

EXPERIMENT 1: Thin-Layer Chromatography and Column Chromatography: Extraction and Separation and Plant Pigments and Common Analgesics

Relevant sections in the text: WADE pages 155-163; 198-205

General Concepts

Chromatography is a common and extremely useful method used to separate and analyze complex mixtures. Using this technique the course of a reaction can be followed, and the products separated and isolated. In this method, the components within the mixture are distributed between two phases: a *stationary phase* and a *mobile phase* (which moves through the stationary). Chromatography works on the principle that different compounds will have different solubilities and adsorption to the two phases between which they are to be partitioned. The material to be separated is placed onto the stationary phase and is then carried along by the mobile phase. The components of the mixture are absorbed by the stationary phase to different degrees and it is thus the various rates of migration for each component on the adsorptive materials that allows separation; the stronger the adsorption the slower the material passes through the system. In this experiment you will learn the techniques of two types of chromatography: column (also known as Adsorption and Partition) chromatography and thin layer chromatography.

Column chromatography will be used to separate the pigments present in spinach leaves, namely the green chlorophylls, orange carotenes and yellow xanthophylls. The pigments will be isolated from the leaves by solvent extraction and then separated by the two types of chromatography.

Prior to performing a bulk separation and isolation using column chromatography, analysis of the plant extract by thin-layer chromatography (TLC) will be performed in order to find the best solvent system to be used in the large-scale separation. TLC with plant extracts produces several spots; easily identified by colour. More commonly in organic chemistry is the separation of complex mixtures of colourless components. In these cases other methods of visualization must be used. In this experiment, you will also perform TLC analysis of common analgesic drugs to determine which components are present. The organic components in these drugs are colorless and produce colourless spots on the TLC which require alternative techniques for identifying spots. Ultraviolet/visible absorption spectroscopy will be used to detect TLC spots that are otherwise colourless. During the course of Chemistry 273a and 283g you will learn other methods of visualization.

Principles of Chromatography

The separation of the components of a mixture depends on the phase each component remains in and the rate at which each travels. The stationary phase does not move, while the other mobile phase travels past the fixed (stationary) phase. Due to the interaction of the various functional groups present in the solute molecules with the stationary phase, each compound travels at a different speed. The strength with which an organic compound binds to an adsorbent depends on the strength of the following types of interactions: ion-dipole, dipole-dipole, hydrogen bonding, dipole induced dipole, and van der Waals forces.

Different types of chromatography use various types of stationary and mobile phases. In this experiment, the solid phase is silica gel while the mobile phase is an organic solvent (may be a single type or a mixture of solvents). This means that the compounds to be separated must choose between being absorbed to the solid silica gel or moving along in the organic solvent. The silica gel is either packed into a column or adhered to a sheet of glass or plastic, depending on the type of chromatography.

When a column is used, the compound mixture is placed on top and the solvents are run down the column separating the mixture along the way. With the silica gel on a plate, the compounds are placed close to the bottom and the mixture is separated as the solvent travels up by means of capillary action. Since silica gel is a porous form of SiO_2 , the surface of gel contains Si-OH and Si-O-Si functional groups. With silica gel, the dominant interactive forces between the adsorbent and the materials to be separated are of the dipole-dipole type. Highly polar molecules interact fairly strongly with the polar Si-O bonds of these adsorbents and will tend to stick or adsorb onto the fine particles of the adsorbent while weakly polar molecules are held less tightly. Weakly polar molecules thus generally tend to move through the adsorbent more rapidly than the polar species. Figure 1 illustrates the general column chromatography set-up and Figure 2 illustrates the movement of a compound mixture in a column and their separation and isolation.

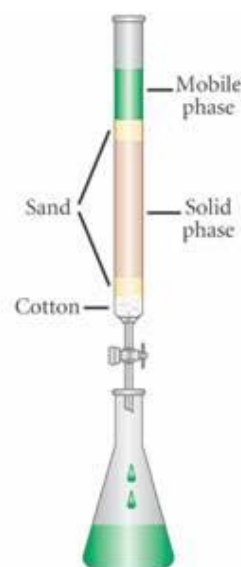


Figure 1.
Column Chromatography set-up. (from Fox and Whitesell)

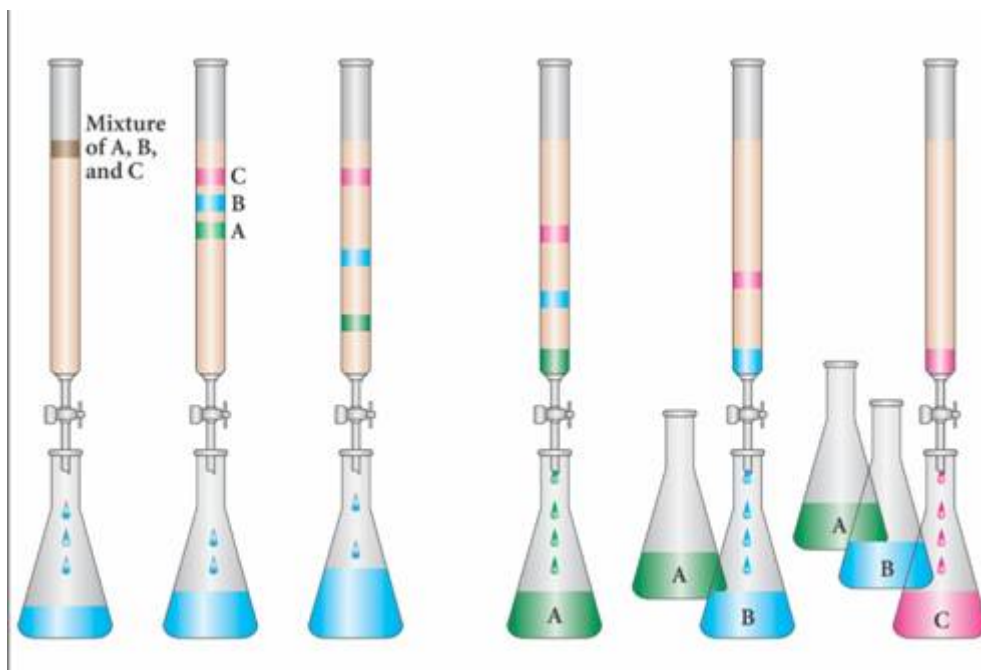


Figure 2: Illustration of a column chromatography separation of a mixture of 3 components, A, B and C. Compound A is the least polar and compound C is the most polar and the separation occurs as solvent is allowed to flow through the stationary phase. (Figure from Fox and Whitesell)

Another factor that establishes the rate at which a compound travels past silica gel is the polarity of the solvent. A polar solvent will compete for silica absorption sites, disallowing the compounds to do so. This promotes a faster rate at which *all* compounds travel. The order in which the compounds move remains the same, while moving faster as the polarity of the eluent (solvent system) increases.

Column Chromatography

A column is prepared by placing a small plug of glass wool in the bottom of the cylindrical glass column, followed by a small layer of sand. The column is then packed with the solid adsorbent phase (silica gel). A slurry of adsorbent in solvent is prepared with the same solvent later used in TLC analysis. The slurry is carefully and slowly poured into the column after it is partially filled with solvent in order to prevent disturbance of the sand. The solvent is allowed to drain as the silica packs tightly. Once the solvent just barely becomes level with the silica (without drying it!), another small layer of sand is applied carefully without disturbing the silica.

The compound mixture is dissolved in a minimum amount of solvent (same as in the column) and very carefully added to the top of the column using a Pasteur pipette. After allowing the compound to absorb into the column, solvent is continually added to the top of the column until each band resolves and is carefully collected.

With coloured substances, the bands may be directly observed and collected as they run off the column. However, with colorless compounds, the development can be observed by collecting many small fractions of the eluting solvent and testing each by thin layer chromatography.

Thin Layer Chromatography (TLC)

TLC involves the same principles of separation as column chromatography but the apparatus and technique for development is different. Instead of a column, the silica (or alumina) is adhered to a plate of plastic or glass. A capillary spotter is used to apply the dissolved sample onto the plate about 1 cm from the bottom (a line with *pencil* is drawn). Once the solvent has evaporated, only the sample remains. The plate is carefully placed into a closed developing chamber, which has a shallow layer of solvent that does *not* submerge the spot. The chamber is lined with a folded piece of filter paper to ensure a uniform and saturated atmosphere of solvent vapour.

The plate is removed when the solvent front has reached about 0.5 cm from the top, and is quickly marked with pencil. The capillary action of the solvent causes the initial spot to be separated into individual components that may be visualized by colour identification or with the following techniques for colourless compounds:

- (i) Irradiation with ultraviolet light
- (ii) Reversible staining with iodine vapour (formation of brown spots which fade)
- (iii) Spraying with a reagent that irreversibly colors the spots, e.g. H_2SO_4 , KMnO_4

Figure 3 illustrates the general principles showing the separation of the same mixture described for column chromatography in Figure 2.

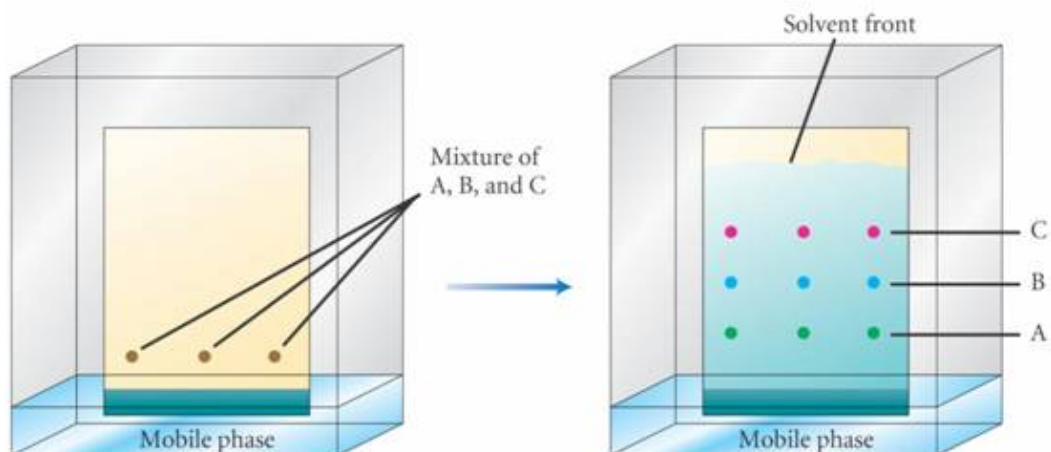


Figure 3: Thin-Layer Chromatography, illustrating the separation of the same mixture described for column chromatography in Figure 2.

The rate at which a compound moves in respect to the solvent front, the retention factor (R_f), is characteristic of that compound under standard conditions. The R_f value is calculated by dividing the distance each spot has traveled (measured from the pencil line to the middle of the spot) by the distance the solvent front traveled from the pencil line. This is illustrated in Figure 4.

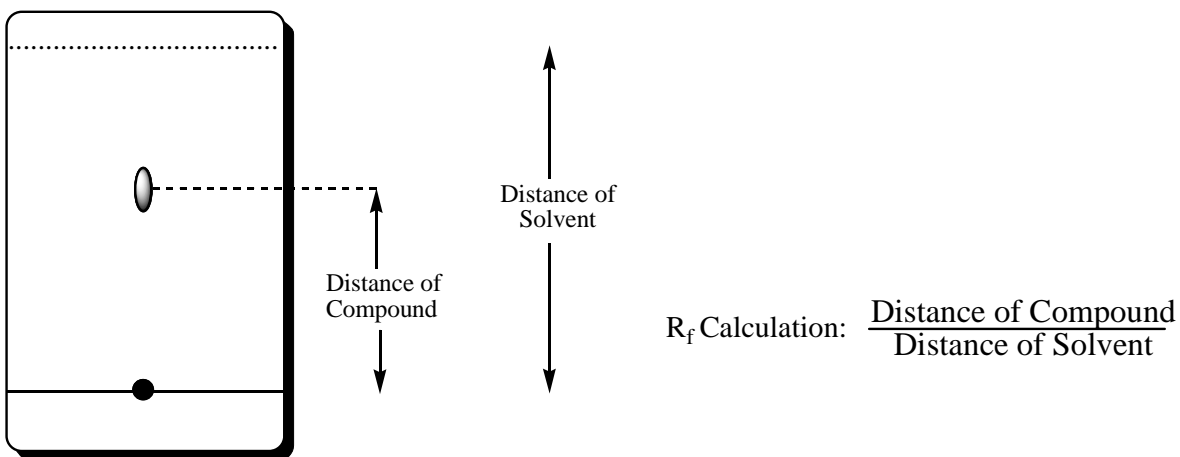


Figure 4: Illustration of a Developed TLC Plate and R_f Calculation

The advantages of TLC are the very small quantities of sample required and the great ease and rapidity with which it is resolved. For these reasons, it is often used to monitor the progress of a reaction by running the crude sample beside the reaction sample on the same

plate. It is also used to determine the best developing or eluting solvents for subsequent separation by column chromatography. The following are some common uses of Thin-Layer Chromatography:

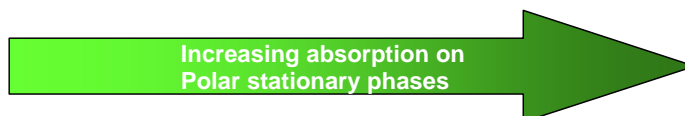
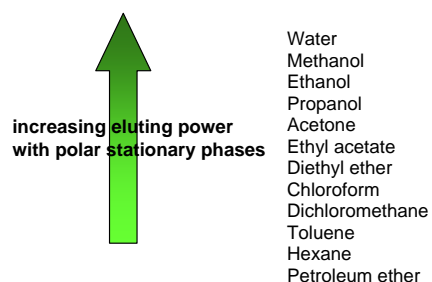
1. To determine the number of components in a mixture.
2. To determine the identity of two substances.
3. To monitor the progress of a reaction.
4. To determine the effectiveness of a purification.
5. To determine the appropriate conditions for a column chromatographic separation.
6. To monitor the progress and separation achieved by column chromatography.

Factors Affecting Resolution of Separation

(i) Adsorbents: Weight ratio of adsorbent to sample is important to obtain accurate separation. If too much sample is applied, the active adsorbing sites will be saturated and the column will be flooded, resulting in poor separation. In most cases, a ratio of 20 to 1 is satisfactory but sometimes up to 100 to 1 is necessary. Even the ratio of column height to diameter is important; about 8 to 1 is considered optimal.

(ii) Solvents: The solvent (or solvent mixture) is important to the compound separation, keeping in mind that the more polar the solvent, the faster the compounds move. In some cases, a solvent system may increase in polarity by gradually changing the composition of the solvent mixture.

(iii) Functional Groups: Compounds with highly polar groups are strongly adsorbed and eluted less readily than less polar (or polarizable) compounds. The strength of adsorption for compounds having the following types of polar functional groups decreases in the order listed below. However, variations may occur depending on the overall structure of each specific compound.



RH, R-X, alkenes, R-OCH₃, R-CO₂R', R¹R²C=O, RNH₂, R-OH, RCO₂H

LIQUID-LIQUID EXTRACTION OF ORGANIC COMPOUNDS

Laboratory experiment #2 has an emphasis on liquid-liquid extraction techniques, but we will use a quick extraction at the beginning of Lab 1, thus the technique is briefly explained below.

The extraction or separation of neutral organic compounds from inorganic, ionic or otherwise water-soluble compounds is based on differential solubilities. Many organic compounds are of low polarity and preferentially dissolve in solvents of low polarity, whereas inorganic salts and highly polar organic compounds preferentially dissolve in solvents of high polarity, such as water. If the proper choice of solvents is made, one organic and water, then the two solvents make an immiscible pair (i.e. they will not mix or dissolve each other), and form layers – like oil and vinegar salad dressing! If a solid compound is then introduced to the solvents, and shaken vigorously, it will tend to dissolve to a greater degree in the solvent that is closest in polarity. In this scenario, inorganic salts and highly polar organic compounds (like sugar) may be separated from compounds of low polarity. Once the compounds have been partitioned between the solvents, the layers are separated by use of a separatory funnel and the solvent can be removed (by evaporation) to yield the solids (or high-boiling liquids).

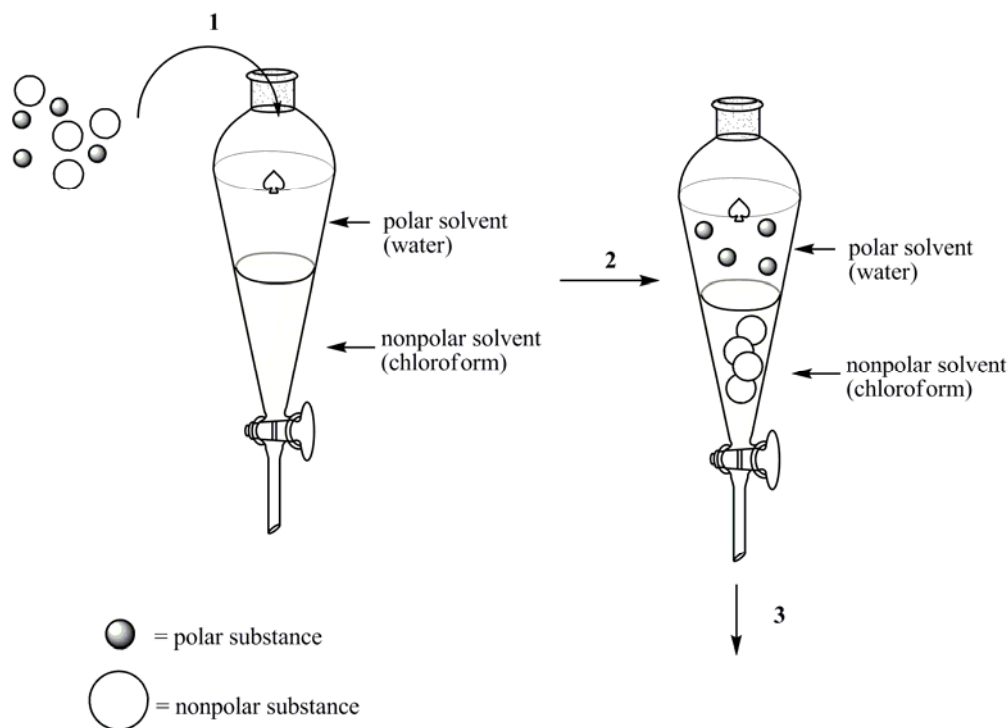


Figure illustrating the principles of solvent-solvent extraction. 1) mixture of compounds introduced to solvent pair, 2) compounds partition into solvent of like polarity, 3) drain individual layers to isolate the separated compounds.

Apparatus and Method

Extraction is made possible since water and organic solvents are immiscible, which form two distinct layers (based on their specific density) in the separatory funnel. The glass bulb has a stopcock and stem at the bottom, as well as a plug at the top. The whole device is supported with a ring clamp covered in rubber tubing.

The solution is poured into the separatory funnel with the stopcock closed and a beaker under it just in case of a leak. A small amount of extracting solvent is added to the funnel (the flask should never be more than $\frac{3}{4}$ full) and with the upper opening and stopcock closed, the funnel is shaken with both hands. The opening should always be facing away from you into the fume hood away from others (Figure 1).

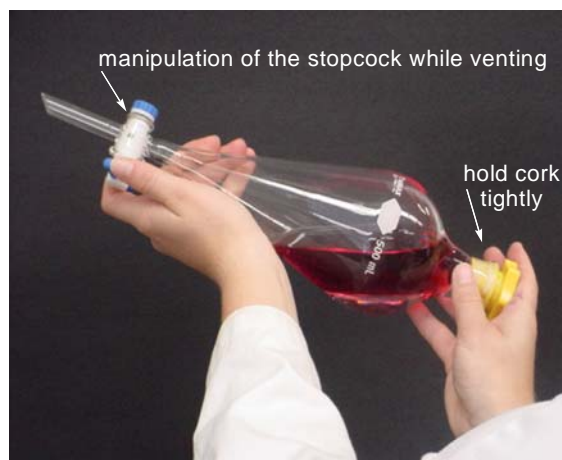
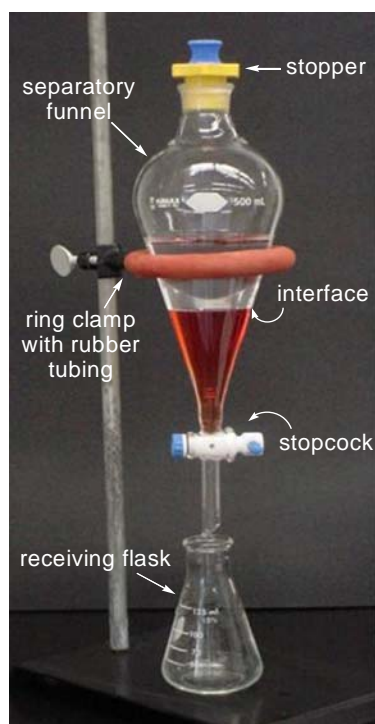


Figure 1: The Separatory Funnel

Shaking the flask is important since it maximizes the surface area of each component and allows intimate mixing. Every couple seconds the stopcock is slowly opened while pointing up to “vent”, which allows built-up pressure to be released. The flask is put back on the ring clamp (with the stopcock closed!) and the top plug removed. The two layers are allowed to settle and the bottom layer is removed at a slow rate into a labeled flask. Just before the last couple drops of the lower layer are collected, swirl the funnel to collect any compound that may have been on the walls. **Collect any last amounts of the lower layer and remove the top layer by pouring it out from the top.** (bottom layer always out of the bottom, top layer always out of the top)

Never discard any layer until you are certain you do not need it. If you are unable to identify which is the organic or aqueous, drop a small amount of water into one and if it dissolves it is aqueous. Normally, three extractions are performed to have an efficient extraction. To remove any last traces of water in organic solvent, anhydrous magnesium or sodium sulfate is used before removing solvent by rotary evaporation.

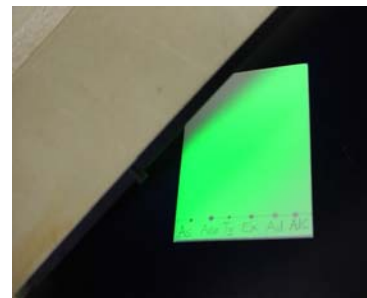
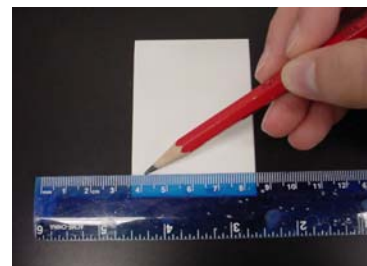
Part A: TLC Analysis of Analgesic Drugs

Experimental Objectives:

- Introduction to solvent-solvent extraction.
- To learn the technique of TLC and the visualization of colourless components.
- To resolve the components of commercial painkillers using TLC and identify the components found in each.

Procedure:

- Obtain one tablet of the following common drugs and record the name in your notebook: (i) Aspirin[®], (ii) Tylenol[®], (iii) Excedrin[®], (iv) Anacin[®], (v) Advil[®]
*Half of a tablet is sufficient – share the other half.
- Your TA will help you coordinate with other students at your bank of fumehoods to share and receive the other solutions for TLC analysis.
- Add 10 mL of chloroform into the mortar and dissolve thoroughly.
- Add 10 mL of water and thoroughly mix.
- Add the mixture to a small separatory funnel and isolate the organic fraction. Dry with a small amount of anhydrous sodium sulfate and use the supernatant for the TLC analysis.
- Obtain a TLC plate. Draw a light pencil line about 1 cm from the end of the chromatographic plate.
- By sharing with the people around you, spot the plate with the 5 different analgesics. **Use a separate capillary tube for each sample**, so that you do not cross contaminate. Make each spot as small as possible (preferably less than 5 mm in diameter). Examine the plate under the ultraviolet (UV) light to see that enough of each compound



has been applied; if not, add more.

- Prepare a developing chamber as indicated in the picture using a 250 mL beaker as the chamber, a half-piece of filter paper inside, and foil or plastic wrap to cover.
- Pour the eluent, 95:5 mixture of Ethyl Acetate/Glacial Acetic Acid, into the beaker to a depth of under 1 cm (~10 mL). Place the prepared TLC plate in the developing chamber, ensuring the solvent level is below the pencil line.
- After the solvent has risen to near the top of the plate (about 0.5 cm from the top), remove the plate and mark the solvent front with a pencil. Allow the solvent to evaporate from the plate in the fumehood and then examine it under UV light.
- Outline the spots with a pencil. The spots may also be visualized by putting the plate in an iodine chamber for a couple minutes.
- Sketch the plate in your notebook and calculate the R_f values for each spot.
- Determine the ingredient(s) of your analgesic contains based on the R_f values below.



The entire procedure is summarized in Figure 5.

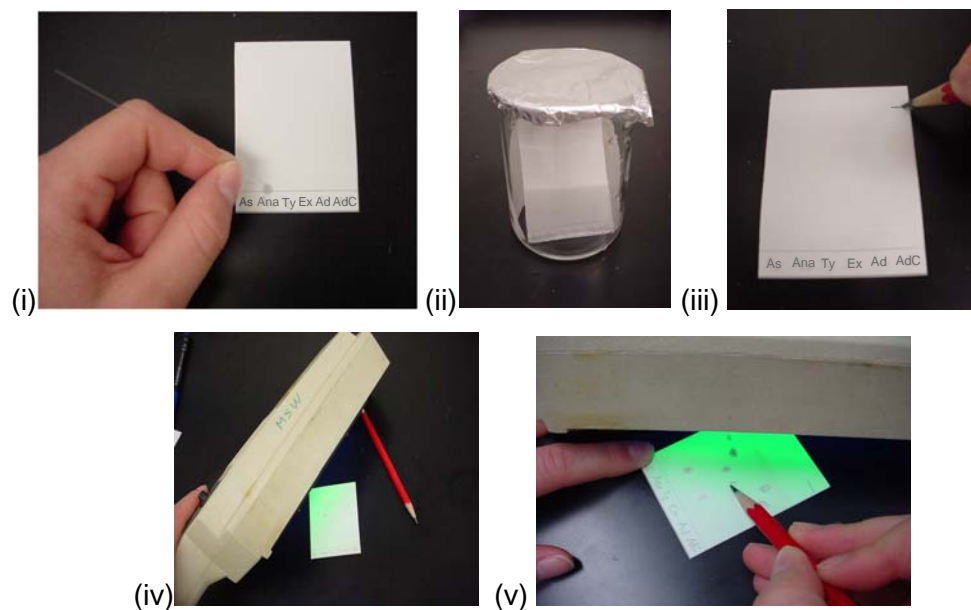


Figure 5: (i) Spotting the labelled TLC plate; (ii) Eluting in the Developing Chamber; (iii) Marking the Solvent Front (iv) Visualizing the Spots with the UV Lamp; (v) Marking Each Spot.

TLC Troubleshooting (these hints will be useful throughout your undergraduate/graduate career)

With practice and experience, you will come to find that TLC is a very easy and useful technique. Suggestions are given below on how to solve common problems you may come across with TLC:

(i) The compound runs as a streak rather than a spot.

- The sample was most likely too concentrated. Run the TLC again, but try either diluting the sample or applying less sample to the plate. Your sample may also contain many components or your experiment did not proceed as expected, which could cause the spots to appear as a streak.

(ii) The sample runs as a smear or an upward crescent.

- Strongly acidic or basic compounds (amines and carboxylic acids) may appear as a smear. With the addition of a few drops of ammonium hydroxide (amines) or acetic acid (carboxylic acids) to the eluting solvent, the spots should appear clearer.

(iii) The sample runs as a downward crescent.

- This shape is characteristic of disturbed adsorbant caused during spotting. Hold the capillary spotter very carefully while applying the sample, ensuring the compound is only located on the desired spot by visualizing the plate under UV light before development.

(iv) The solvent front does not run in a straight line.

- Without a straight solvent front, R_f values cannot be accurately calculated. This problem can be solved by ensuring the edges of the TLC plate are smooth and not chipped by running your fingers along the sides and bottom of the plate. Also, make sure that the plate is not touching the sides of the container (or the filter paper) as the plate develops.

(v) Many random spots are seen on the plate.

- An organic compound such as solvent may have dropped on the plate. If the capillary spotter chipped or broke while applying the spots, excess sample may have splashed on the plate. Also, if pen was used on the plate, blue spots would appear.

(vi) Few or no spots are seen on the plate.

- Most likely, an insufficient amount of compound was applied. Try concentrating the solution or spot it several times in one place, allowing the solvent to dry between applications. If the solvent level in the chamber was deeper than the spotting line, the eluent would have dissolved the samples, preventing them to travel up the plate. Also, try visualizing the plate with iodine if the spots did not appear under UV light. Lastly, your experiment may not have proceeded as expected.

EXPERIMENT 1A: DATA SHEET

TLC of Analgesics

Name:	
Demonstrator:	

Due before the start of your laboratory the week of September 28, 2009.

1. Draw a representation of your TLC plate after it has eluted and calculate the R_f values.

2. Identify the components of each of the analgesics from the tlc and complete the following table. (place an **X** if the drug contains the medical ingredient)

Components of Several Commercial Painkillers

		Medical Ingredient and its R_f Value			
		Acetylsalicylic Acid (R _f = 0.6)	Acetaminophen (0.46)	Caffeine (0.17)	Ibuprofen (0.67)
Analgesic	Anacin				
	Aspirin				
	Excedrin				
	Tylenol				
	Advil				

3. Look up and draw the chemical structures of each of the organic medical ingredients listed in the Table below (use any source you can find). Do the relative Rf values make sense to you? Explain your reasoning.

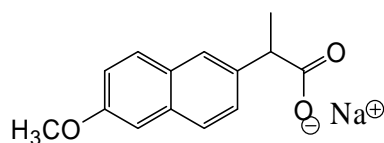
Medical Ingredient	Rf	Chemical Structure
Acetylsalicylic Acid	0.6	
Acetaminophen	0.46	
Caffeine	0.17	
Ibuprofen	0.67	

Rationale for relative Rf values:

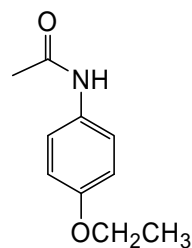
4. Drugs exert their effects by binding to specific proteins and receptors. To be recognized by the receptor, the molecule must contain certain atoms positioned at the right distances with respect to each other.

Besides all being powerful analgesics (pain-killers) some of these drugs also act as antipyretics (fever reducer) and/or anti-inflammatory agents (reduces swelling). For example ibuprofen acts as an analgesic, antipyretic and anti-inflammatory, but acetaminophen has little value as an anti-inflammatory.

Given the above statements what drug effects do you expect from the drugs naproxen and phenacetin whose structures are shown below? Explain briefly your reasoning.



naproxen

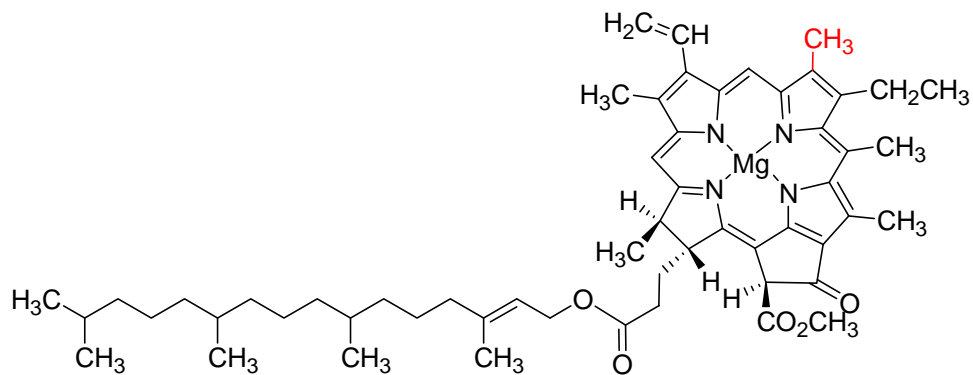


phenacetin

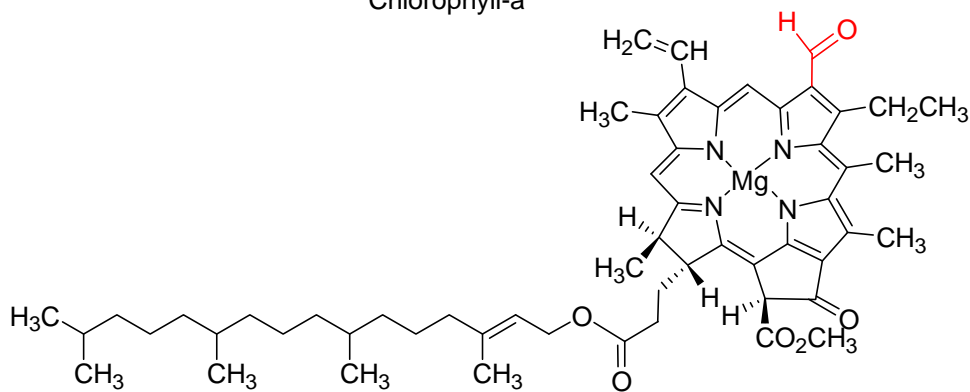
Week 2

Part B: Column Chromatography and TLC Analysis of Spinach Extract

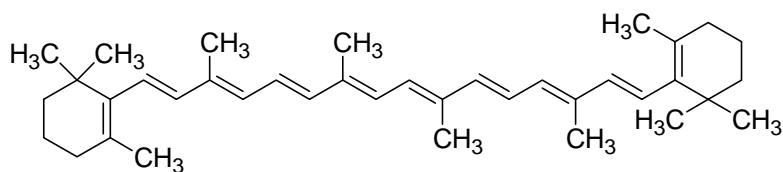
Column chromatography will be used to separate the following pigments found in spinach extracts. Some of these are shown in Figure 6.



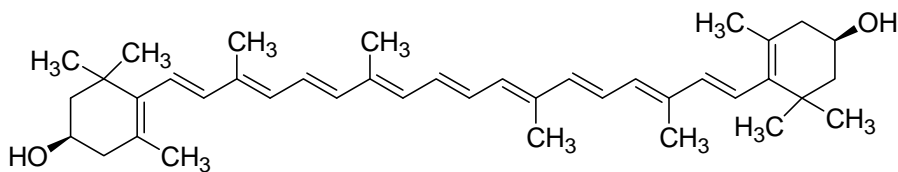
Chlorophyll-a



Chlorophyll-b



β-carotene



Lutein (a xanthophyll)

Figure 6: Structures of some of the pigments found in spinach leaves.

Experimental Procedures

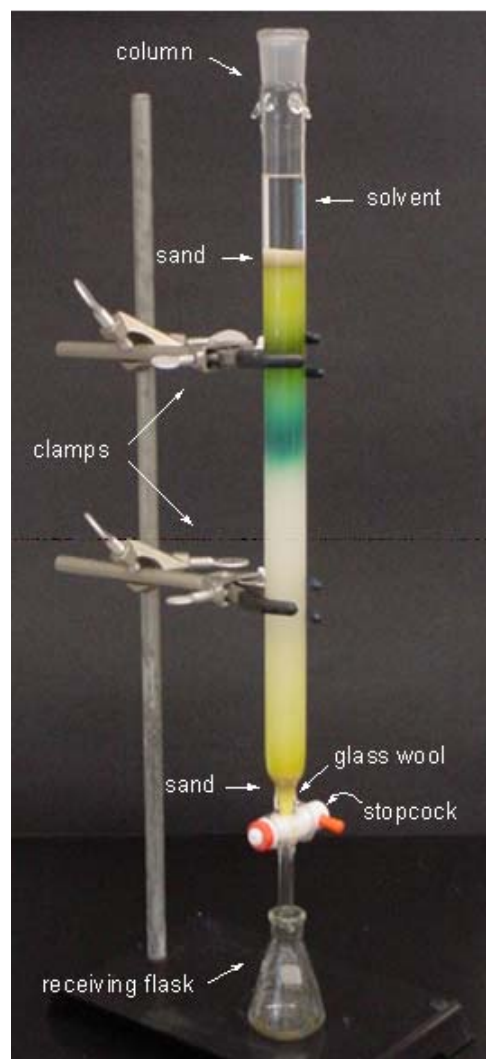
(i) Column Preparation

- Obtain a 60 cm chromatography column, 40 mL of deactivated silica gel and 250 mL of the developing solvent mixture (petroleum ether (bp 60-80°C)/acetone; 8:2).
- Prepare a slurry of the silica adsorbent with 70 mL of solvent in a 250 mL Erlenmeyer flask.
- With a piece of glass tubing push a small plug of glass wool or cotton into the constriction at the bottom of the column. Clamp the column in a vertical position and add a 1 cm layer of sand on top of the glass wool or cotton.
- Ensuring that the stopcock of the column is closed, pour in 15 mL of solvent. After settling, quickly decant all of the slurry through a funnel into the column.
- Open the clamp at the bottom and allow solvent to drain (2-3 drops/second) while tapping the walls of the column with the ends of a folded piece of rubber tubing.

NOTE: The majority of packing occurs in the first 5 minutes and regular tapping of the column is particularly important in this period. However, over the next 20-30 minutes as the solvent drains out, additional settling occurs which can be monitored by marking with a felt tip pen or masking tape. Extraction of pigments can be completed during this time.

DO NOT allow the silica to run dry!

- Once the solvent level is within 6 cm of the top of the adsorbent, the packing should be essentially complete. Carefully add a 1 cm *level* layer of sand on the adsorbent.
- Drain off excess solvent until its level is precisely on top of the sand (no lower!) and close the clamp.



(ii) Extraction of Spinach Pigments

- Shred about 2-3 medium sized leaves of fresh spinach and crush the shreds in a clean porcelain mortar containing a little sand and about 15 mL methanol.
- Filter the methanol solution into a 250 mL separatory funnel (with the stopcock closed!) supported by a ring clamp through a funnel and filter paper. Press the spinach as free of solvent as possible. Grind the macerated spinach again with a mixture of 10 mL of methanol and 25 mL of petroleum ether (bp 30-60°C).
- Filter the mixture again into the separatory funnel. Re grind the spinach collected in the filter with a second portion of 15 mL of methanol and 25 mL of petroleum ether (bp 30-60°C), and again filter the mixture into the separatory funnel.
- Wash the petroleum ether layer (which contains the pigments) by adding 25 mL of water to the separatory funnel. With the stopcock closed and the top corked, invert the funnel and point the stopcock away from you and into the fume hood. Gently shake several times, and vent by slowly opening the stopcock while the flask is still inverted to relieve pressure. Shake and vent an additional two times.
- Allow the layers to settle with the flask on the ring clamp and remove the top cork. Emulsions may be broken up with a little aqueous NaCl. Slowly allow the bottom aqueous layer to collect in a labelled Erlenmeyer flask. Add another 25 mL of water to the organic layer in the separatory flask and complete the removal of methanol by washing again. Combine the ether layers into a labelled Erlenmeyer flask and dry by adding a small (1-2 g) quantity of anhydrous sodium sulfate. After swirling and allowing to dry for 5-10 minutes, gravity filter off the drying agent and concentrate the pigment solution by evaporation to a volume of about 2 mL.

NOTE: This sample is used for TLC and Column Chromatography

(iii) Development and Elution

- Saving sufficient pigment extract to complete the TLC analysis, carefully transfer the remaining extract to the top of the sand layer with a dropper. Avoid wetting the upper walls! Drain off the solvent until the pigment solution is just below the top of the sand. Rinse the walls with about 1 mL of fresh solvent and drain until the level is once again below the top of the sand. Repeat the rinsing of the walls until the solvent above the Silica Gel is virtually

colorless.

- Very carefully fill the column with fresh solvent, and allow solvent to drain at a rate of 2 drops/second.

NOTE: TLC analysis can be performed while development, but never allow the silica to run dry!

- Observe the separation of bands as the column develops. When the edge of the first band (yellow) reaches the lower part of column make a drawing of the various bands. Label the various bands and their colour. Compare the separation with that observed by TLC with the same solvent.
- Collect the three fractions (yellow, blue-green, and green) by replacing the 250 mL flask with a 125 mL Erlenmeyer flask just before a new band is eluted. As soon as the band is collected, replace the flask with the original containing just clear solvent. Change the flask whenever a new band is about to run off. Clear solvent may be discarded in the proper container.
- Concentrate each fraction to a small volume by evaporation (**rotoevaporator**) for analysis by TLC.

(iv) Thin Layer Chromatography (TLC) with Spinach Extract

- Obtain a TLC Silica Gel plate and lightly draw a line about 1 cm from the bottom with a pencil and ruler. With a short length of capillary tubing, dip it into the original spinach extract sample. The solution will be drawn up the tubing by capillary action. Touch the tip of the capillary against the plate at the line and solution will drain onto the coating. Apply a sample of each other fraction isolated in the same way, repeating application to each spot until each is about 2 mm in diameter.
- Prepare a developing chamber by lining the wall of a 4-oz bottle with a folded 12.5 cm filter paper. Add sufficient developing solvent (5-8 mL) to saturate the paper liner and form a layer in the bottom of the bottle about 4mm deep at the shallow centre.
- Develop the pigment extract in each of the following solvent mixtures:
 - (i) petroleum ether (bp 60-80 °C)/acetone (9:1)*
 - (ii) petroleum ether (bp 60-80 °C)/acetone (8:2); already prepared
 - (iii) petroleum ether (bp 60-80 °C)/acetone (7:3)*

*Make about 10 mL quantities as needed.

- Carefully lower the spotted plate into the bottle and stand it on the bottom centre against the

wall, ensuring that the spots are above the solvent level.

- Recap the bottle and observe the development of the solvent and sample mixture up the plate. When the solvent front reaches ~5 mm from the top, remove and make a pencil marking of the solvent level before it quickly evaporates.
- Record the color, intensity and location of each spot visible. Then note the presence of any additional spots by standing the plate in a bottle containing a few crystals of iodine for a few minutes. Quickly outline any additional spots with a pencil.

(v) Ultraviolet/Visible Spectra

- The electronic absorption spectrum of each fraction in cyclohexane over the range 350-700 nm, will be provided to you.
- Include a discussion of the visible spectrum in your report.

For this laboratory you will write a report of no more than 5 pages.

Be sure to include in your results/discussion section the following:

- What is your conclusion with respect to the number of pigments in spinach? Which pigment is which?
- What is your estimate of the minimum number of components in the spinach extract based on these observations? Draw your three plates and calculate the R_f of each spot under each condition. Explain which solvent system is best and why.
- Explain the relative rate of elution from the column (and relative R_f values by TLC) on the basis of their chemical structures
- Discussion of the UV-vis spectra.
- This technique is very useful for separating reaction mixtures. Explain briefly how this technique can be used to separate a mixture of colorless components.
- Other things that your TA will mention