

Separation of Spinach Pigments by Column Chromatography

Objective:

To separate and identify the major pigments found in spinach leaves—such as β -carotene, chlorophyll a, and chlorophyll b—using column chromatography and to analyze the separated components using thin-layer chromatography (TLC).

Introduction:

Column chromatography is a separation technique based on differential partitioning between a mobile phase and a stationary phase, similar to other chromatographic methods. In this process, the stationary phase—often a solid adsorbent like alumina or silica—interacts with the components of a mixture as the mobile phase (a liquid solvent) is passed through it. Compounds move through the column at different rates depending on their relative affinities for the stationary and mobile phases.

A wide range of interactions can influence this separation, including hydrogen bonding, dipole-dipole forces, electrostatic interactions, Van der Waals forces, size exclusion, and affinity binding. When using a polar stationary phase such as silica or alumina, compounds are separated primarily by polarity. More polar compounds interact more strongly with the stationary phase and elute more slowly, while non-polar compounds move more quickly. The general elution order for functional groups on a polar stationary phase is:

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

One of the main advantages of column chromatography over other methods (e.g., TLC, GC, or HPLC) is its capacity to separate larger quantities of material and the flexibility to adjust the mobile phase during the separation process. Modifying solvent polarity, pH, or ionic strength can selectively elute certain components while leaving others behind, making this technique especially useful for complex mixtures and biological samples.

In this experiment, silica is used as the polar stationary phase (See Figure 1). As the mobile phase is changed to increasingly polar solvents, compounds are eluted based on their relative polarity. More polar solvents more effectively compete with the analytes for binding sites on the silica, causing even strongly retained compounds to elute.

Thin Layer Chromatography (TLC) is a specific type of chromatography that uses a thin, uniform layer of **silica gel** or **alumina** coated on a solid backing such as glass, metal, or plastic. In TLC, the silica or alumina acts as the stationary phase. Often, this layer contains a fluorescent dye that glows under UV light to help visualize the results. The mobile phase is a suitable solvent or solvent mixture. As the solvent ascends the TLC plate, different components travel different distances based on their affinity for the stationary and mobile phases. The experiment is typically stopped when the solvent front has nearly reached the top of the plate to maximize separation. The position of the solvent front is then immediately marked before it evaporates.

If your only goal is to determine the number of components in the mixture, you can simply count the spots. However, TLC can also be used for compound identification by calculating **R_f values** (*retention factor* values) [Fig 1]. Under identical conditions, the R_f value

for a substance should remain constant. However, changes in temperature, solvent composition, or TLC plate materials can affect these values. Keep this in mind when using R_f values to identify unknown compounds

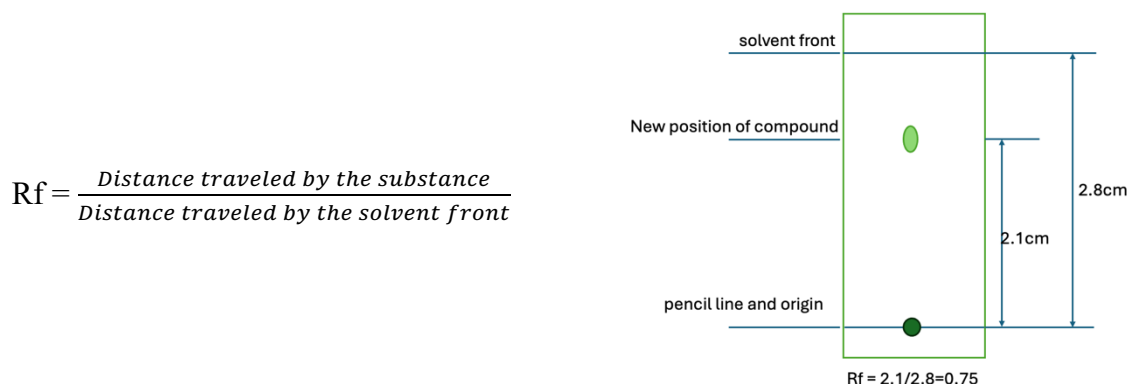


Figure 1: Calculating R_f value on a TLC plate

Molecular Structure and Polarity of Major Pigments from Spinach

Spinach contains several naturally occurring pigments, each with distinct molecular structures and polarities that influence their behavior during chromatographic separation. The major pigments include **chlorophylls**, **carotenoids**, and **xanthophylls**. These differences in structure—particularly in the presence of polar functional groups and the extent of conjugation—account for their differing affinities for polar or non-polar stationary and mobile phases (**Fig 2**).

1. Chlorophylls (Chlorophyll a and b)

Chlorophylls are green pigments that play a central role in photosynthesis. Both chlorophyll **a** and **b** contain a large, conjugated **porphyrin ring** structure with a centrally coordinated **magnesium ion (Mg^{2+})**, as well as a long, non-polar **phytol tail** that anchors the molecule in the thylakoid membrane.

- **Chlorophyll a** has a methyl group ($-CH_3$) at a specific position on the ring.
- **Chlorophyll b** has a formyl group ($-CHO$) in place of that methyl group.

This small structural difference makes chlorophyll **b** **slightly more polar** than chlorophyll **a**, due to the more polar nature of the aldehyde group.

Polarity Ranking:

Chlorophyll b > *Chlorophyll a* (both moderately polar due to the polar porphyrin ring and polar groups)

2. Carotenoids (e.g., β -Carotene)

Carotenoids are long-chain hydrocarbons composed of repeating **isoprene units** with extensive **conjugated double bonds**, giving them their characteristic orange/yellow color and strong light-absorbing properties. The primary carotenoid in spinach is **β -carotene**.

β -carotene is a purely **non-polar** molecule. It contains only carbon and hydrogen atoms with no polar functional groups, which makes it highly soluble in non-polar solvents and gives it minimal interaction with polar stationary phases like alumina or silica.

Polarity:

Very **non-polar**

3. Xanthophylls (e.g., lutein and violaxanthin)

Xanthophylls are oxygenated derivatives of carotenoids. While they have a similar conjugated backbone to β -carotene, they also contain **hydroxyl (-OH)** or **epoxide (-O-)** groups, which increase their **polarity** relative to non-oxygenated carotenoids.

For example:

- **Lutein** has two hydroxyl groups.
- **Violaxanthin** has both hydroxyl and epoxide groups.

These polar functional groups allow xanthophylls to form hydrogen bonds, making them more polar and less mobile on polar stationary phases than β -carotene.

Polarity Ranking:

Violaxanthin > Lutein > β -Carotene

Overall Polarity Order of Spinach Pigments (from most polar to least):

Xanthophylls (**yellow**) > Chlorophyll a and b (**green**) > β -Carotene (**yellow-orange**)

This polarity gradient explains the order in which pigments elute in column chromatography and appear in thin-layer chromatography. Polar compounds (like xanthophylls and chlorophylls) interact more strongly with polar stationary phases and move more slowly, while non-polar compounds like β -carotene move faster and elute earlier when non-polar solvents are used.

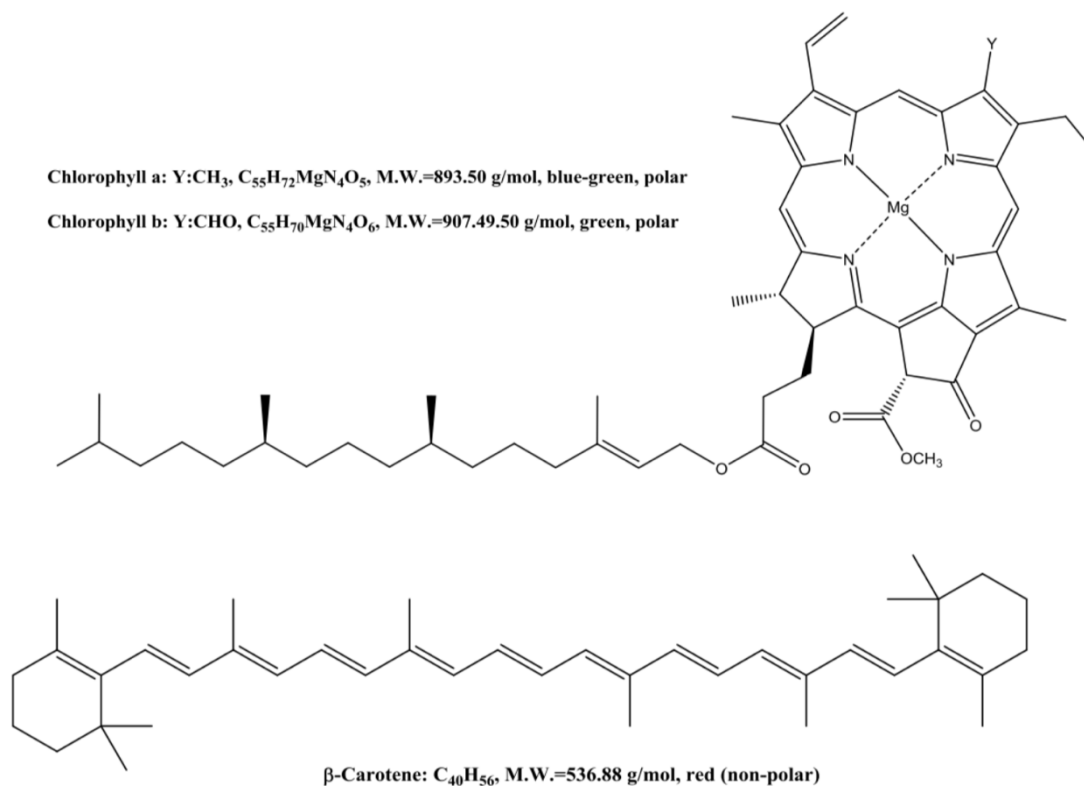


Figure 2: Molecule Structure for Chlorophyll a, Chlorophyll b, and β -Carotene.[1]

Equipment:

- Fresh spinach leaves
- Disposable Pasteur pipettes
- Small amount of glass wool
- Sand
- Mortar and pestle
- Test tubes to collect separated fractions
- Ring stand with a small clamp
- Pipette bulb

Chemicals:

- Silica gel (stationary phase)
- Heptane:Acetone (1:1) – Extraction solvent
- Heptane:Acetone (9:1 and 1:1) – Mobile phase
- Anhydrous sodium sulfate – Drying agent
- Spinach leaves (fresh)
- Distilled water

Procedures:

1. Extract pigments from spinach leaves

Using a small mortar and pestle, thoroughly grind four spinach leaves (avoid using stems or thick veins) in a mixture of 4 ml of heptane and 4 ml of acetone. Transfer the liquid extract to a centrifuge tube using a Pasteur pipet and swirl the extract gently with an equal volume of water. **Do not shake the centrifuge tube**, because emulsions are easily formed, and these can be a source of great frustration to you. Remove and discard the aqueous layer. Wash the organic layer with water two more times to remove any water-soluble materials that are present in the leaves. Add several spatula-tips full of anhydrous sodium sulfate. After 5-10 mins, transfer the solution from the drying agent to an Erlenmeyer flask. If the solution is not deeply colored, concentrate it using a gentle stream of air to remove some of the solvent.



2. Preparing the Pasteur pipette column

- Clamp a clean, dry Pasteur pipette vertically to a ring stand.
- Use a long, thin object to gently push a small plug of glass wool to the bottom of the narrow end of the pipette. Do not pack it too tightly, as this will impede the flow.
- Add a small layer (about 0.5 cm) of sand on top of the cotton plug. This creates a level base for the silica gel.
- Add the dry silica in small portions into the top of the Pasteur pipet. Gently tapping or vibrating the column as you go. This helps to remove air pockets and ensures the silica packs down tightly, creating a uniform bed. Continue adding silica until it fills the pipet to about an inch below the top.
- Add a final, thin layer of sand to the top to protect the silica surface.
- Carefully add solvent (9:1 heptane: acetone, the mobile phase) to the top of the silica gel. Use a pipet bulb to gently push the solvent through the silica to speed up the process. Make sure the solvent level never drops below the top of the silica gel. Continue adding solvent until the silica bed is uniformly wet and settled.



3. Performing the chromatography

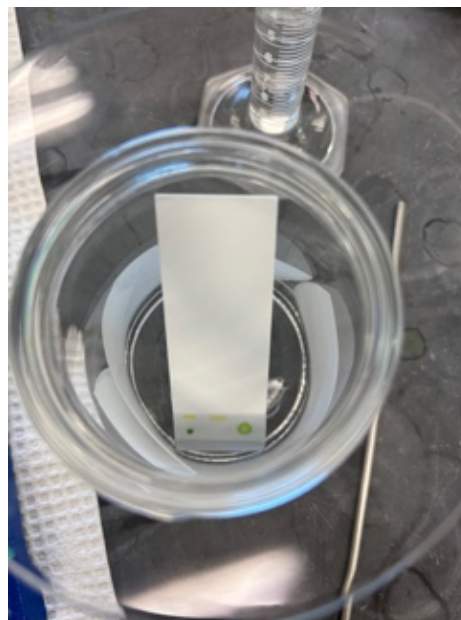
- a. Use a pipette to carefully add a small amount of the spinach extract to the top of the column. As soon as the last of the extract has soaked into the sand layer, use a fresh pipette to add a small amount of the less polar solvent (9:1 heptane: acetone) and allow it to just touch the top of the sand. This concentrates the pigments at the top of the column.
- b. Eluting the first fraction (carotene): Fill the pipette with the less polar solvent mixture (9:1 heptane: acetone). Use a pipette bulb to apply gentle, consistent air pressure to force the solvent through the column. You should see a yellow band (carotene) begin to move quickly down the column.
- c. Collecting the fractions: Place a clean test tube under the column. As the yellow band reaches the bottom, collect it in the test tube.
- d. Eluting the second fraction (chlorophylls): Once the yellow band has been collected, change the collection tube. Refill the column with a more polar solvent mixture (1:1 heptane: acetone or 100% acetone) and continue eluting. The green chlorophyll bands will begin to move and elute more slowly due to their higher polarity.
- e. Collecting the chlorophylls: Collect the green bands in a new test tube as they elute from the column.



4. Performing the TLC (Thin Layer Chromatography)

- a. Prepare the TLC Plate:
Draw a light pencil line near the bottom of the TLC plate, about 1 cm from the bottom edge. Make small tick marks on this line for each sample to be spotted.
- b. Spot the Sample:
Use a capillary tube to apply a small, concentrated spot of the sample at each tick mark. Allow the spot to dry completely.
- c. Prepare the TLC Chamber:
Pour a small amount of the mobile phase solvent (the ratio between heptane and acetone may vary) into a developing chamber, ensuring the solvent level is below the spots on the plate. Cover the chamber to saturate the atmosphere with solvent vapor.
- d. Develop the Plate:
 - Carefully place the TLC plate into the chamber using forceps, ensuring the **spotted side is down, and the spots are above the solvent level.**

- Cap the chamber immediately to prevent solvent evaporation.
 - Observe as the mobile phase travels up the stationary phase by capillary action.
- e. Visualize the Spots:
- When the solvent front is about 1cm away from the top of the plate, remove the plate and mark the solvent line with a pencil.
 - Allow the plate to dry completely.
 - Visualize the separated spots using a UV lamp (if the compounds are UV-active) or a suitable staining reagent.
- f. Analyze the Results:
- Each separated component appears as a distinct spot.
 - The Retention Factor (R_f) value for each spot is calculated by dividing the distance traveled by the sample by the distance traveled by the solvent front.



Importance References:

[1] Pavia, D.L., Lampman, G.M., Kriz, G.S., and Engel, R.G. Introduction to Organic Laboratory Techniques: A Microscale Approach 3rd Edition Saunders College Publishing: New York, NY, 1999.

Column Chromatography

<https://www.youtube.com/watch?v=R491QOnkmCs>

TLC

[https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_\(Analytical_Chemistry\)/Instrumentation_and_Analysis/Chromatography/V._Chromatography/A._Introducing_Chromatography%3A_Thin_Layer_Chromatography](https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumentation_and_Analysis/Chromatography/V._Chromatography/A._Introducing_Chromatography%3A_Thin_Layer_Chromatography)