CHROMATOGRAPHY

Chromatography relies on physical interactions between molecules to separate a mixture. A chemical reaction does not occur. Instead, the substances in the mixture have structural differences that determine how each substance will interact with the stationary phase, and how well that substance will dissolve in a solvent (also called the eluent or mobile phase). Chapter 3 of *Chemistry in Context* discusses the property of molecules called polarity, which is one important property that is involved in chromatographic separations.

During a chromatographic separation involving column chromatography, a mixture in solution is applied to the top of a vertical column of a packed stationary phase. The vertical column is typically made of a glass tube with a valve at the bottom that opens and closes. The mobile phase (or eluent) typically may be any organic solvent, water, or dilute water solution of another solvent. As solvent is added to the top of the column, the various components of a mixture will dissolve in the solvent, or will be attracted to the solid phase. Any component dissolved in solvent will travel down the column. Any component attracted more to the solid phase will remain on the column. The old adage "like dissolves like" holds true for chromatography.

A carefully chosen combination of stationary phase and mobile phase will provide for each component of a mixture to be somewhat soluble in each phase. Thus, each component will be attracted to the solid phase, then will dissolve into the mobile phase, then back onto the solid phase, after traveling down the column some distance. Subtle differences in structure can mean big differences in the time it takes for two components of a mixture to travel down a column. A substance that is more like the stationary phase, is more attracted to this phase, and spends more time on the column. This means less time dissolved in the solvent, and less time traveling down the column. A substance that is more like the eluent (mobile phase), spends more time dissolved in the mobile phase, and reaches the bottom of the column sooner. Substances with different colors can be observed to separate into different fractions, or colored bands, on a chromatography column. The separate bands of color can be collected at the bottom of the column, as solutions of different components of the original mixture.

Stationary phases such as cellulose paper, or silica or alumina, are relatively polar. Silica can be coated with hydrocarbon compounds that impart a nonpolar nature. Mobile phases vary widely, from common organic solvents such as acetone or hexane, to water and water solutions with a variety of pH's, to inert gases that travel through columns at high speeds and temperatures. Carefully choosing combinations of stationary and mobile phases can provide suitable conditions for the separation of so many different types of substances, that chromatography is now widely employed in research and industrial laboratories all over the world. For instance, purification of pharmaceutical products often requires chromatography.

Chromatography of Plant Pigments: Column Chromatography of Spinach Extract

Spinach appears dark green. This color is actually a combination of several different substances, each with a slightly different color: β-carotene and chlorophylls. β-carotene is converted to Vitamin A in our bodies. In solution of a hydrocarbon solvent such as petroleum ether, β-carotene appears yellow. Crystals of the pure solid are red. Since this substance is beneficial when ingested, it has been adopted as a food dye, to impart various shades of red or yellow to foods. Chlorophylls of several types are separated as a group of compounds that appear green in solution. Chlorophylls are the essential green components of photosynthesis.

A careful choice of stationary phase and mobile phase will enable the separation of a yellow solution of β-carotene and a green solution of several chlorophylls from spinach leaves. Column chromatography will be used, with a stationary phase that is a form of aluminum oxide called alumina. Alumina is relatively polar.

The mobile phase required for this separation involves two solvents: petroleum ether, a mixture of low-boiling hydrocarbons, and acetone, a common solvent often found in nail-polish remover. The structures of β-carotene and one form of chlorophyll are provided.

Procedure for Separating β-carotene and Chlorophylls from Spinach

Extracting the Spinach Pigments

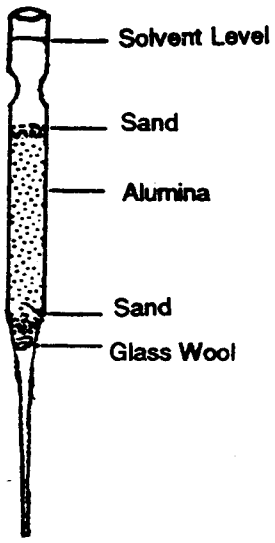
1. Place about one teaspoon of freshly thawed spinach in a mortar, and add about 25 mL of solvent that is composed of 80% petroleum ether, 20% acetone.

2. Grind up the spinach until the solvent takes on a green color. Decant the liquid into a large test tube.

Preparing a Chromatography Column

3. Obtain a disposable, glass Pasteur pipette. Soak a small swab of glass wool (about the size of a pea) in petroleum ether, and stuff it from the top of the pipette column to the bottom end, gently with a piece of wire. Be sure the glass wool is firmly in place, where the pipette narrows, but do not pack it too tightly (The solvent must flow through the glass wool. Also, pushing on the glass wool might crack the pipette.)

Disposable Pipette Column



4. Have a beaker or other large container from your lab drawer ready to catch solvent as it flows through the column you are preparing. Hold the pipette column over the beaker, to catch the solvent. Add petroleum ether to the top of the column, until it is nearly filled. Quickly add just enough sterilized sand to give a 3 mm layer on top of the glass wool

5. Add alumina slowly into the petroleum ether in the column, until the column is nearly filled. Tap the column with a pencil to ensure even packing of the alumina. If necessary, add more petroleum ether to maintain the level of solvent well above the level of alumina; this prevents the formation of bubbles in the column. Add alumina until about 1.5 - 2 inches of column remains empty at the top. The alumina column should be about 4-6 cm high. (Your lab instructor can help you determine how much alumina to add).

6. To the top of the alumina layer, add enough sterilized sand to provide a 3 mm layer. At this point, the column should be dripping petroleum ether at the rate of about 1 drop per second.

7. When your column is prepared, allow the solvent to drip out until the sand on top of the column is slightly covered with a thin layer of solvent. To the top of the column, add about 1 mL of the prepared spinach extract (from step 2), using a clean pipette.

8. Wait until the level of the spinach extract falls to the level of the sand, then add petroleum ether to the column, keeping the level well above the top sand layer. Continue to add petroleum ether to the column, maintaining the level well above the top sand layer. A band of yellow material will begin to separate and travel down the column, as you add more petroleum ether.

9. Use a clean, dry test tube to collect the yellow solution as it flows off the chromatography column. Set this yellow solution aside, to be analyzed later.
10. When all the yellow material has been removed from the column, add acetone to the top of the column and a band of green chlorophyll will appear, and will begin to move down the column.

11. Continue to add acetone to the top of the column, maintaining the level of the acetone well above the top sand layer, until all the green material has traveled down the column.

12. Collect the green solution in a clean, dry test tube. Keep the green solution separate from the yellow solution.

13. Any excess solvent at the top of the column should be poured into the Used Organic Solvent container, in the large hood at the back of the lab. The glass pipette should be disposed of in the blue and white glass disposal boxes at the front of the room. DO NOT PLACE GLASS IN THE TRASH CANS!!.

Analysis of the Spinach Pigments

Observe the colors of the two fractions collected from your chromatography column, and note which fraction was eluted from the column first. Note also which solvent eluted which color.

Spectrometric Determination of β-carotene and Chlorophylls

1. Read the directions on page 64, before using the Bausch and Lomb Spectronic 20s which are available in the lab. Be sure to turn on the Spec 20 at the beginning of each lab period, to allow it to warm up.

2. Obtain 2 cuvettes from the stockroom (room 141). These cuvettes resemble test tubes, but are made of special glass. Each cuvette will have a blue or white marking near the neck of the tube, which your lab instructor can help you identify. Please keep track of the cuvettes, and return them to the stockroom at the end of each lab period.

3. Place 3-4 mL of your yellow solution in one cuvette. Combine the yellow fractions from several groups, if you do not have enough solution. The yellow fraction contains the β-carotene. The cuvette does not need to be filled to the top. Wipe off the outside of the cuvette after filling.

4. The second cuvette will be used to contain a background or "blank" for your determinations. The blank will change for the green and yellow fractions, since the pigments were eluted (washed off the column) with different solvents. The yellow fraction contains the β-carotene; this fraction was collected in petroleum ether. Fill the second cuvette with 3-4 mL of petroleum ether, taking care to wipe off the outside of the container.

5. Take your cuvettes with you to the Spec 20, placed in a test tube stand or beaker. Set the wavelength dial on the top right of the Spec 20 to 680 nm.

6. Follow the instructions given by your TA or in SuperChem Lab on how to operate, zero and blank the Spec 20. At each wavelength, zero, blank, and then take the sample(s) reading before proceeding to the next wavelength.
7. Record the %Transmittance for the yellow solution at 10nm increments from 680nm down to 400nm. Petroleum ether will be used as the blank for the yellow solution.
8. At each wavelength convert the %Transmittance values to Absorbance using the following formula:

   Absorbance = 2.00 – (log %Transmittance)

9. Repeat steps 3 through 8 for the chlorophyll (green) solution, except that acetone will be used for the blank instead of petroleum ether. The chlorophylls are in acetone solution since that is the solvent used to elute the chlorophyll from the column.
10. Plot a graph of %Transmittance vs Wavelength and Absorbance vs Wavelength for the *B*-carotene. and for the chlorophylls. Place both samples in the same graph using excel so that two graphs will be produced (%T vs wavelength and A vs wavelength).

Chromatography of Plant Pigments **Data Page 1**

Name _____ Date _____

Lab Partner _____ Lab Instructor _____

Which color reached the bottom of the column first? _____
What solvent (eluent) were you using to elute this pigment? _____

For each test solution, record the % Transmittance reading from the dial on the Spec 20 in the column beside the wavelength. Record the calculated Absorbance in the third column, next to the % Transmittance.

Wavelength (nm)	% Transmittance	Absorbance
680		
670		
660		
650		
640		
630		
620		
610		
600		
590		
580		
570		
560		
550		
540		
530		
520		
510		
500		
490		
480		
470		
460		
450		
440		
430		
420		
410		
400		

Chromatography of Plant Pigments **Data Page 2**

Name _____ Date _____

Lab Partner _____ Lab Instructor _____

Which color reached the bottom of the column second? _____
What solvent (eluent) were you using to elute this pigment? _____

Record the % Transmittance reading from the dial on the Spec 20 in the column beside the wavelength. Record the calculated Absorbance in the third column, next to the % Transmittance.

Wavelength (nm)	% Transmittance	Absorbance
680		
670		
660		
650		
640		
630		
620		
610		
600		
590		
580		
570		
560		
550		
540		
530		
520		
510		
500		
490		
480		
470		
460		
450		
440		
430		
420		
410		
400		