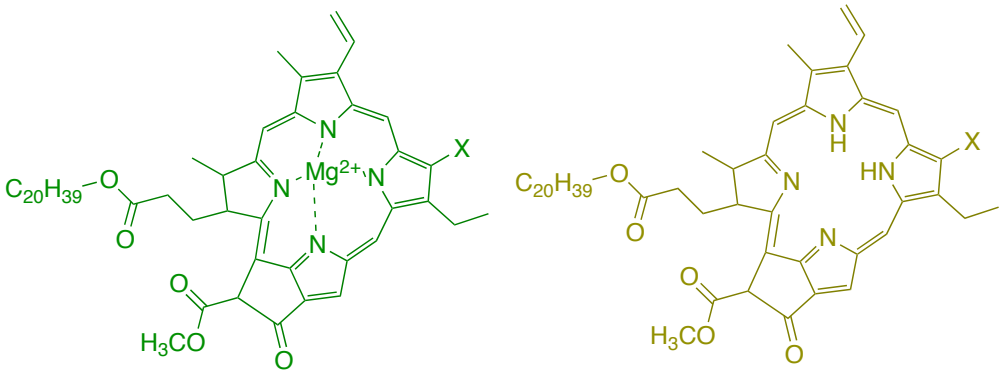
Experiment 4 – TLC Analysis of Spinach Pigments

Reading Assignment
Mohrig Chapter 18

Thin layer chromatography (TLC) is a form of qualitative analysis called **adsorption chromatography** used almost daily in synthetic organic chemistry labs. The composition of a mixture can be assessed relative to known standards and separation of the mixture is roughly based on polarity. In brief, a small portion of the sample is placed on the **stationary phase** (polar silica) and is carried up the TLC plate by the **mobile phase** (solvent). *A polar compound will interact more with the polar stationary phase and will not travel as far up the plate. Conversely, a less polar compound will move farther up the plate.* Notice that this technique is not terribly different from GC. Separation is based on polarity in both cases – relative boiling points can be estimated from polarity. Mobile and stationary phases are still employed, except that the states of matter are different. The principles of TLC will later be applied to *column chromatography*, where larger sample volumes will be physically separated and isolated based on polarity of the components. In this lab, students will use solvents of different polarity (mobile phases) to determine the optimal separation of the pigments found in spinach extracts.

The most prominent types of plant pigments are **chlorophylls, carotenoids, flavanoids,** and **tannins.** Chlorophylls contain a ring system formed by four pyrroles linked by four methine bridges, a Mg^{2+} ion in its center, and a long nonpolar hydrocarbon chain ($C_{20}H_{39}$). Chlorophylls are cousins of other biologically important molecules such as vitamin B_{12} and the heme found in hemoglobin; they all have a tetrapyrrole ring system. There are two main types of chlorophylls present in higher plants, **a** and **b**. Chlorophyll **a** is more abundant than chlorophyll **b** by a ratio of 3 to 1. The only structural difference between them is that a methyl group in **a** has been replaced by a formyl group, CHO, in **b**.



Chlorophyll a, X = CH_3
Chlorophyll b, X = CHO

Pheophytin a, X = CH_3
Pheophytin b, X = CHO

Figure 1. Structures of chlorophylls and pheophytins.

Chlorophylls are found in the chloroplast in association with small proteins. These protein-chlorophyll complexes are crucial in photosynthesis. The chlorophyll molecule acts as an antenna for visible radiation. The light absorbed is used to make carbohydrates and oxygen using carbon dioxide and water as raw materials.

Chlorophylls are labile compounds. In the presence of acids the central Mg^{2+} ion is easily replaced by protons and **pheophytins** are produced. In the summer months the leaves produce and degrade chlorophylls at a fast rate. Every fall, as the daylight dwindles, less chlorophyll is produced but its degradation continues, giving way the colors of autumn: yellows, reds, purples, and browns. These colors are largely due to the other leaf pigments, such as carotenes, flavins, and tannins (**Figure 2**), which are masked by an abundance of chlorophyll during the summer months.

Carotenes are polyunsaturated hydrocarbons that belong to the family of terpenes. Carotenes have 40 carbon atoms per molecule. They are found in the chloroplasts in association with chlorophylls and proteins where they, too, play an important role in photosynthesis. They are auxiliary pigments in the light-harvesting process and protect the chloroplasts against photooxidation.

β-Carotene, found in carrots, sweet potatoes, and green leaves, is one of the most abundant members of the family, and the precursor of vitamin A. Other members include α-carotene, an isomer of β-carotene with only half of its vitamin A activity, and lycopene, a red pigment found in tomatoes and watermelons that has no vitamin A activity. Carotenes are easily oxidized, especially in processed food, and must be protected from light and air to maintain their dietary value. The oxygen-containing products derived from carotenes are called **xanthophylls.** Xanthophylls and carotenes form the **carotenoid** family. The most abundant xanthophylls found in green leaves are lutein, violaxanthin, and neoxanthin (**Figure 2**).

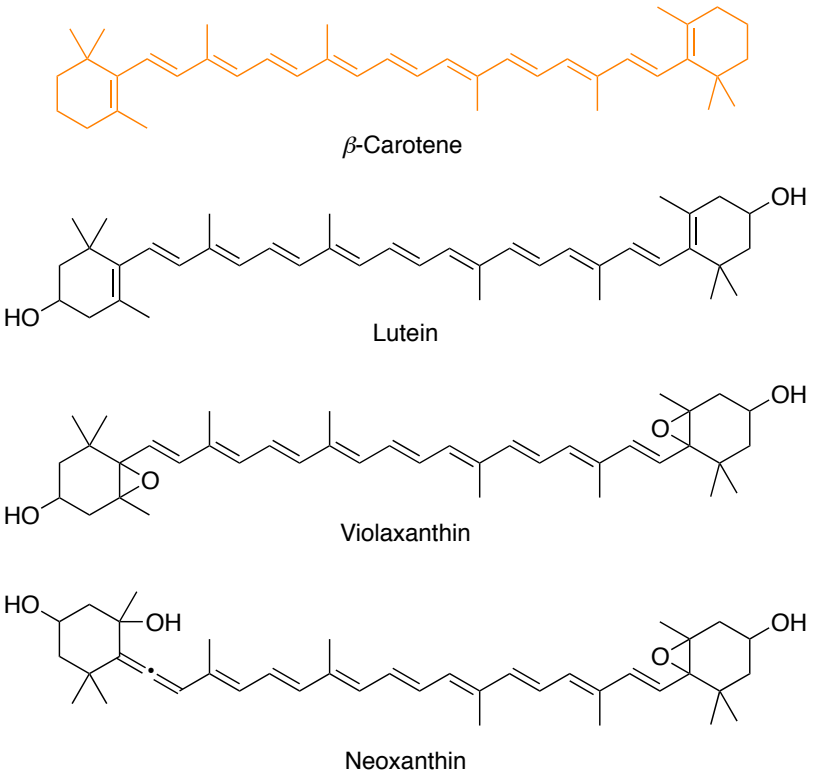


Figure 2. Structures of select carotenes and xanthophylls.

In this experiment students will isolate pigments from spinach leaves via solid-liquid and liquid-liquid extraction. The procedure given below can be applied with minor modifications to the extraction of chloroplast pigments (chlorophylls and carotenes) from almost all types of leaves. It can also be used to isolate the pigment from red, yellow, or green bell peppers. As already mentioned, the pigments from green leaves normally include carotenoids (β-carotene, lutein, violaxanthin, and neoxanthin), chlorophylls (a and b), and pheophytins (a and b). Pheophytins may be present in the leaves, but they may also be generated during the extraction process. As the cell membranes are broken, naturally occurring acids from the cytoplasm and other cell organelles come in contact with chlorophylls (from the chloroplasts), producing pheophytins.

The extraction is performed by first crushing the leaves with methanol, and then with a mixture of methanol and hexanes (two immiscible solvents). The first extraction with methanol breaks the cell membranes and removes water. This extract is discarded because it is poor in pigments. The second extraction with methanol-hexanes removes the pigments that go preferentially to the hexane layer. Methanol helps in detaching the pigments from their cellular complexes with proteins.

The extracts will be analyzed using TLC plates with silica gel as the stationary phase. You will investigate four different mobile phases and determine their power in separating the **chlorophylls (green), the xanthophylls (yellow), the pheophytins (green-gray), and the carotenes (yellow-orange).** You will observe that not all the mobile phases are able to move the pigments from the origin and other mobile phases will move only certain components.

Notebook Preparation

- *Purpose*: one-sentence description plus the scheme, which begins with the spinach leaves. Include the extraction solvents over the arrow (read below) and write the names of the potential pigments on the right side of the arrow.
- *Reagent Table*: hexanes, methanol, and ethyl acetate (bp, density, and hazards). Qualitative analysis in this experiment – no need to include masses. Include a list of materials (chemicals, glassware, equipment, etc.) underneath the table.
- *Procedure*: Hand-written, step-wise procedure in your own words; diagrams, pictures, flow chart encouraged
- *Safety & Clean-up* – Copy the table at the end of the procedure into your notebook.

Bring your own spinach leaves (roughly a large handful). Any unused spinach brought into the lab must be discarded (don't expect to take it home and eat it!).

PROCEDURE – students work in pairs

Vegetable Extract

Weigh 10 g of fresh spinach leaves and chop them using scissors. Place the spinach in a large mortar, add 12 mL of methanol, and crush the leaves with the pestle for about 3 minutes. This can get messy if you're careless so use common sense in your crushing technique! With the aid of the pestle or a spatula, squeeze the spinach against the side-wall of the mortar to remove as much methanol as possible. Transfer the liquid using a pipet into a 250-mL Erlenmeyer flask labeled "methanol-water" and set aside. The contents of this flask will eventually be discarded.

The following steps should be performed in a fume hood. Extract the remains of the leaves with a mixture of 15 mL hexanes and 5 mL methanol, crushing the tissue with the pestle for about 2-3 minutes. The extract should be deep green. Leaving behind as much solid as possible, use a pipet to transfer the liquid (a mixture of hexanes and methanol) to a 100-mL beaker. Set up the vacuum filtration apparatus and filter quickly in the fume hood, using an additional 2 mL of hexanes to aid the transfer. Collect the filtrate directly in a small filter flask and discard the filter paper. Clean the funnel immediately before proceeding to the next step or it will make any future experiment turn green! Use a small amount of methanol as instructed by your TA to initially rinse the funnel into the waste before washing in the sink.

Still in the fume hood, transfer the filtrate into a screw-cap test tube and add 5 mL of water. Shake, vent, and allow the layers to separate. If a deep green upper organic layer is not apparent, add 5 mL of hexane, being careful not to overflow the test tube. Remove the lower aqueous layer using a long-stem pipet with bulb and transfer into an Erlenmeyer flask labeled "methanol-water." Wash the remaining hexane layer with 5 mL of water (add 5 mL of water, shake, vent, separate). Collect the aqueous layer along with any emulsion present in the "methanol-water" flask. Add a small spatula-ful of granular sodium sulfate to the test tube dry the organic layer. Cap the test tube and swirl occasionally. After about 5 minutes, filter the suspension by using a clean and dry micro-funnel (about 2.5 cm in diameter) and a cotton plug. Collect the filtrate in a dry 50-mL round-bottom flask. Using a rota-vap, evaporate the solvent until the volume is approximately 2-3 mL. The color of the final extract should be deep green. With the aid of a Pasteur piper, transfer the liquid to a labeled scintillation vial or test tube.

TLC Analysis

Locate, but DO NOT REMOVE, the TLC jars in the fume hood with the names of the solvents to be used: Hexanes; 9:1 Hexanes-Ethyl Acetate (9:1 Hex/EtOAc); 7:3 Hexanes-Ethyl Acetate (7:3 Hex/EtOAc); Ethyl Acetate. Keep the jars covered with their lids except when opening to add your plate. ***Students are not permitted to add or remove any solvent from the TLC jars.*** Consult TA for assistance with adjusting solvent levels if necessary.

Obtain four silica gel TLC plates (2.5 x 6.6 cm). *Be extremely careful not to bend the plates or to scratch the silica surface (the chalky side). Handle plates by the edges.* Label them with the name of solvent on the very top of the plate with light pencil markings. Make two small pencil marks on either side of the plate to indicate the origin on the adsorbent side of the plate at a distance of about 1 cm from the bottom edge (see lecture handout).

Determining the Optimum Number of Applications

The "9:1 Hex/EtOAc" plate will be used to determine the optimum number of applications needed to visualize the separation of pigments. On this plate, the sample will be applied in three different lanes, each one with a different number of applications (1, 4, and 7). Dip a narrow capillary tube (not a melting point tube) in the extract; the liquid will rise through capillary action. Apply the liquid to the plate by gently and *briefly* touching the plate with the tip of the capillary tube. Raise the capillary tube to stop the flow of liquid when the diameter of the spot is about 1-2 mm. The spot should be very tight and small and at least 0.5 cm from the side edge of the plate. On the same plate, apply a second and a third spot at a distance of about 0.5 cm from each other. The number of applications for the second spot should be 4, and for the third spot 7. Allow the solvent to evaporate between applications. Failure to do so will result in very large spots.

Using tweezers, hold the plate next to the jar to ensure the spots are above the solvent level of the 9:1 Hexanes-Ethyl Acetate jar. If the spots are too low, the sample will dissolve in the solvent and contaminate other experiments. *Gently lower and place* (DO NOT drop) the TLC plate in the chamber with 9:1 Hexane-Ethyl Acetate. *Without moving the jar*, allow the solvent to run up the plate to a height about 0.5-1 cm from the top of the plate. ***Keep TLC chambers covered and in the fume hood***. Remove the plate from the chamber (use tweezers). *Immediately mark the solvent front with pencil* and allow the solvent to evaporate in the fume hood.

Sketch the TLC plates to scale in your lab notebook, paying special attention to the colors of the spots. Are they yellow, pale green, deep green, gray, orange? Notice any color spot at the origin. This analysis should be done without delay because the colors of the spots fade very quickly (within hours). Determine how many applications gave you the best results, or in other words, clear and well-delineated spots without streaking. If all spots are too faint to be seen, the extract should be concentrated by further evaporation of the solvent. If the spots run with tails, too much sample has been applied. Either reduce the number of applications or dilute your extract with hexanes.

Analyzing your Extract

Spot the extract on the remaining plates, using the optimal number of applications determined on the 9:1 plate. *Develop all the plates at the same time (to the best of your ability).* Calculate the R_f values for each pigment in each solvent. Refer to the top of page E4-3 to identify the pigments by color. Decide which solvent gives the best separation. Be sure to write down in your notebook all the data that are necessary to reproduce your R_f values (stationary phase, solvent, number of applications). Draw the plates in your notebook and indicate colors. Check first with your TA then dispose of all plates in the solid waste. Students who keep their plates will lose points. Dispose of your TLC plates and **answer the in-lab questions before leaving the lab.**

<table>
<tr><td colspan="2"><u>Clean-up and Waste</u>

This has the potential to be a very messy experiment if you're not careful. You may not be able to see the dark green extracts on the black counters, but it's there!
Clean and dry your work stations.
When you dry with paper towels, make sure it's a clean wipe, not a green wipe!
That's how you know it's clean ☺</td></tr>
<tr><td>General Clean-up</td><td>-Thoroughly wipe down bench tops
- Wash glassware and mortar & pestles (see below) with soap & water. TA's will finish clean-up with methanol.
- Clean vacuum filter funnel immediately after using.</td></tr>
<tr><td>Organic Solvents – TLC Waste</td><td>- Contents of "methanol-water" flask
- Mobile phase from TLC chambers (only if asked)</td></tr>
<tr><td>TLC – Solid Waste</td><td>- All used TLC plates (return unused to TA)
- Sodium sulfate and cotton plug
- Capillary tubes
- Pipets
- Let remains of spinach leaves dry in hood in mortar, then put in waste, NOT in sink</td></tr>
<tr><td>Reclaimed Hexanes</td><td>- Contents of the rota-vap trap</td></tr>
<tr><td colspan="2"><u>Safety</u>: Hexane and ethyl acetate are flammable. Keep TLC jars in the fume hoods.</td></tr>
</table>

Pre-lab Questions

1. Compare and contrast GC and TLC according to the following criteria:
   - State the classification of each chromatographic method;
   - Explain the differences in stationary and mobile phases (states of matter);
   - State the property that each type of separation is based on;

2. Arrange the following solvents/solvent mixtures in order of increasing polarity: hexanes, ethyl acetate, 7:3 hexanes-ethyl acetate, 9:1 hexanes-ethyl acetate.

3. Suppose a non-polar compound is spotted on a TLC plate. Which solvent from #2 will move the compound the farthest from the origin? Which solvent from #2 will move the compound the least from the origin?

4. What would happen if a TLC plate was placed in a jar where the solvent level was above the level of the origin (sample spot)? What should you do if you inadvertently spotted your plate too low (aside from consulting your TA)?

5. Why should ink be avoided in marking TLC plates?

6. Arrange the following compounds in order of increasing polarity: lutein (L), neoxanthin (N), β-carotene (C), violaxanthin (V). Which should display the highest R_f? Which should display the lowest R_f?

In-Lab Questions

1. What roles does methanol play in the first two extractions?

2. Draw the TLC plates both in your lab notebook and then again in your final report. Indicate colors (brownie points for using crayons or colored pencils in the report) and identify as many pigments as possible on each plate (chlorophylls, xanthophylls, pheophytins, and carotenes – see p E4-3). Recall that not all pigments will appear on all plates.

3. Calculate the R_f values for each spot on each plate and tabulate your results. Include the pigment identification (in-lab #2) for each spot. There is no need to calculate the R_f of a 'smear', but note in the table that a smear did occur. As you re-create the following table in a word processing document, it may be appropriate to 'merge' cells when spots overlap. Alternatively, you can list the same R_f value for several pigments if they overlap.

Table x. TLC Analysis of Spinach Pigments

Solvent → ↓ Pigment	Hexanes (R_f)	9:1 Hex/EtOAc (R_f)	7:3 Hex/EtOAc (R_f)	EtOAc (R_f)

* More rows may be needed.

4. Briefly discuss the overall separation power of the solvent using the points below based on your results. Consider the polarity of the solvents and pigments in your discussion.

(a) Explain the differences observed in the plates run with hexanes and 9:1 hexane-ethyl acetate.
(b) Was ethyl acetate a good solvent to separate the pigments?
(c) Which solvent was best to separate just the carotenes from the other pigments?
(d) Which was the optimal solvent for separation of the most pigments (greatest number of individual spots observed)?

Abstract

Consult the technical writing guidelines to write a draft of the abstract after completing the in-lab questions and before leaving lab, time permitting. Either way, plan ahead to get help with the abstract by going to TA office hours.

The main result in this experiment is determination of the **optimal TLC solvent**, being the one that separated spinach extract into the most spots. Retention factors (R_f) should not be included in the abstract. Use one sentence to comment on whether results were as expected – what polarity of solvent would you expect to best separate the spinach pigments based on their polarity?

Adapted from…Palleros, D. R. "TLC Analysis of Vegetable Extracts," Experimental Organic Chemistry, 2000. Wiley: Hoboken. p. 190-196.

Exp 4 Extraction and Analysis of Pigments From Spinach Leaves Name _____

Section Day _____ Time _____ TA Name _____

CHEM 8L GRADING RUBRIC – Use as cover page for report

SECTION	INSTRUCTOR COMMENTS	POINTS ASSIGNED
IN-LAB QUIZ		**/ 5**
LAB REPORT		
ABSTRACT One paragraph, four-six sentences: Purpose, procedure, main result(s), and conclusion(s).		/ **20**
INTRODUCTION Each pre-lab question is addressed complete sentences. Structures and calculations are hand-written, where appropriate.		/ **30**
RESULTS The main results are stated, as outlined in the in-lab questions, using complete sentences.		/ **40**
NOTEBOOK PAGES Proper format: reaction scheme, chemical info table, procedure, waste and clean-up procedure.		/ **20**
NEATNESS AND ORGANIZATION Proper order and format (see syllabus for full descriptions of each section).		/ **5**
LAB TECHNIQUE & CLEAN UP Lab space left clean, proper technique, instructions followed, checked in with TA before leaving.		/ **5**
	LAB REPORT TOTAL	**/ 125**