Lab #5:
EXTRACTION AND SEPARATION OF PLANT PIGMENTS

Purpose of the lab:
The purpose of this lab activity is for the student to learn about extraction and chemical separation technology. Specifically, the student will learn how to do a liquid phase-extraction and Thin Layer Chromatography in order to separate a mixture of molecules.

Method:
In the first step of this lab, the student will remove some of the water from spinach using a methanol rinse. In the second step, liquid-phase extraction will be used to extract pigment molecules from the partially dehydrated pulverized spinach. In the third and final step, the pigment molecules will be separated from each other using thin layer chromatography.

PROCEDURE
1. Vigorously crush about 10 spinach leafs with a mortar and pestle for 30 seconds.

2. Add 30 mL of methanol and continue to crush the spinach-methanol mixture for 30 seconds. Pour off as much of the methanol extract as possible and discard it in the waste container in the hood. It is ok if a small amount of crushed spinach is lost in the waste.

3. Locate the nearest fire extinguisher. There is a fire extinguisher near three of the four corners of the lab, so one should be nearby. Pick it up, study it, and decide exactly what you would do if you needed to use it. If you have any questions, ask the instructor. Replace the fire extinguisher on its mount on the wall.

4. Working under your bench-top hood to contain the vapors, add 25 mL of methanol and 35 mL of petroleum ether to the spinach in the mortar (Caution: petroleum ether is extremely flammable).

5. Crush for two minutes, and filter the liquid with vacuum as follows:
   • Use a 250 ml side-arm flask, Buchner funnel, and a piece of #3-filter paper in the funnel. Clamp the vacuum flask to a ring-stand. Clamps are in the common drawers.
   • Turn on the vacuum then pour the spinach-liquid mixture from the mortar into the funnel.

6. Place the liquid that you collected in to a clean 125 mL Erlenmeyer flask.

Figure 1. Filtration apparatus.
7. Add 25 mL of water to the liquid in the 125 ml Erlenmeyer flask from step 6. Stopper, then shake slowly (try not to get foaming) for 30 seconds, and then allow the two liquid layers to separate. At this point you should have a dark, intensely green, clear layer on top and a lighter green, cloudy layer on the bottom. (If you don't have a dark green layer on top, add 10 mL of petroleum ether and stopper, then shake again. If that doesn't work, consult with the instructor.)

8. Use a Pasteur pipette (located on the counter) to transfer about 1 ml of the dark green, top layer to your smallest, Erlenmeyer flask. Be sure the flask is dry; do not pre-rinse it.

9. Obtain two chromatography slides. You will be working in pairs. Each student should spot one slide. Spot using the capillary tubes provided and develop the slides in the capped jars as instructed below.

9.1. The spots must be small and dark. Spot the slide about 3 times (in the same place): ¾ of an inch from the bottom of the slide, waiting about 30 seconds between each spotting so the previous spot dries. DRAW A LINE NEXT TO YOUR SPOT (NOT TOUCHING THE SPOT) SO YOU WILL BE ABLE TO CALCULATE THE R_f VALUES LATER.

9.2. Use the developing troughs at the end of the lab benches. Place both partners’ slides in the trough, the developing solvent will work best if you keep the lid on trough. Note your slot number so you do not exchange your TLC slide with someone else’s.

9.3. Stop the development when the solvent comes to within 1.5 inches of the top of the TLC slide or when you can clearly see good separation of 5 spots. Immediately draw a line near the top of the slide where the solvent reached for calculation of R_f values.

DISCUSSION/DATA

The visible spots are, in order of decreasing R_f values, due to β-carotene (orange to orange-yellow with the highest R_f value), pheophytin a & b (grey, the pheophytin b spot is often too weak to be seen), chlorophyll a & b (green to blue-green), and the xanthophylls (yellow with the lowest R_f value). Xanthophyll is a general term that encompasses many different compounds. They are molecules of β-carotene that have been oxidized, so they contain many OH groups. Pheophytin a & b are identical to chlorophyll a & b except the Mg ions are replaced by hydrogen ions.
Measure the distance traveled by each spot and by the solvent. START ALL MEASUREMENTS FROM WHERE THE SLIDE WAS ORIGINALLY SPOTTED. Make a drawing in the box (below and to right) of your TLC slide, identify the spots, and record the distance travelled for the solvent and for each pigment molecule in the data table below. The pigment molecules are ordered from the top of your slide to the bottom, with spot #1 being the spot that travels the furthest (β-carotene). Note that you may not be able to see spot #3, #7, #8 because of the small amounts present in most spinach samples.

**DATA:**
*If you do not see all these spots, just write “not visible” for those not seen. Start all distance measurements, including the distance the solvent traveled from your original spot.*

<table>
<thead>
<tr>
<th>Spot</th>
<th>Molecule</th>
<th>Distance traveled(cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>solvent</td>
<td>distance the solvent traveled from your original spot</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>top spot, highest on slide</td>
<td>β-carotene color = yellow/orange</td>
</tr>
<tr>
<td>2</td>
<td>Pheophytin a color = grey</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Pheophytin b color = grey if visible</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Chlorophyll a color = blue/green</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Chlorophyll b color = green less intense than chlor. a</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Xanthophyll 1 color = yellow</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Xanthophyll 2 color = yellow if visible</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Xanthophyll 3 color = yellow if visible</td>
<td></td>
</tr>
</tbody>
</table>

Start your Rf measurements where you originally spotted the slide. Then measure to the center of each spot.

**Drawing of developed slide goes here:**

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Calculate the R<sub>f</sub> values:

<table>
<thead>
<tr>
<th>Spot</th>
<th>Molecule</th>
<th>Calculation</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>β-carotene</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>color = yellow/orange</td>
<td>note: pay attention to significant figures and units</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Pheophytin a</td>
<td>color = grey</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Pheophytin b</td>
<td>color = grey if visible</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Chlorophyll a</td>
<td>color = blue/green</td>
<td></td>
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<td>Chlorophyll b</td>
<td>color = green less intense than chlor. a</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Xanthophyll l</td>
<td>color = yellow</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Xanthophyll 2</td>
<td>color = yellow if visible</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Xanthophyll 3</td>
<td>color = yellow if visible</td>
<td></td>
</tr>
</tbody>
</table>

**QUESTION**

1) Silica gel (the coating on your TLC slide) is very polar and the developing solvent is quite non-polar. Based on your TLC slide data (R<sub>f</sub> values), which of the plant pigments that you see on your slide is the most polar? **Explain.**