

# Isolation of Chlorophyll and Carotenoid Pigments from Spinach

Adapted from: Pavia, D.L., Lampman, G.M., Kriz, G.S., and Engel, R.G. *Introduction to Organic Laboratory Techniques: A Microscale Approach 3<sup>rd</sup> Edition* Saunders College Publishing: New York, NY, 1999.

## Technical and Theoretical Skills

In this assignment you will learn

- Isolation of a natural product
- Extraction
- Column Chromatography
- Thin-layer chromatography

## Introduction

The leaves of plants contain a number of colored pigments generally falling into two categories, chlorophylls and carotenoids. The green chlorophylls a and b, which are highly conjugated compounds capture the (nongreen) light energy used in photosynthesis. Carotenoids are part of a larger collection of plant-derived compounds called terpenes. These naturally occurring compounds contain 10 (=monoterpene i.e., limonene, thujene, carene, geraniol, menthol, terpineol), 15 (=sesquiterpene i.e., zingiberene, farnesol), 20 (=diterpene i.e., taxadiene, steviol, phytol, retinol), 25 (=sesterterpenes, i.e., geranylarnesol), 30 (=triterpene i.e., squalene, lanosterol) and 40 carbon atoms (=tetraterpene i.e., lycopene, carotene), which suggest that there is a compound with five carbon atoms that serves as their building block. Their structures are consistent with the assumption that they were made by joining together isoprene units, usually in a “head to tail” fashion. Isoprene is the common name for 2-methyl-1,3-butadiene. The branched end is the “head” and the unbranched is the “tail”. That isoprene units are linked in a head to tail fashion to form terpenes is known as the isoprene rule. Lycopene (shown on the right), the compound responsible for the red coloring of tomatoes (3 mg/100 g) and watermelon (4.5 mg/100 g), and  $\beta$ -carotene, the compound that causes carrots (8 mg/100 g) and apricots (2 mg/100 g) to be orange, are examples of carotenoids.  $\beta$ -Carotene which cleaves to form two molecules of vitamin A when it is ingested, is the major dietary source of this vitamin. Carotenoids act as antioxidants and anti-inflammatories in the human body. Retinol, also called vitamin A, plays an important role in vision. Lutein and zeaxanthin are associated primarily with eye health

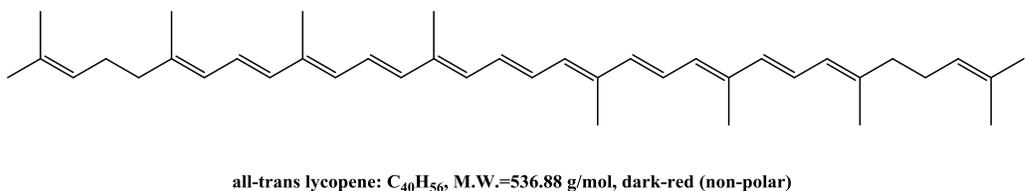
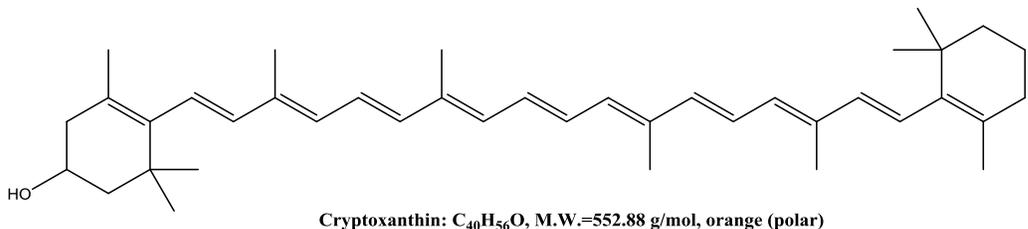
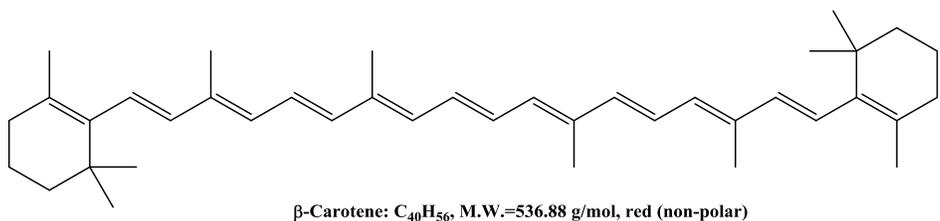
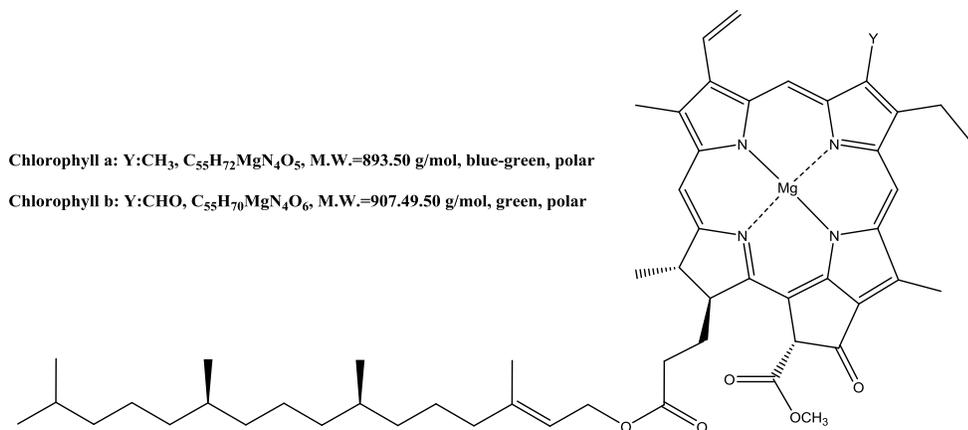


Spinach leaves, which the students will use in this assignment this quarter, contain chlorophyll a, chlorophyll b and  $\beta$ -carotene as major pigments as well as smaller amounts of other pigments such as xanthophylls. The xanthophylls, which are oxidized versions of carotenes, and pheophytins, which look like chlorophyll except that the magnesium ion is replaced by two hydrogen atoms. In this assignment the student will isolate and separate the spinach pigments using differences in polarity to effect the separation. Since the different components are colored differently, the separation is easily followed visually.

## Molecular Structure and Polarity

The structures of the major components are given below. Note that the  $\beta$ -carotene, which is a hydrocarbon, is nonpolar. By contrast, both chlorophylls contain several polar C-O and C-N bonds and also a magnesium ion chelated to the nitrogen atoms. The distinctions between the chlorophylls, which are more polar than  $\beta$ -carotene is slight: chlorophyll a has a methyl group ( $Y=CH_3$ ) in a position where chlorophyll b has an aldehyde ( $Y=CHO$ ). This makes chlorophyll b slightly more polar than chlorophyll a. Pheophytins are chlorophyll molecules without the  $Mg^{2+}$ -ion and two nitrogen atoms protonated instead.

After you extract the pigment mixture from the leaves into a hexane solution, you will take advantage this polarity difference to separate the mixture using column chromatography.



## Column Chromatography

Column chromatography involves the separation of compounds by the same mechanism as other chromatographic techniques, *i.e.* differences in partitioning between mobile and stationary phases (See "Chromatography" in your text).

The stationary phase serves as an adsorbent through which the mobile phase is passed. Many compounds with varying functional groups may be used as the stationary phase and several types of interactions can aid in developing the desired separation (*i.e.*, hydrogen bonding, dipole-dipole, interactions, electrostatic interactions, Van der Waals forces, size exclusion, affinity, etc.). The following sequence illustrates the general affinity of the functional groups towards a polar stationary phase like silica:

ionic > acids/bases > amides > alcohols > ketones > aldehydes > esters > ethers > halides > unsaturated hydrocarbons > saturated hydrocarbons

The major advantages of column chromatography are its ability to handle larger amounts of material (compared to GC, TLC and HPLC) and the ability to change the eluting solvent throughout the course of the separation. This allows one to remove one component while a desired product remains essentially unmoved. A change in the mobile phase moves the desired product through the column, which may include changes in solvent polarity, changes in pH, or changes in ionic strength. The last two are used largely in biological separations (proteins, peptides).

By varying the stationary phase and by changing the mobile phase, an efficient separation may be achieved. In this assignment, alumina serves as the stationary phase, which is considered a polar stationary phase because of the presence of oxygen and hydroxyl groups on the surface. Thus, the more polar components are held to the polar alumina more tightly and, therefore, move through the column more slowly. Increasing the polarity of the solvent moves all components faster but has the largest effect on polar compounds because their solubility increases significantly.

Finally, you will analyze and identify the pigment fractions using thin layer chromatography, which also separates by polarity.

## Experimental Procedure

### Period 1: Isolation of Pigment from Spinach Leaves

Weigh about 1.0 g of fresh spinach leaves (avoid using stems or thick veins). Cut or tear the spinach leaves into small pieces and place them in a mortar along with 0.5 g of anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) and 2.0 mL of acetone. Grind with a pestle until the spinach leaves have been broken into particles too small to be seen clearly and mix well with  $\text{Na}_2\text{SO}_4$ .

If too much acetone has evaporated, you may need to add an additional portion of acetone (0.5-1.0 mL). Using a Pasteur pipette or spatula, transfer the mixture to a centrifuge tube. Rinse the mortar and pestle with 2.0 mL of cold acetone, and transfer the remaining mixture to the centrifuge tube. Cap tightly. Centrifuge the mixture being sure to balance the centrifuge first (**Make sure to return the centrifuge tube to the TA at the end of the meeting!**).

Add ~2.0 mL of hexane to the centrifuge tube, cap the tube, and shake the mixture thoroughly. Next, add 2.0 mL of water and shake thoroughly with occasional venting. Centrifuge the mixture to break the emulsion, which usually appears as a cloudy green layer in the middle of the mixture. The pigment layer is the top layer, which should be dark green. Most of the acetone will dissolve in the water.

Using a dry Pasteur pipette, carefully separate layers and transfer the top organic layer (a dark-green hexane solution of spinach pigments) into a clean test tube. Add another 1.0 mL of hexane to the centrifuge tube that contains the aqueous layer, cap the tube and centrifuge the mixture to break the emulsion. Separate the layers again and add the top layer to the test tube.

The dark-green hexane solution of spinach pigments in the test tube may contain traces of water that must be removed before separating the components through chromatography (Why?). To dry the solution, add 0.5 g of anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) to the hexane solution. Cap and gently swirl to allow the sodium sulfate to contact all parts of the hexane. After standing for 5 minutes, use a clean, dry Pasteur pipette to transfer the liquid into another clean test tube. Label this test tube with an *E* for **extract** so that you do not confuse it with the test tubes you will be working with later in this experiment.

Add about 0.5 mL hexane to rinse the hydrated sodium sulfate and transfer this liquid to the same test tube, *E*.

### Column Chromatography of Spinach Pigments

Clamp a clean, dry Pasteur pipette vertically and push a very small plug of cotton to the bottom of the pipette. Weigh out about 1.25 g of alumina. Pack the column with alumina and gently add a little less than one half cm of sea sand to the top of the column. Tap gently to achieve a flat surface.



Once the procedure is started, it should not be stopped: **The alumina must be kept wet with solvent all the time.** Since the column does not have a stopcock to stop the flow of solvent during the procedure, all the solvents must be at your workspace before starting the process. In separate labeled containers obtain 15 mL hexane, 15 mL of 70 % hexane-30 % acetone solution, 15 mL acetone and 15 mL of 80 % acetone-20 % methanol solution. Also, label six test tubes 1 through 6 and a beaker as “waste solvent.”

First, transfer about 0.5 mL of your spinach extract (from test tube, *E*) to a small vial for use later in the thin layer chromatography analysis.

Then, when you are ready to begin the column separation, place the waste solvent beaker under the column and add about 3.0 mL of hexane to the top of the column. As the alumina is wetted, the hexane will flow into the beaker. When the solvent level drains to the top of the sand add your spinach extract to the top of the column.

As the extract drains onto the alumina, the pigments should begin to separate into a yellow carotene band and a green chlorophyll band. If a separation is observed, add an additional 4 mL of hexane and continue collecting solvent in your waste solvent beaker until the yellow band reaches the

bottom of the column and the solvent draining out turns yellow. Replace the waste beaker with test tube #1. Continue to add hexane until the yellow band passes through the column switching to test tubes #2, #3, etc as necessary until the yellow eluant becomes clear. Then, replace the test tube with the waste beaker.

If the yellow band has not begun to separate from the green band after the initial 4 mL of hexane has drained through the column, add 4 mL of the next more polar solvent (70 % hexane-30 % acetone). When changing solvents, do not add the new solvent until the level of the last solvent is almost at the top of the alumina. When the appropriate solvent is found add this solvent until the yellow band is completely removed from the column.

Once the yellow band has eluted from the column, add several ml of the next more polar solvent when the level of the last solvent is almost at the top of the see sand. If the green band moves down the column, continue to add this solvent until the green band is eluted completely from the column. If the green band does not move, change to the next polar solvent. Collect the green band in a clean test tube. Cover and save these fractions for the next lab period when you will study the visible spectrum and TLC of the green and yellow fractions.

*If it is necessary due to time constraints, the vial can be labeled with your name, capped, and stored until the next laboratory period.*

## **Period 2: Characterization**

### **1. Thin Layer Chromatography:**

Before you can carry out the thin layer chromatography (TLC) on your fractions and original extract, you must save about 3-mL of the chlorophyll and  $\beta$ -carotene fractions for UV-visible spectra. For the TLC you must concentrate the separated fractions. Pass a gentle stream of air over the solutions in a hood to evaporate the solution to solvent about 1 mL of yellow and 1 mL of green solution. If you have several test tubes of the same pigment, combine the contents as you evaporate so that you end up with just one vial of yellow carotenes and one vial of green chlorophylls.

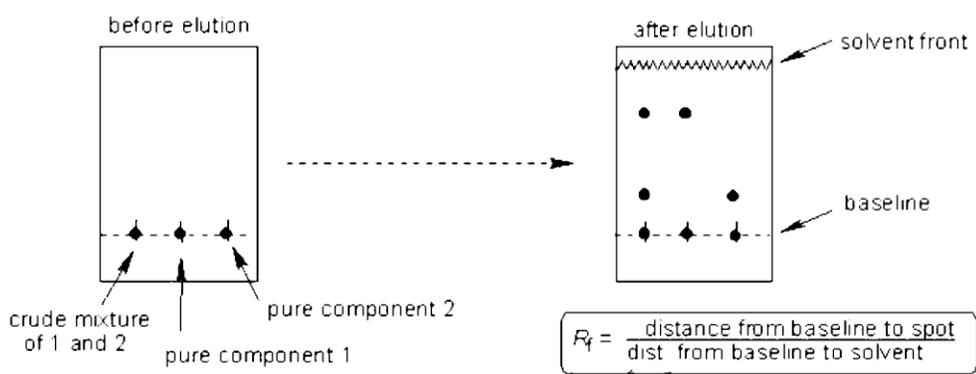
Gently draw a horizontal pencil line about 1 cm from the bottom of a thin layer chromatography (10 cm x 4 cm). As shown in the figure below, spot your three solutions (E, yellow and green) on this line. Use a separate microcapillary tube for each solution. Fill each capillary by dipping it in the solution and gently touch it to the plate to empty it.

Use several short touches to empty each capillary so that the spots will be small (1-2 mm in diameter). If the spots look light in color, re-spot on top of the original spots until the spots are fairly dark. Allow the spots to dry. Obtain a chromatography jar, and to the jar add about 10 mL of developing solvent (70 % hexane-30 % acetone) then place the TLC plate in the developing jar with the spotted end at the bottom of the jar and allow the solvent to rise up the plate undisturbed until about 80 % of the plate is wet (**The start line has to be above the solvent level**). Remove the plate and quickly draw a pencil line across the plate to mark the farthest reach of the solvent.

This is called the solvent front. Allow the wet plate to dry in the hood. Circle the visible spots in pencil since the colors may fade over time. **Transfer the diagram into your lab notebook and take a picture with your cell phone as well.**

### Retardation factor ( $R_f$ ):

Different compounds should move different distances on your TLC plates; however the exact distance a particular compound moves depends on how far the solvent is allowed to rise up the plate. The further the solvent moves, the further the spots travel. To take this variation into account, the ratio of the two distances is calculated and reported. This ratio, called the retention factor ( $R_f$ ) allows comparison with reported values and other people's work. Specifically, the retardation factor is defined as the fractional distance the center of the spot moves compared to the distance travelled by the solvent front. The  $R_f$ -value for a compound is dependent on the developing solvent and the composition of the adsorbent on the chromatography plate.



The appearance of a TLC plate for the separation of a two component mixture.

**For your post-lab report**, calculate  $R_f$ -values for each spot on your plate. Spots with the same  $R_f$ -values within experimental error and the same appearance are likely to be the same compound. **Please check the handout for the specific guidelines for the report.**

Include a sketch of **your** TLC plate in your notebook. Your plate, however, may be considerably more complex in appearance than the example above.

Depending on the spinach sample, the conditions of the experiment, and how much sample was spotted on the TLC plate, you may observe other pigments. These additional components can result from air oxidation, hydrolysis, or other chemical reactions involving the pigments discussed in this experiment. Be sure to clearly label the lanes as coming from extract, yellow band or green band and label the colors of as many of the separated spots as possible.

Using the guide below, identify as many spots in the TLC as you can. Determine which pigments were present in the yellow band and which were present in the green band from your column.

***Pigments in spinach in order of decreasing  $R_f$ -values:***

Carotenes (1 spot) (yellow-orange)

Pheophytin a (gray, may be nearly as intense as chlorophyll **b**)

Pheophytin b (gray, may not be visible)

Chlorophyll a (blue-green, more intense than chlorophyll **b**)

Chlorophyll b (green)

Xanthophylls (possibly three spots: yellow)

**2. UV-Visible Spectra:**

Take the UV-visible spectrum of your chlorophyll and  $\beta$ -carotene from  $\lambda=400\text{--}800$  nm.