

Spectrophotometric analysis of photosynthetic pigments

Objectives

- Part 1: prepare extracts of spinach leaf and carrot root pigments (this will be done for you)
- Part 2: measure and compare absorption spectra from spinach leaf and carrot root tissue
- Part 3: separate photosynthetic pigments using thin layer chromatography (TLC), identify purified pigments, and compare their absorption spectra
- Part 4: observe chlorophyll fluorescence

Introduction to photosynthetic pigments

Photosynthetic organisms (plants, algae, and some bacteria) rely on a variety of pigments to capture light energy from the sun. These colored compounds are particularly diverse in the algae, and their prominence in the biology of algae is indicated by the very names of many algal divisions. Indeed, photosynthetic pigments reveal much about the physiology of these organisms.

In green algae and plants, the chlorophyll molecules are responsible for the capture of light energy that can then be used to drive photosynthetic electron transport. The chlorophylls *a* and *b* of green algae and plants are composed of a tetrapyrrole ring that contains Mg^{2+} as a central metal. Examine Figure 1 and note that chlorophyll *a* has a methyl group (CH_3) in the position where chlorophyll *b* has a formyl group (CHO). This chemical difference allows the two molecules to be separated from one another. Other than this seemingly simple difference, these two molecules are identical, including a hydrophobic hydrocarbon phytol tail that allows these molecules to be tightly associated with the hydrophobic region of the thylakoid membrane. Because of the hydrocarbon tails, chlorophylls are soluble in organic solvents and relatively insoluble in water.

The carotenoids are a second class of pigments involved in harvesting light energy. These molecules usually have 40 carbon atoms and are divided into a pure hydrocarbon group, the carotenes, and another group with two additional oxygen atoms, the xanthophylls (see Figure 2). Like chlorophyll molecules, carotenoids are soluble in organic solvents and relatively insoluble in water. The major carotenoids found in higher plants are β -carotene and lutein.

Visible light consists of the wavelengths of the electromagnetic energy spectrum to which the human eye is sensitive, covering approximately the region from 380 to 760 nm. There are also wavelengths to which the human eye is not sensitive, below 380 nm and above 760 nm.

Several plant components, such as the chlorophylls (Figure 1), anthocyanins [the red pigments you saw in onion cell vacuoles in lab exercise 3 (could these function as accessory pigments for photosynthesis? why or why not?)], and carotenoids (Figure 2), absorb light in these wavelengths and appear to be colored. Many colorless molecules can be changed to light-absorbing pigments by using certain reagents (such as the reaction between Biuret reagent and polypeptides). The property of light absorbance allows for both qualitative and quantitative analysis of colored substances. Spectrophotometers are instruments that measure the amount of light absorbed by molecules. They provide convenient and sensitive tools for quantitative analysis.

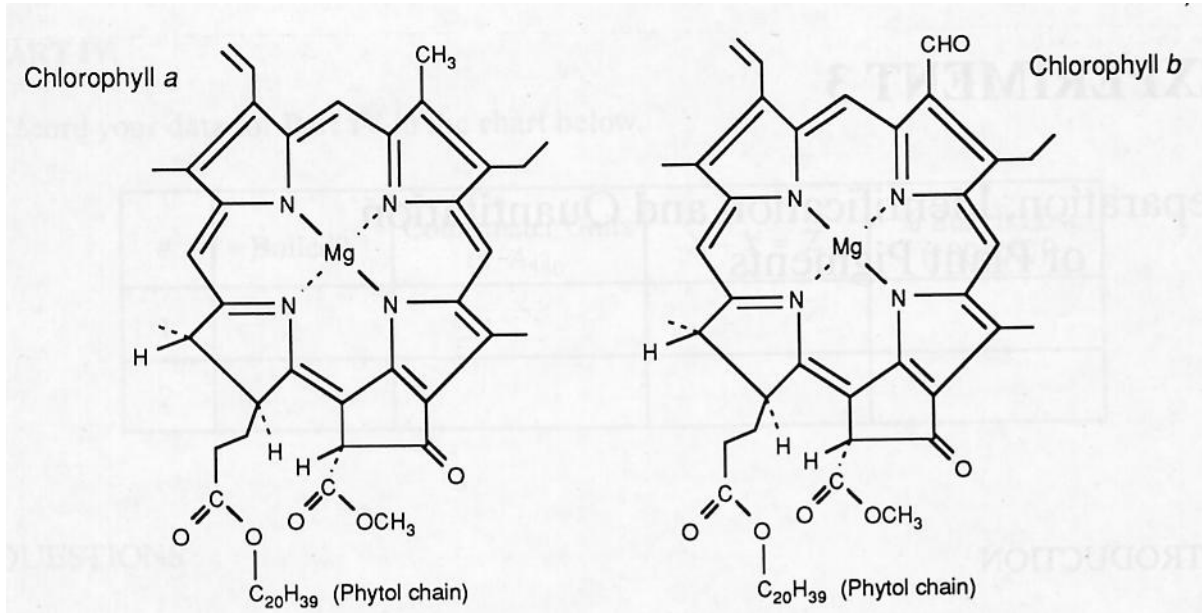


Figure 1: Chlorophyll structure

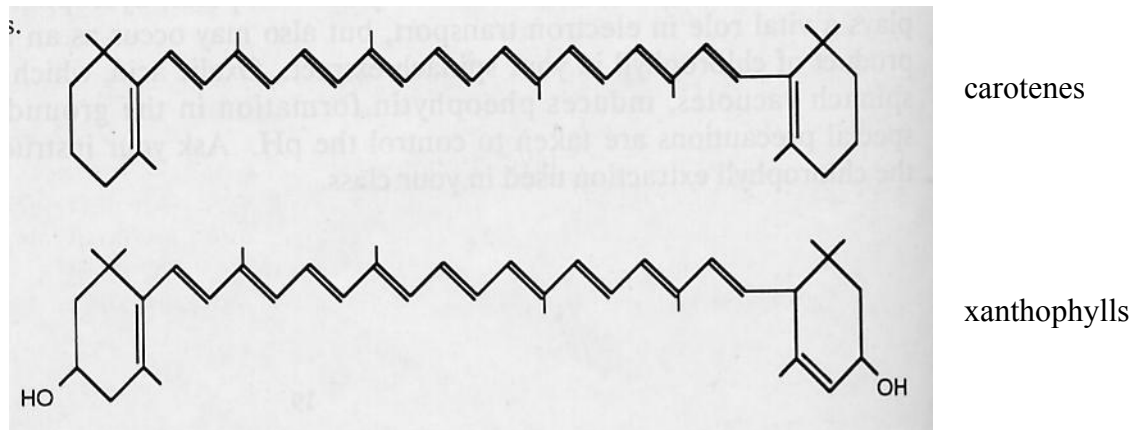


Figure 2. Carotenoid structure

Introduction to chromatography

In this lab, you will extract pigments from carrot roots and spinach leaves. You will prepare and compare absorption spectra for these two pigment extracts. You will then separate the pigments by means of thin-layer chromatography (TLC) and examine the absorption spectra for a subset of the purified pigments.

Chromatography is a technique that can be used to separate and identify a wide range of chemical compounds. The separation of the components of a mixture is a function of their different affinities for a stationary phase (e.g. solid or liquid) and their differential affinity for a moving phase (e.g. liquid or gas). When the stationary phase is solid and the moving phase is liquid, the separation of compounds is governed by their tendency to associate with the mobile (usually a hydrophobic organic solvent) liquid phase or to adsorb onto the solid (usually hydrophilic) surface. The solid phase might be paper, starch, or silica gel.

If the solid is applied in a thin layer to a supporting glass or plastic plate, the method is called thin-layer chromatography (TLC). In TLC, the mixture to be separated is first applied as a spot or a line to the solid phase. Then, the mobile solvent is allowed to pass through the applied compounds along the immobile phase. The compounds will dissolve in and move with the solvent; the movement of the pigments with the solvent along the immobile phase is called the development of the chromatogram.

The distance traveled by a particular compound will depend on its affinity for the hydrophobic (mobile) phase versus its affinity for the hydrophilic (solid) phase. This distance is characteristic for specific set of conditions and may be used to identify the compound. The ratio of the distance traveled by a compound to that of the solvent front is known as the R_f value: unknown compounds may be identified by comparing their R_f 's to the R_f 's of known standards.

Part 1: Preparation of Plant Pigment Extracts (work in groups of 4; with 2 students preparing the spinach extract and 2 preparing the carrot extract; these same groups should follow through with the chromatography and spectroscopy steps). *This part of the lab will be done for you before you come to lab.*

spinach leaves
baby carrots
100% acetone (**flammable**)
Waring blender
2 sets of mortar/pestle
10-mL, 5-mL, and 1-mL pipets
50 mL orange cap tube

pipette pumps
1.5 mL microcentrifuge tubes
microcentrifuge
gloves

review $\mu\text{L}/\text{mL}$ conversions

Balance tubes in the centrifuge (always!). Note that acetone and other the solvents used this week are extremely flammable. Use NO flames or sparks in the lab this week!

Turn on your Spectronic 20 Spectrophotometer so that it can warm up for at least 15 minutes before use.

A. Preparation of spinach extract

1. Trim spinach leaves so that you have only blade tissue; discard the petiole (leaf stem) and midrib (big vein in the middle of the leaves). Place approximately 5 grams of spinach leaves in your mortar.
2. ***Wear gloves to minimize contact with acetone.*** Add 10 mL 100% acetone to the mortar (we can't use the blender for this preparation because it can create a spark that would ignite the very flammable acetone). Vigorously grind tissue using the pestle for about 10 minutes, or until the resulting solution is strongly colored and most of the tissue has been pulverized. You will need to aggressively grind the pestle against the mortar—don't be delicate, but be careful not to splash the acetone.
3. Pour/scrape as much of the ground tissue as possible into a 50 mL-centrifuge tube. Use approximately 5 mL 100% acetone to rinse the mortar – pour this solution into the centrifuge tube until you reach the ~10 mL mark (or use a plastic spoon to transfer the *liquid* only). Cap the tube and invert to mix.
4. Using a 1.0 mL glass pipette, transfer 1.0 mL of homogenate to each of two 1.5 ml microfuge tubes.
5. Clarify the suspension by centrifugation in a microfuge for 3 minutes at maximum speed. This centrifugation will separate the cell debris into a pellet, leaving the solubilized pigments in the supernatant. You'll want to coordinate with other groups to centrifuge at the same time.

Make sure that you balance the tubes in the centrifuge (always!) and be sure the machine is spinning by listening. The little ticking noise is just the timer; the centrifuge itself should sound a bit like an airplane taking off. You may need to press down hard on the lid to get the machine to start spinning. Be patient when it is finished—the lid won't open back up until the rotor has finished spinning (to protect you from being hurt). If the centrifuge lid won't open, check to see that it is plugged in. If it is, wait for the rotor to finish spinning. If it still won't open, ask your instructor.

6. Transfer the clarified supernatants (~850 μ L from each tube) to two fresh 1.5 ml microfuge tubes. Label tubes and store on ice. Dispose of the microfuge tubes containing the cell debris pellets in the ACETONE dry waste container in the fume hood.
7. CLEAN the mortar and pestle by pouring any remaining liquid into the ACETONE WASTE container in the fume hood. Clean the emptied mortar and pestle with water.

B. Preparation of carrot extract

1. Place 5 baby carrots into the blender and puree (yes, it will be very chunky; use a metal spatula to help get the carrot chunks to the bottom of the blender). Measure approximately 10 mL of puree into a clean mortar.
2. ***Wear gloves to minimize contact with acetone.*** Add 8 mL 10% acetone to the mortar (using a glass pipette). Vigorously grind tissue using the pestle for about 8 minutes, or until the resulting solution is strongly colored (you'll need to grind quite strenuously in order to extract the pigments, but be careful not to splash the acetone).
3. While wearing a clean glove, scoop up the orange homogenate in your hand (wearing a glove only!), gently squeeze the extract from the chunks back into the mortar. Using a glass pipette or micropipetor, transfer 1.0 mL of the *liquid* homogenate into each of two labeled 1.5 ml microfuge tubes. Dispose of the squeezed chunks in the acetone dry waste in the fume hood.
4. Clarify the suspension by centrifugation in a microfuge for 3 minutes at maximum speed. Coordinate with other groups to use the centrifuge at the same time.
5. Transfer the clarified solution (~850 μ L each) to fresh 1.5 ml tubes. Label tubes and store on ice.
6. CLEAN the mortar and pestle by pouring any remaining liquid into the ACETONE WASTE container in the fume hood. Clean the emptied mortar and pestle with water.

Part 2: Preparation of Absorbance Spectra from Spinach and Carrot Plant Extracts

pigment extracts from spinach and carrot
Spectronic 20 with 1-cm cuvettes
10 mL pipettes
100% acetone

1. Preparation of the absorption spectrum requires that *some* light be transmitted through your sample. Therefore, you must adjust your sample concentration so that it falls in the appropriate range.
 - For the SPINACH sample, dilute your sample in 100% acetone to achieve an absorbance at 652 nm between 0.3 and 0.4. An estimation of the dilution you will need is 1 mL extract plus 4 mL 100% acetone for a final volume of 5 mL (practice question: what is the dilution factor for this dilution? _____). This dilution worked for your instructors when they practiced doing this lab last week. However, you may have been more or less successful at extracting the pigments from the tissue. Record YOUR dilution here:
 - For the CARROT sample – An estimation of the dilution factor to use is 1/20. You should plan on making 5 mL of this 1/20 dilution (remember that this is 1 part extract to a total volume of 20 parts). How do you do these calculations? Record YOUR dilution here:
2. Once your samples have been diluted appropriately, obtain data for absorption spectra of the carrot and spinach extracts. For each extract, record the absorbance at 10-nm intervals between 380-700 nm. Be sure to blank the instrument each time you change wavelengths. What should you use as your reference or blank? Be sure you answer this question correctly (check with your instructor!). Also, don't forget to zero the spec before you begin. If your spec won't blank at a particular wavelength, skip that wavelength and continue to the next interval.

Instead of blanking the machine twice at all those wavelengths during one lab period, you are strongly encouraged to wait before measuring the absorbances until you have a chromatographically separated pigment to work with. It is recommended that you should blank the machine at a wavelength and measure the absorbance of each of your **THREE samples at that wavelength. Then, change the wavelength, re-blank, and measure the **THREE** samples. And so on and so forth.**
3. Dispose of samples in the ACETONE liquid waste bottle in the fume hood. Place dirty test tubes in the bin labeled for their disposal (also in the fume hood—we don't want to breathe the fumes for the rest of the lab period).
4. Plot the absorption spectra for spinach and carrot extracts using graph paper or Excel.

****You will only be measuring data for THREE of these columns.** Get data for the other columns from other groups in your lab**

each pair of students will measure total spinach, total carrot, and *one* of the purified pigments

Absorbance Spectra Data Table

Dilution	spinach total extract	carrot total extract	TLC-purified pigment: beta-carotene	TLC-purified pigment: chlorophyll a	TLC-purified pigment: chlorophyll b	TLC-purified pigment: violaxanthin <small>non</small>
380nm			none	none	none	none
390						
400						
410						
420						
430						
440						
450						
460						
470						
480						
490						
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510						
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Part 3: Chromatographic separation of photosynthetic pigments.

concentrated (undiluted) pigment extracts from spinach and carrots

TLC plates coated with Silica Gel 60, 2.0cm x 7.0cm

thin paintbrushes, rulers

glass containers with lid

developing solution (Petroleum ether-acetone-chloroform solution (3:1:1 v:v:v))

NOTE: the developing solution is toxic and very flammable!! Use only in the fume hood.

A. Overview

- The acetone extracts contain a mixture of pigments that you will separate by using thin layer chromatography, (TLC).
- Each group will separate extracts of carrot and spinach. However, you will only isolate and construct an absorption spectrum for 1 pigment.

B. Protocol (wear gloves to prevent contamination of TLC plates and to protect yourself from the harmful organic solvents)

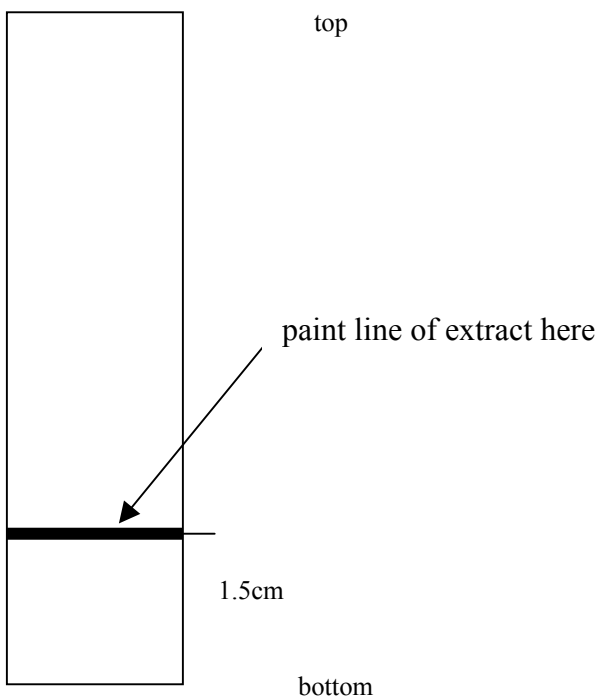
1. Take 2 silica strips (TLC plates), and use a pencil to GENTLY write a label near the top edge that indicates the pigment source: “s” for spinach, and “c” for carrot. (Figure 3). *NOTE: If you press too hard, the silica gel will crack off of the plate—if it does, you will need to get new strips.*
2. Next, using a pencil and a ruler, make a *TINY* mark 1.5 cm from the bottom of the strip (do not draw a line across the TLC plate).
3. Using a thin paintbrush, apply a **VERY THIN LINE** of the appropriate extract across the strip at your mark 1.5 cm from the bottom of the TLC strip. Try to keep the line as thin as possible.
4. Allow the line to dry *completely*; this may take 15-30 seconds (or even more). Once the first line is dry, apply a second line directly on top of the first one. Repeat until a total of 25 application lines have been made. You will have to be **VERY PATIENT** during this step. You may blow gently (be careful not to spit!) on the lines to encourage faster drying, but it is crucial that each application dries before the next application is added.

NOTE: if you don't allow the lines to dry completely between applications, the bands of separated pigments will be blurred.

5. After the 25th (and final application), wait 3 minutes to ensure the strip is thoroughly dry.
6. While you are waiting during this final drying step, set up 2 developing chambers by.
 - a. Add a small squirt of developing solvent into each of 2 chromatography chambers (only in the fume hood!).

NOTE: The level of the solution should be LESS than 1 cm (below the height of the spotted pigments) from the bottom of the chamber.
 - a. Cap the chamber (still in the hood).

7. Use forceps to lower one prepared TLC plate into each chamber and then re-cap the chamber (in the fume hood.) **DO NOT** MOVE THE CHAMBER AFTER ADDING THE TLC PLATE (if you do, the solvent will splash around and your chromatogram will not develop properly).
8. After the chromatograms have been started, watch the different pigments begin to separate as the solvent fronts move up the strips. This will take approximately 5-15 minutes.
9. (Read step 10 and be ready to proceed before doing step 9.) REMOVE the strip from the chamber when the solvent front comes within 1 cm of the top of the strip. **DO NOT** LET THE SOLVENT REACH THE END OF THE STRIP!!! Remove the strip only in the fume hood, because it will be stinky (and the fumes will be harmful for you to breathe). Be sure to replace the cap on the chamber to prevent the escape of more fumes. Leave the capped chambers in the fume hood when you are done.
10. **IMMEDIATELY** after removing the strip, use a pencil to mark the solvent front (the solvent will evaporate in approximately 2 seconds and you will NOT be able to see where it was if you haven't marked it). Continue with analysis of the strips as quickly and soon as possible, because the pigments will fade quickly. When the strip has dried, you may remove it from the fume hood to continue your analyses.
11. When all the chromatograms are complete, pour any remaining developing solvent into the **TLC WASTE** container located in the fume hood. DO NOT rinse the empty chromatogram jars with water.



C. Identifying Photosynthetic Pigments

1. Identifying characteristics.

As described in the handout introduction, angiosperms contain two broad classes of pigments: the chlorophylls and the carotenoids. Different pigments absorb light at different wavelengths, and hence differ in color (why?). Because of their different chemical structures, pigments also vary in their solubility in water and in their degree of polarity and absorbance to silica gel. This information is qualitatively described in the following table:

Table I. Characteristics of photosynthetic pigments

Pigment group	Pigment	Color	Solubility	R _f value
xanthophylls	neoxanthin	yellow	least soluble	0.09
xanthophylls	violaxanthin	yellow		0.21
xanthophylls	lutein	yellow		0.40
chlorophylls	chlorophyll b	yellow-green		0.46
chlorophylls	chlorophyll a	green-blue		0.60
chlorophylls	pheophytin	gray	↓	0.72
carotenes	beta-carotene	yellow-orange	most soluble	0.98

2. Analyzing the TLC data

- On the worksheet, sketch the banding patterns and solvent front you obtained for each chromatogram.

NOTE: You should do this as soon as possible after the strips are removed from the developing jars because some of the fainter bands may fade quickly.

- Measure the distance traveled by the solvent and the distance traveled by each pigment band. Record your data in the chart on the worksheet. Note that you may not find all four pigments in each of the extracts. If you do not, how will you determine which pigment is which?
- Answer the questions regarding the TLC data on your worksheet.

D. Determining the absorption spectrum of an isolated pigment.

1. Now that each of the pigments has been purified, it is possible to examine the characteristics of the individual pigments. Each group will determine the absorption spectra of one (or two, depending on the time available and the number of students per lab section) purified pigment.
2. Each group will be assigned a pigment to work on for this portion of the lab. Label two 1.5 ml microfuge tubes to identify your pigment of interest.
3. Identify your assigned pigment on the TLC strip and then use a razor blade to scrape the silica gel from the plate onto a piece of weighing paper. Pool the silica gel scraping from all the TLC plates in your lab section (the pigment from just one TLC plate will not be at a high enough concentration for you to measure its absorption spectrum). Your instructor may have additional scrapings to supplement those in your lab section.
4. Transfer the pooled silica powder into a 1.5 ml microfuge tube and add 1.0 mL of 100% acetone. Mix well.
5. Collect the silica gel in the bottom of the tube by centrifugation in the microfuge for 1 min at full speed. Notice the color of the silica gel. What color is it? _____
6. Use a 1.0 mL glass pipette, transfer the supernatant of purified pigment into the appropriate cuvette.
7. Determine the absorbance spectrum of your pigment from 380 nm to 700 nm. What should you use as your blank and why?

8. Using the graph paper available or Excel, plot the absorption spectra for the pigments that were successfully separated by thin layer chromatography.

BC2004 Lab Exercise 6. Data analysis.

You may work as a group to answer the questions and do the analyses shown below. You will not turn in this lab except as part of your lab notebook at the end of the semester, but be sure you understand the answers to the questions and how to analyze the data. You will be responsible for the material from this lab on next week's exam.

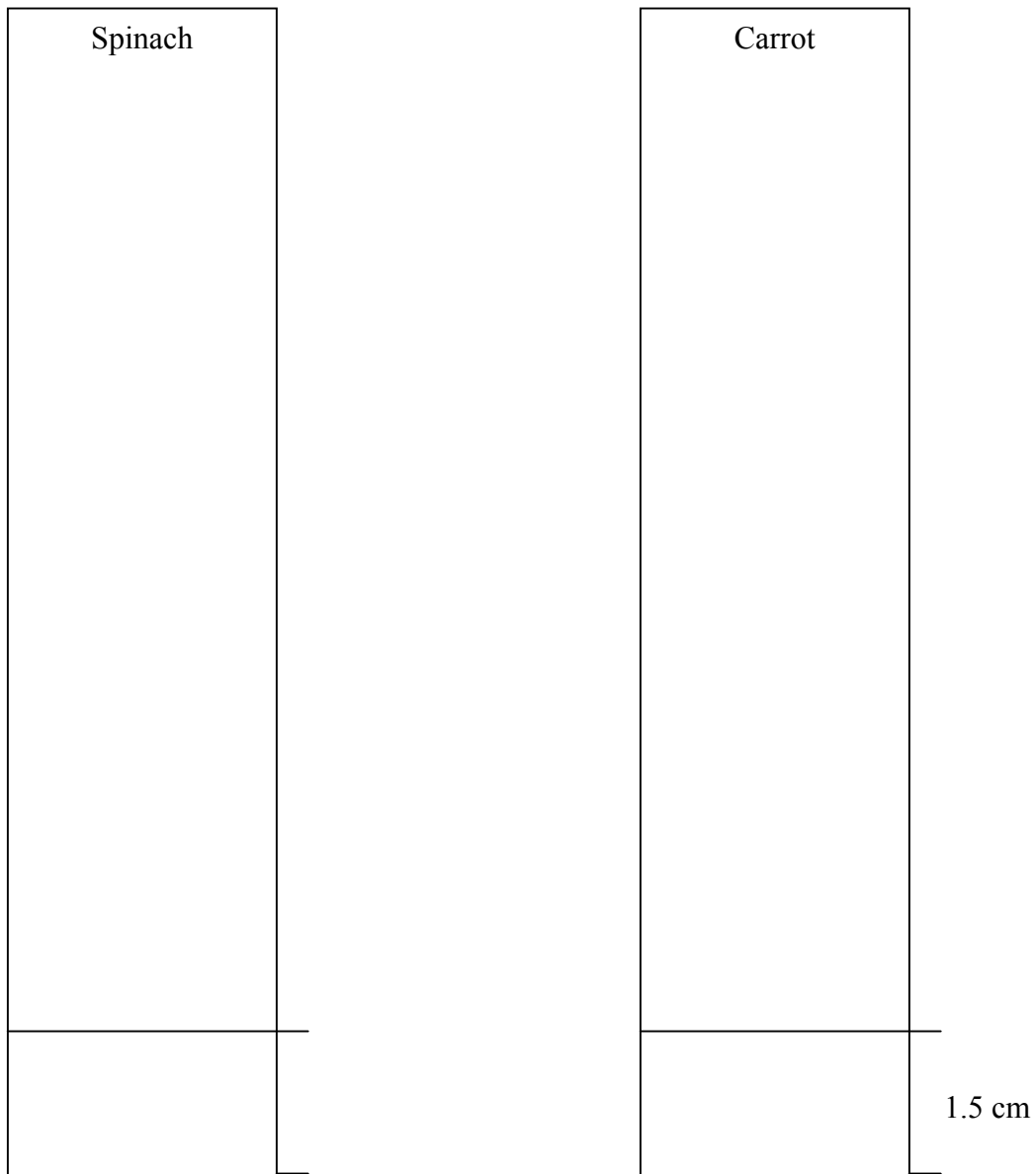
Part 2: Preparation of absorption spectra

1. Attach your properly labeled absorption spectra for carrot root and spinach leaf extracts. You may plot both spectra on one graph if you like. (*Remember to always label ALL axes of every graph you ever make! Always include units (unless, of course, there are no units, as for absorption).*)
2. Briefly compare the results you obtained for the two plant extracts.
3. In the chlorophyll *a*, chlorophyll *b*, and total chlorophyll analyses in lab exercise 5 last week, why didn't you need to perform some sort of correction for carotenoid content? (you won't need all this space)

Part 3: Chromatographic separation of photosynthetic pigments

3C.

1. Sketch the solvent front and the bands you observed on your chromatograms and label them based on the information shown in Table I. Also include the color of each band. Are all the bands nice, clear straight lines? If not, what might account for the distortion?



2. Measure the distance traveled (from the starting pencil line), and calculate the R_f for each pigment. (You may not have 7 pigments—begin at the top of the TLC plate and record data for each band below the solvent front.)

$$R_f = \text{distance traveled by pigment} \div \text{distance traveled by the solvent}$$

		Distance traveled		R_f value	
		Spinach	Carrot	Spinach	Carrot
Solvent front	Name of Pigment				
Pigment 1 (top)					
Pigment 2					
Pigment 3					
Pigment 4					
Pigment 5					
Pigment 6					
Pigment 7 (bottom)					

1. How do your values of R_f compare to those shown in Table 1?
2. Why are R_f 's useful to know? Speculate on whether they would be the same if you used a different developing solvent? Why or why not?
3. Did you have any difficulty determining which pigments were which? Why or why not?

3D: Absorption spectra of purified pigments

1. Prepare properly labeled absorption spectra for the TLC-purified pigments.
2. How do these spectra compare to those of the total plant extracts?
3. Describe the role of chlorophyll a and its accessory pigments in the light-dependent reactions of photosynthesis. How do the light-dependent reactions of photosynthesis relate to the light-independent reactions? Where (specifically) do each of these sets of reactions occur?