzing noise the <Setup..> and <Zero> should be outlined in black.

4) Select <Setup...>. In the dialog box for this mode:

    A) set X Mode to Start 800, Stop 400

    B) set Y Mode Abs,

    C) Y min -5, Y max 5

    D) Display Options to Individual Data

    E) Scan Controls to Fastest

5) Select the Baseline tab at the top of Setup dialog box.

6) Select Baseline correction.

7) Select OK

8) Select Baseline and insert a blank sample OF PURE SOLVENT for which you substance is dissolved in, then select OK

9) Replace the blank with your sample.

10) Select <Start>

11) In set floppy into 3.5" Floppy into drive, and select the file to save to this drive. Give the file a name

12) Name you sample, select <OK>

13) Go to file save and select, <Save Data As...>,

    A) give a file name

    B) change the file type to "Spreadsheet Ascii (*.CSV)

    C) select the 3.5" Floppy into drive to save

    D) select <SAVE>

14) Open your data in EXCEL and use the graphing mode to plot the data.

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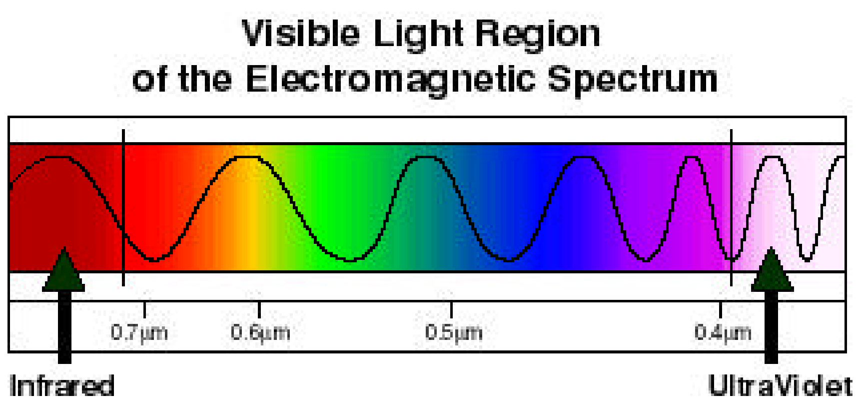


RESULTS/DISCUSSION (include the following in your formal conclusion)

1. Include graphs of the four spectra for chlorophylls a and b, carotene and xanthophyll
1. Include structures for each pigment. Paste the structure of each compound into the appropriate cell. If necessary do the following <Insert>, <Paste Special>, <Picture> and turn off [float over text]
2. Tabulate the wavelength at absorbency maxima for each of the four pigments.
3. State the color(s) of visible light each pigment absorbs.
4. What color does each pigment appear to our eyes? In other words, state the color(s) of visible light each pigment transmits.

Pigment	Chlorophyll a	Carotene	Chlorophyll b	Xanthophyll
Structure	(import from SigmaAldrich web site)	(import from SigmaAldrich web site)	(import from the Galactic web site)	(import from the SDBS, Japanese web site)
Wavelength(s) of absorbency maxima				
The color of visible light the pigment absorbs:				
To our eyes the pigment appears (give color)				

5. Structurally what are the major differences between chlorophyll a and b?

6. From the SigmaAldrich structures for **chlorophyll a and b**, what does the shorthand notation for “Phytyl" structurally represent? Draw the Phtytl fragment in ISIS draw and import its structure.

7. Structurally what are the major differences between **carotene and xanthophyll** (caratenoids)?

8. Structurally what are the major differences between the chlorohylls and the caratenoids?

9. Structurally what are the major differences between the chlorohylls and the caratenoids?

10. Comment on the polarities of petroleum ether, methyl alcohol, diethyl ether, acetone, and water. Which is most polar? Which is least polar? Which organic is most soluble in water?

	pet. ether	methanol	diethyl ether	acetone
Relative polarity of solvent				
Solubility in H_2O				

11. Indicate the solubility characteristics of each in the following solvents.

	pet. ether	95% methanol	diethyl ether	30% methanolic KOH
carotene			not measured	
chlorophyll a			not measured	
xanthophyll				
chlorophyll b				

12. Clearly identify which layer contained which pigment in the final separation.

Plant Pigment Separation Results Table:

Plant Pigment	Extraction Part	Layer	effective extracting solvent
carotene	A	top	
chlorophyll a	A	bottom	
xanthophyll	B	top	
chlorophyll b	B	bottom	

13. Based on your answers in 8 and 9, explain why the solubility characteristics of each pigment are the way they are in the indicated solvent. Use the drawing template and box the best answer in each.

a. Structurally all four pigments are more (polar, nonpolar) than (polar, nonpolar) because all four are more soluble in (petroleum ether, acetone) used in the extraction.

b. Structurally **chlorophyll a** and **carotene** are (more, less) polar than **chlorophyll b** and **xanthophyll**

c. Experimentally **chlorophyll a** was determined to be more (polar, nonpolar) than **chlorophyll b** because **chlorophyll a** was determined to be more (soluble, insoluble) in 95% methanol.

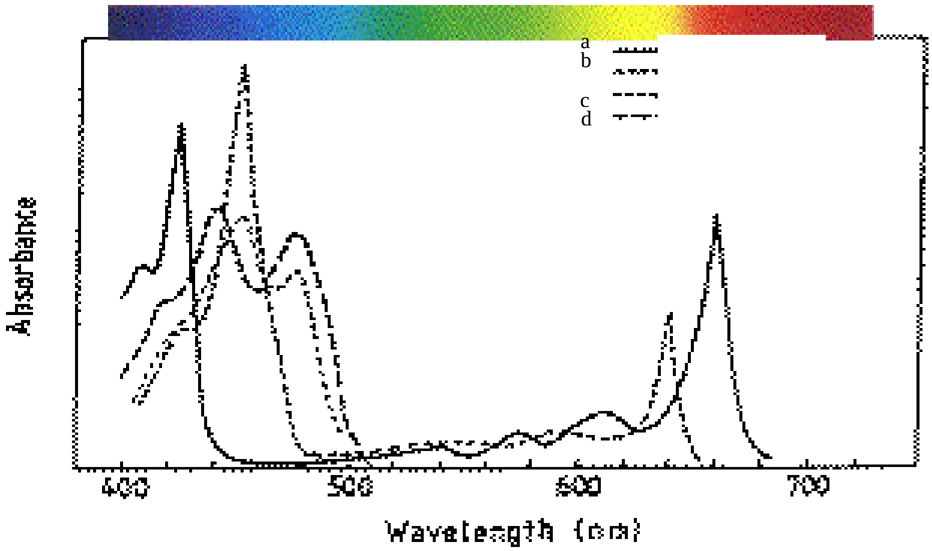
d. Experimentally **carotene** was determined to be more (polar, nonpolar) than **xanthophyll** because **carotene** was determined to be more (soluble, insoluble) in 95% methanol.

e. Experimentally **chlorophyll a** was determined to be more (polar, nonpolar) than **carotene** because **chlorophyll a** was determined to be more (soluble, insoluble) in 30% methanolic KOH.

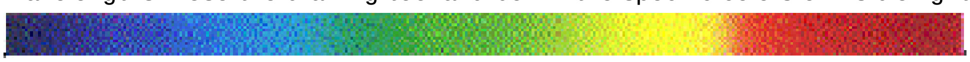
f. Experimentally **chlorophyll b** was determined to be more (polar, nonpolar) than **xanthophyll** because **chlorophyll b** was determined to be more (soluble, insoluble) in diethyl ether.

14. Compare your various absorption spectra the actual spectrum for each compound super imposed below. Match the four compounds to the UV-visible spectra legend given below:

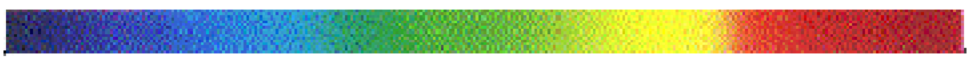
a.	b.	c.	d.



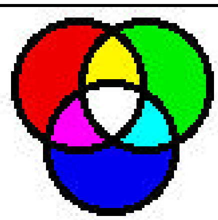
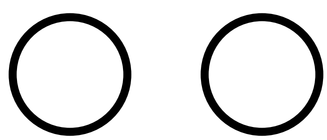
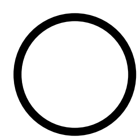
15. **Chlorophylls a and b** can only absorb very specific amounts of visible light. What colors are associated with those wavelengths? Use the drawing tool and box in the specific colors of visible light.



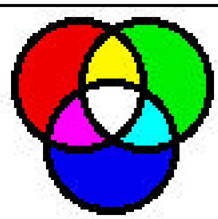
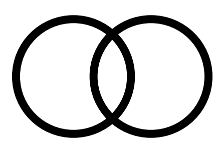
16. **Carotene and xanthophyll** can only absorb very specific amounts of visible light. What are those colors associated with those wavelengths? Box in the specific colors of visible light.



17. White light consists mainly of red, blue, and green wavelengths. **Chlorophylls a and b** strongly absorb wavelengths of __________ light and __________, leaving behind __________ wavelengths gives the color __________. Thus **chlorophylls a and b** appear __________ to our eyes.

White light, R + G + B = W	Chlorophylls a and b filter out what color? Fill in the colors.	What color is left remaining?
		

18. Accessory pigments, **carotene and, xanthophyll**, strongly absorb wavelengths of __________ light and ultraviolet light, leaving behind __________ and __________wavelengths which mix to form __________. Thus **carotene and xanthophyll** appear __________to our eyes. Color in the circles.

White light, R + G + B = W	Carotene and, xanthophyll filter out what color(s)? Fill in the color for circle on the right.	What colors are left remaining? The center ellipsoid should show a mixture of the two colors.
		

19. Most plants have **chlorophyll pigments a and b**. What is the importance of each pigment?

20. Some plants contain **carotene and xanthophyll** in addition to chlorophyll pigments a and b. What might be the role of the accessory pigments, carotene and xanthophyll? What is the importance of each pigment?