

Experiment 2

Extraction of Chlorophyll and Carotenes from Spinach and Analysis by Thin Layer Chromatography

From an adaptation for the short laboratory by Ms. Alexis Rich and Dr. Christine Rich, University of Louisville, Louisville, KY; modified by Dr. Toni Bell, Bloomsburg University

INTRODUCTION:

Ever wonder how scientists isolate those natural products that wind up leading to modern wonder drugs? I mean who would have thought to test the bark of Pacific yews for that wee bit of compound (taxol) that could stymie breast cancers? Likewise, who would believe that checking out seawater along sewage outlets on the Sardinian coastline would eventually lead to the cephalosporin antibiotics? Get this: lots of pharmaceutical leads come from stuff in our dirt.

Sometimes these discoveries are truly serendipitous; other times scientists are merely following up on leads provided by a region's culture. For centuries, some natives of the South American jungles have been getting a mellow high from licking the skins of certain frogs. When researchers finally checked it out, a new class of opioid peptides emerged...and the discovery of more frogs in other places (surprisingly, no one had ever thought to lick them before) which secreted different opioid peptides. I can't help being amused by that one....it's that mental picture of distinguished, bearded scientists in lab coats scouring the wilds for frogs to lick. To sober up, I get a different mental picture: one of those scientists clutching his chest in pain after licking a South American poison dart frog. This frog's skin secretions contain one of the most lethal toxins known. Just a little lick is enough to cause fatal cardiac arrest.

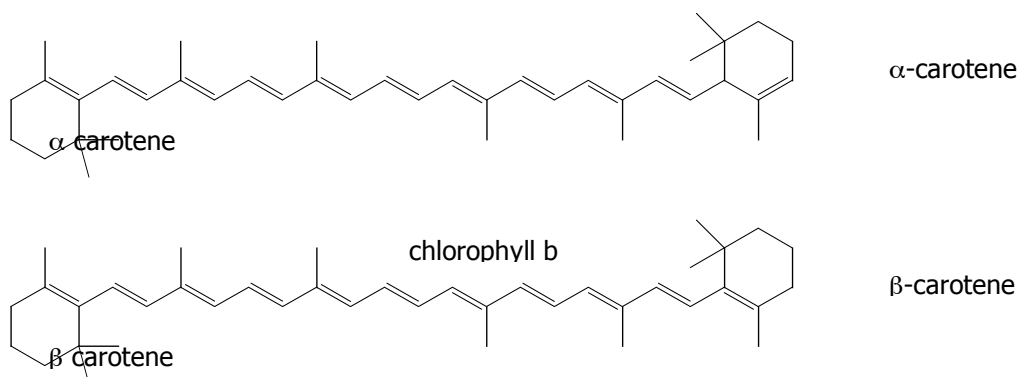
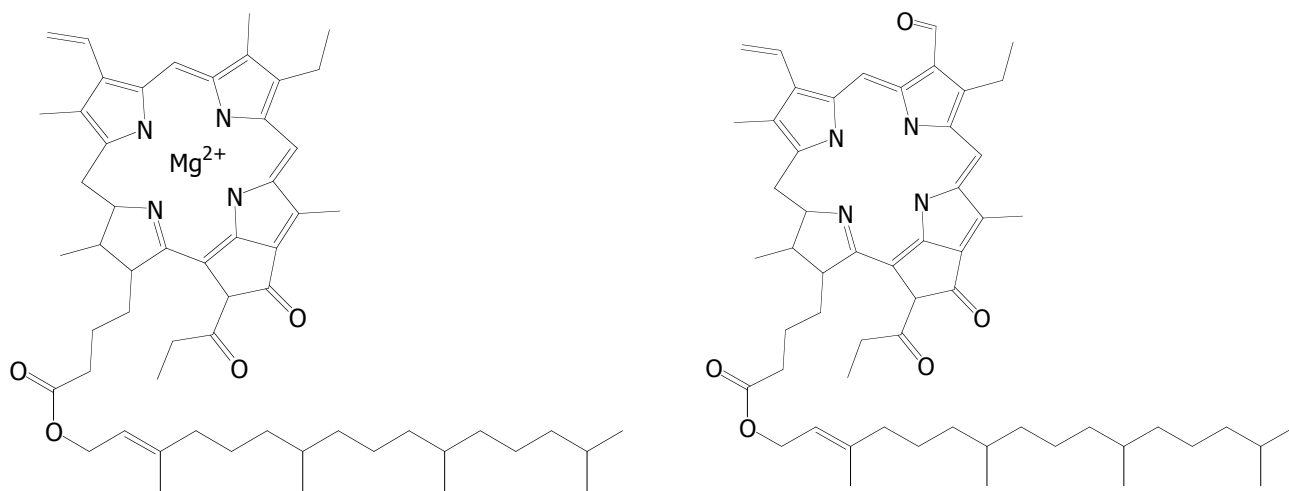
What's all this have to do with the next two labs? Well, go back and read the opening question. The most common approach to isolating these bioactive natural products is extraction.

Extraction-Although spinach isn't as exciting as amphibious skin secretions, it's a nice, non-toxic way for you to see how it all works. Think about all the stuff you know is in spinach: cellulose, iron (not as much as Popeye would have you believe), water-soluble vitamins, chlorophylls and carotenes. We're only interested in the last two. The structures of the chlorophylls and carotenes are shown on the next page. Compared to the other components mentioned, these are relatively non-polar organic substances. Since 'like dissolves like', they will be soluble in organic solvents like dichloromethane or acetone. Therefore, we should be able to selectively extract these compounds into organic solvent and leave the other compounds behind.

To do the extraction, you will first grind up the spinach in a little bit of acetone. We want to get as much of the chlorophylls and carotenes out of the leaves as we can. Unfortunately acetone will dissolve almost anything, including the stuff you don't want. The green acetone with the spinach compounds is called the "extract."

Chlorophylls and carotenes do not dissolve very well in water, but they dissolve like crazy in hexane. The other compounds do not dissolve well in hexane, but dissolve well in water. Like water and oil, water and hexane 'do not mix.' If you mix them together, the hexane will form a layer on top of the water (like oil does) because hexane is less dense than water. The differences in compound solubility in water and hexane, and the fact that water

and hexane form layers, will allow you to separate the chlorophylls and carotenes from the water-soluble compounds. After vigorous shaking to mix the layers temporarily, you will allow them to separate. The lovely green chlorophylls and yellow carotenes will leave the water at the bottom to dissolve in the hexane layer at the top. After pipetting off the hexane layer, isolating the chlorophylls and carotenes, you will see how many chlorophylls and carotenes you have and be able to characterize them by thin layer chromatography.



Thin Layer Chromatography—Once you have your hexane extract containing the products of interest, how could you quickly determine that the compounds are in there? And if so, how many? Run TLC, that's short for thin layer chromatography. You will put a little spot of your extract on a plastic plate coated with silica gel. Silica gel is a very polar substance. Then, you will stand the plate in a container with a mixture of solvents. In this case, the solvents will be quite non-polar. The solvents will begin to travel up the plate, like a wick. Some of the compounds in your mixture will be more polar and will stick to the spot on the silica. Other compounds in your mixture will be less-polar to differing degrees and will travel up with the solvent. Since there are many levels between totally polar and totally non-polar, the compounds can be separated by polarity. The more affinity a compound has for the solvent, the farther up the plate it will travel.

Before I close this discussion, I need to point out that these techniques are not used only in the context of natural product chemistry. You'll see that extractions are routine procedures during the work-ups of all manner of organic synthesis reactions. TLC has multiple applications. For instance, it is often used to monitor the progress of a reaction or to separate very small amounts of substance.

MATERIALS NEEDED:

fresh spinach leaves
acetone
5" Pasteur pipette
1-15mL centrifuge tube with a screw cap OR a test tube with a tight-fitting cork
distilled water
hexane
small beaker for waste
boiling chip or stick
anhydrous Na_2SO_4
sample vial
a pencil
a ruler
cotton OR glass wool
silica gel TLC plates-microscope slide sized
filter paper
TLC chamber
UV lamp

EXTRACTION PROCEDURE:

1. Weigh out approximately 0.5 g of fresh spinach leaves (don't use stems) and record the mass. Tear the leaves into confetti-sized pieces and place them into a mortar. Add about 1.0 mL of acetone and grind the leaves with a pestle until the acetone turns a bright, deep green. You may add more acetone as necessary.
2. Using a 5" Pasteur pipet, transfer the liquid to a centrifuge tube. Don't be too concerned if a small amount of sludgelike gunk is also transferred, it will be extracted later. Rinse your mortar and pestle with another 1.0 mL of acetone and transfer this to the centrifuge tube also.
3. If you have more than 1.5 mL of acetone in the centrifuge tube at this point, it's a good idea to reduce the volume via evaporation in a warm sand bath. Don't forget the boiling chip or boiling stick or it will spatter. Allow the extract to cool to room temperature before the next step.
4. Add 2.0 mL of hexane and 2.0 mL of distilled water to the extract in the centrifuge tube, cork it and shake gently. Occasionally, DIRECT THE TUBE AWAY FROM YOURSELF AND OTHERS, and vent. Soon, there should be a cloudy, light green lower water layer and a clear, bright green top hexane layer. Be sure to identify which is the water and which is the hexane

layer. Allow the tube to stand a few minutes undisturbed to maximize separation of these layers.

5. Using a 5" Pasteur pipet, carefully draw off the water layer and transfer it to a small waste beaker. Remember to depress your pipet bulb before you insert the pipet into the centrifuge tube.

6. Add another 2.0 mL of distilled water to the hexane layer in the centrifuge tube as a wash. The new water layer should be very nearly colorless even after shaking and allowing the layers to separate. Again, draw off the bottom water layer and combine it with the first one in the waste beaker.

7. Although water and hexane 'do not mix'...reality is that a little bit of the water will stay in the hexane. You can tell there is water in the hexane layer; it may be a little cloudy. You must 'dry' the hexane layer by adding some anhydrous Na_2SO_4 . A few microspatula scoops is usually sufficient. Na_2SO_4 soaks up the water as it becomes hydrated. However, you can be sure you've added enough when additional Na_2SO_4 no longer clumps when swirled in the extract and your extract is not at all cloudy. Ask your professor for help with this.

8. To remove the drying agent and filter your extract: prepare a filter pipet by inserting a small cotton plug into a 5" Pasteur pipet. Using a micro clamp, secure the pipet on a ring stand and place a clean collection vial below the stem of the pipet. Transfer your extract via pipet to the filter pipet. The cotton plug will prevent any drying agent from contaminating your extract as it drips into the vial. To complete the transfer, rinse the centrifuge tube containing the drying agent with another 1.0 mL of hexane and filter this also.

THIN LAYER CHROMATOGRAPHY:

1. Prepare the TLC chamber by placing a strip of filter paper up the side. Make 10 mL of hexane:acetone (7:3, v/v) mixture. Pour the solvent mixture over the filter paper into the beaker until it is about 0.5 cm deep. Place the lid on the chamber. This does two things: A. it keeps all the solvent from evaporating and B. it allows the air inside the chamber to become saturated with solvent. You don't want those volatile solvents evaporating from the plate as they travel up do you?

****Instructor's note:** Even slight changes in the composition or contamination of the developing solvent will lead to differences in R_f values. Don't let the chambers sit for long periods of time between plates. If a chamber gets contaminated, prepare a fresh one.**

2. Obtain a TLC plate and VERY lightly draw a pencil line (no ink!) about 1 cm from the bottom. *If you press too hard, the silica will come off...in which case you will have to get a new plate!* Using a capillary tube, make a spot of your extract on the pencil line. You may have to let the spot dry and then spot it again if it isn't dark enough. Carefully place the spotted plate into the chamber and replace the lid. *(If you drop the spot into the solvent, you will have to spot a new plate.)*

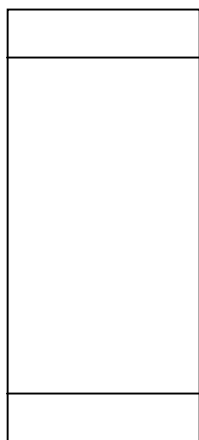
3. You will immediately see the solvent start to travel up the plate. The line of solvent moving up is called the 'solvent front.' Once the solvent front is within 1 cm of the top of the plate, remove the plate and quickly mark the solvent front with a pencil.
4. Although most of the spots are easily visualized by the naked eye, use the UV lamp to insure that you are noting all possible spots. Circle each spot in pencil. Lightly label each spot (A, B, C,...).
5. Determine R_f values for all of your spots. This will give you quantitative values for comparison. Measure the distance from the starting line to the solvent front. Then, measure to the center of each spot. Divide the center spot distance by the solvent front distance; this is the R_f value. The higher the R_f value, the less polar the compound.
6. Try to match them to the compounds shown below (listed in order of decreasing R_f values):
 - carotenes (1-2 yellow-orange spots)
 - pheophytin A (gray, intense)
 - pheophytin B (gray, may only be visible under UV)
 - chlorophyll A (blue-green, intense)
 - chlorophyll B (green)
 - xanthophylls (as many as 3 yellow spots)

Name(s) _____

Section _____ Date _____ Due Date _____ Score _____

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1. Mass of spinach _____
2. To the right, sketch the layers in centrifuge tube.
Clearly label the water and hexane layers.
3. Why must you occasionally vent the tube during shaking?
4. Draw a sketch of your TLC plate, labeling the spots. Then, fill in the table.



TLC Plate Sketch

Spot	Rf	Probable Identity

5. Were there any spots you couldn't identify?
6. Which compound is the most polar? Least polar? How do you know?
7. What do you think would happen if you used ink to mark to spotting line?