EXPERIMENT 1

SEPARATION AND PURIFICATION OF NATURAL PRODUCTS

SUGGESTED READING:

- Smith, J.G, *Organic Chemistry, 4th Edition,2014, McGraw Hill, Sections 1.12-1.13, 2.3-2.8, 3.3-3.5.*
- Zubrick, The Organic Chem Lab Survival Manual 1st Ed., pp. 7-20, 21-38, 71-78, 91-107, 111-127, 189-191, 197-208. *(.pdf on Blackboard Learn*)
- Annex I ……..Melting point apparatus
- Annex III ……Extraction
- Annex IV ……Chromatography

PURPOSE

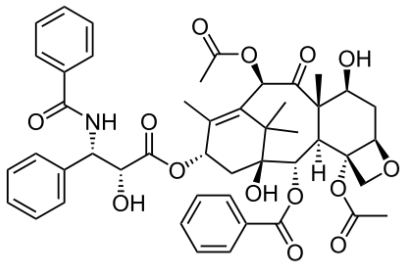
This experiment will provide a review of the techniques necessary to isolate and purify a product once a chemical reaction has finished. Three important techniques will be reviewed: **extraction, thin layer chromatography [TLC]**, and **sublimation**. Extraction and thin layer chromatography will be explored in the separation of pigments from spinach leaves, while extraction, TLC, and purification via sublimation will all be explored to isolate caffeine from tea leaves. These techniques are integral in any organic chemistry lab and will be used often in the rest of this course and in further organic chemistry lab courses.

INTRODUCTION

The purification of chemical compounds is very important in order to properly characterize the compound and use it for further reactions and avoid contaminants. When extracting interesting natural products from plant and animal sources, chemists go through a painstaking process to ensure that the product obtained is pure. These natural products often provide starting points for the synthesis of new medicinal compounds, such as Taxol shown below in Figure 1.1. There are many different techniques used to purify organic compounds and, in this experiment, we will review two techniques taught in CHM1321: extraction, and thin layer chromatography (TLC), and use a cold finger; a bench-top sublimation technique.

Most purification techniques take advantage of the difference in physical properties between compounds. Extraction uses **two immiscible solvents** (usually water and an organic solvent, such as ethyl acetate or dichloromethane) to separate organic compounds. Compounds with high water solubility (most ionic compounds and very polar organic compounds) will end up in the aqueous phase, while the compounds with low water solubility (most organic compounds) remain in the organic phase. These two layers can then be separated and the products isolated.

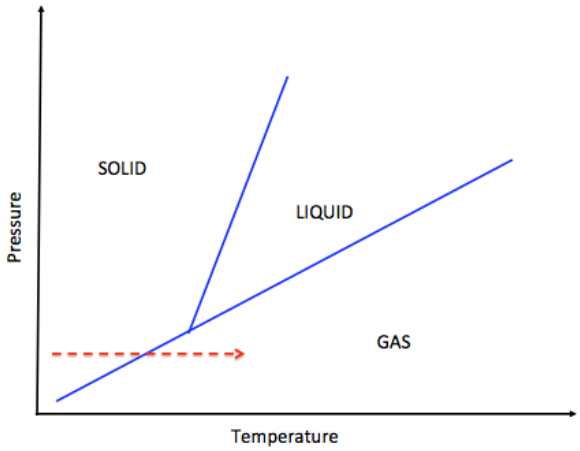
Figure 1.1: Taxol is a complex natural product and anti-cancer drug isolated from the bark of the Pacific yew tree.



Further, It is possible to separate compounds by taking advantage of their reactivity to acids and bases. Some compounds can react with an acid or a base to become ionic salts, making them soluble in the aqueous phase. Once isolated, the ionic salt is returned to the neutral state upon reaction with an acid or a base, rendering the molecule insoluble in water and easily isolated. Further details on extractions and reactive separations are available in Appendix III.

Once a target compound has been isolated, it can be purified by sublimation. Sublimation is the physical process in which a material transitions from the solid to the vapour phase without melting or going through a liquid state. If a compound possesses a high melting point as well as an appreciable vapour pressure under standard conditions (1 atm, 25˚C), it can be isolated from other non-volatile substances by condensation. Dry ice is a common example of material that sublimes in everyday life, where this solid form of carbon dioxide evaporates as it warms to room temperature without getting wet. Sublimation is used to freeze-dry coffee and food for camping and space travel. The phase diagram for caffeine is presented in Figure 1.2, where the dashed arrow indicates the phase transition at standard pressure.

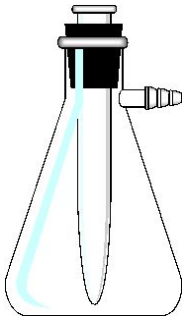
Figure 1.2: Phase diagram of caffeine



Sublimation in the laboratory can be accomplished using an apparatus called a 'cold finger'. A cold finger, or test tube typically filled with ice, sits in a side-arm flask containing a loose, crude

solid. As the flask is heated, molecules vapourize and condense as fine crystals on the surface of the cold finger, optimally positioned about 1 cm above the base of the flask. Applying reduced pressure can speed up the process.

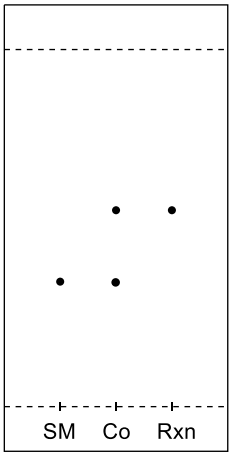
Figure 1.3 Cold finger apparatus used for sublimation



Once the target compound has been isolated, thin layer chromatography, or TLC, can be performed to observe if any impurities are present in a product. Chromatography is used to separate a mixture according to the differences in distribution of its constituents between two phases. A mixture is dissolved in a liquid, or mobile phase, while the solution is passed over a stationary phase. The different components of a mixture will have a different affinity for the mobile or stationary phase, thus allowing the various compounds to be separated from one another. This technique takes advantage of the difference in polarity of molecules. TLC employs a plate covered with a very polar silica gel ($SiO_2 \cdot xH_2O$) as a *stationary phase*. The compounds in question are spotted onto a plate (~ 1 cm from the base) by dissolving the mixture in a solvent, typically 1 – 2 mg/mL, and using capillary tubes to fix the mixture onto the stationary phase. The plate is then placed in a developing jar containing a small volume (~ 10 mL) of developing solvent. This developing solvent acts as the mobile phase, moving up the plate via capillary action and pulling the analytes along with it. The distance a compound moves along the plate is dependent on its polarity. Molecules with polar groups can bind to the silica gel in two ways; through hydrogen bonding and through dipole-dipole interactions. The strength of the interaction of the compound with the solid phase is the sum of these two interactions. The rate of migration depends on the interaction of each compound with the two phases. Generally, more polar compounds interact more strongly with the polar silica gel and therefore migrate more slowly than less polar compounds. Less polar compounds generally migrate faster than more polar compounds.

Thin layer chromatography can also be used to monitor the progress of a reaction. A TLC plate is created with the starting material spotted in one lane and the reaction mixture spotted in another lane. A third lane is set up by spotting both the starting material and the reaction mixture together on the plate. Once the plate has been developed, the disappearance of the starting material over time can be observed, as shown in Figure 1.4. A more complete description of TLC, as well as how to report separations by calculation of a retention factor or R_f value, is available in Appendix IV.

Figure 1.4: A sample TLC plate showing a complete consumption of the starting materials (note the disappearance of the spot corresponding to the SM in the Rxn lane).



SM = Starting Materials

Co = co-spot of SM and Rxn

Rxn = Reaction mixture

In summary:

(1) The silica gel, the stationary phase, is polar: more polar compounds interact more strongly with this phase than less polar compounds.

(2) Increasing the polarity of the mobile phase (solvent) causes ALL compounds to migrate further on the plate.

(3) The compounds, once separated, can be visualized on the TLC plate using ultra-violet light or a staining method.

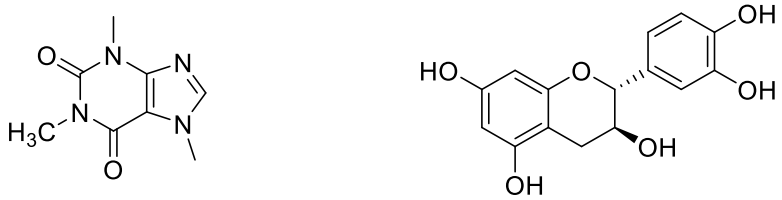
Observing the melting point of a solid is a quick and simple way to verify the purity of a product. When a chemical product is pure, the melting point range is very small, often less than 2 °C. Any amount of impurity that is present in the molecule, no matter how small, will lower its melting point by a few degrees and broaden the range. The impurity causes defects in the crystalline lattice of the molecule, making it easier to overcome the intermolecular interactions that hold the molecule as a solid.

PART A: EXTRACTION AND RECRYSTALLIZATION OF CAFFEINE FROM BLACK TEA

Tea is a popular beverage second only to water in worldwide consumption. Tea leaves are native to the southern regions of China, where it was discovered as a drink and medicine around 2737 BC. Tea leaves are composed of cellulose, chlorophyll, polyphenols, tannins, and caffeine. The medicinal properties of tea come from caffeine, as well as the antioxidant activity of polyphenols such as catechin and theaflavin. Black tea results from the oxidation of polyphenols during the fermentation process, while green tea is non-fermented. As a result, green tea possesses a greater concentration of polyphenols over that in black tea. When tea is brewed, the polyphenols and

caffeine are dissolved in the hot water, while the cellulose and other plant materials remain insoluble. Caffeine can be extracted from tea by using an organic solvent like dichloromethane. Caffeine is soluble in dichloromethane whereas the weakly acidic polyphenols are not. The industrial decaffeination of tea and coffee is done using either dichloromethane or chloroform as an extracting solvent.

Figure 1.5: The structure of caffeine and catechin (a polyphenol) found in tea leaves.



Caffeine
MW = 194.19 g/mol
m.p. 238 °C

Catechin
MW = 290.27 g/mol
m.p. 175 - 177 °C

Caffeine is a highly addictive alkaloid that is a stimulant for the central nervous system. It is found in many other plants, such as coffee beans and cocoa, and added to many drinks as a stimulant. Health Canada recommends no more than 400 mg/day for the average adult and 300 mg/day for young women of childbearing years. The caffeine content in various beverages is provided in table 1 below. Note that some coffee shops sell containers of up to 961 mL!

Table 1: Caffeine content in various beverages

Beverage	mg/250 mL
Coffee	80 – 125
Decaf coffee	2 – 6
Black tea	30 – 75
Green tea	20 – 50
Coca-Cola	32
Red Bull	80

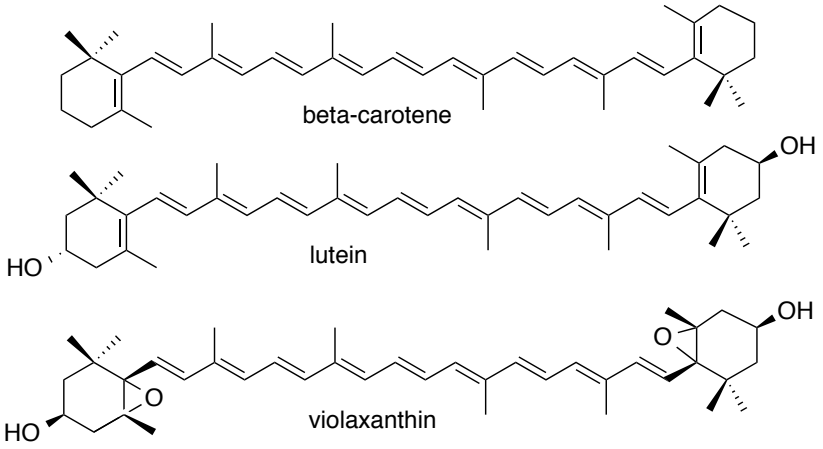
Caffeine can be extracted from tea using hot water; however various tannins will also dissolve into the aqueous solution. Alternatively, an organic solvent like dichloromethane can dissolve caffeine while the weakly acidic polyphenols remain in the aqueous phase. Industrial decaffeination of tea leaves and coffee beans undergo a similar process typically using chloroform as an extracting solvent.

In this experiment, caffeine will be extracted from a strong cup of tea using dichloromethane. The caffeine will subsequently be purified by sublimation, and its purity verified by TLC and melting point.

PART B – EXTRACTION OF CAROTENOIDS AND CHLOROPHYLLS FROM SPINACH

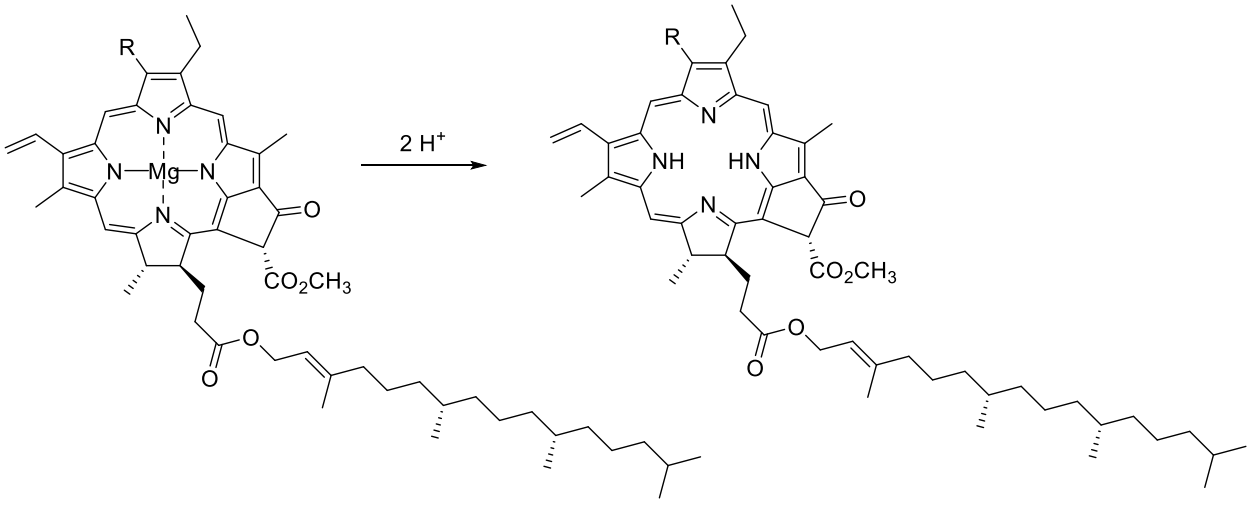
Plant leaves are composed of cellulose and pigments, where a number of pigments can generally be classified into carotenoids and chlorophylls. Carotenoids belong to a family of terpenes composed of repeating isoprene, or 2-methyl-1,3-butadiene units. Lycopene, found in tomatoes and watermelon, is the carotenoid responsible for the red colour. β-carotene is the pigment that provides the yellow-orange colour in carrots and apricots. Another common carotenoid is lutein, a xanthophyll responsible a yellow pigmentation in egg yolks and in the leaves of most green plants. The chemical structures of various carotenoids are illustrated in figure 1.6.

Figure 1.6 - Structures of various carotenoids found in spinach leaves



Chlorophylls such as chlorophyll *a* (blue-green) and chlorophyll *b* (green) are the compounds responsible for the green colour in plant material. These highly conjugated molecules play an active role in photosynthesis by capturing light *via* its porphyrin ring. Solar energy is trapped by chlorophylls and converted to chemical energy, namely electrons shuttled to other parts of the plant to be stored as sugars and starches. Chlorophylls, as presented in Figure 1.7, are sensitive to strong acids, which displace the magnesium of the porphyrin ring to form pheophytins. Pheophytins are responsible for the olive-coloured pigments present in vegetables once they begin to rot, or have been cooked for too long.

Figure 1.7: Conversion of chlorophylls to pheophytins



Chlorophyll

R = CH_3 Chlorophyll a
R = CHO Chlorophyll b

Pheophytin

R = CH_3 Pheophytin a
R = CHO Pheophytin b

The carotenoids and chlorophylls will be isolated from fresh spinach leaves by liquid-liquid mini-extraction, and the chromophores will be subsequently separated and identified using thin layer chromatography by exploiting the differences in polarity of the described molecules. A number of pigments of various colours may be isolated, but you are responsible for the identification of the three carotenoids and four chlorophylls discussed above.

MANIPULATION

All transfers of liquids and solids should be as quantitative as possible. For example, in the transfer of the original solution to the separatory funnel some of it invariably remains in the small Erlenmeyer flask. To recover most of this, rinse the flask with a small amount of solvent and add this extra volume to the separatory funnel.

Use the right size of equipment! In this laboratory course, this aspect is not always possible because you do not have all sizes of each type of equipment. Nevertheless, do not attempt to re-crystallize a sample from 10–20 mL of solvent using a 500 mL flask; a 50 mL or 25 mL flask would be much more appropriate. An Erlenmeyer flask is the appropriate vessel for re-crystallization (not a beaker).

SAFETY

- Evaporation of all organic solvents must be done in the fume hood.
- Read the instructions in the annex on the proper use of the extraction funnel before starting the experiment. Do not hesitate to ask your TA for help.
- Before the experiment, wash your glassware with water and then acetone in order to avoid contamination.

* **You should always wash your hands before leaving the lab** *

Work carefully!

It is recommended that each partner take the lead on one part of the experiment, communicating and sharing all observations and results ***before leaving the lab****. You are each responsible for submitting a report describing both parts A and B of this experiment.*

EXPERIMENTAL PROCEDURE

Part A: Extraction and recrystallization of caffeine from black tea

1. Pour 50 mL of distilled water into a beaker and place it on a hot plate. Place a watch glass over the beaker and bring the water to a boil.
2. Obtain two (2) tea bags and record their mass together, assuming that the average mass of an empty tea bag is 0.120 g. DO NOT REMOVE THE TEA FROM THE BAGS.
3. Place the tea bags into the boiling water and turn off the heat on the hot plate. Stir the bags occasionally for the next 15 minutes as the hot plate cools to room temperature.
4. Discard the tea bags, and add 2.0 g Na_2CO_3 to the steeped tea. Stir with a Teflon-coated stirring rod until the solid has dissolved.
5. Transfer the solution into a 125 mL separatory funnel supported on a wooden support rack, and add 10 mL of dichloromethane to the separatory funnel, noting which layer is the aqueous and which is the organic. Never fill an extraction funnel more than ¾ full!
6. Rinse the beaker with an additional 5 mL of dichloromethane to maximize transfer and add this to the separatory funnel.
7. Shake the funnel *gently* and vent it frequently. Return it to the support rack and allow the layers to separate. Two brown, turbid phases may form. This is an undesired emulsion.
8. If an emulsion is present, add 10 mL dichloromethane and continue the extraction.
9. If you still have an emulsion, add 15 mL saturated NaCl solution (brine), and wait for the layers to separate.
10. Draw off the organic layer into a 125 mL Erlenmeyer flask. With the aqueous layer still in the separatory funnel, repeat the extraction with 2x10 mL dichloromethane. Combine the extracted organic phases in an Erlenmeyer.
11. Carefully remove the aqueous phase into a beaker and leave to the side (do not discard any phases, washings until you are sure of what you need and what you do not), and clean the separatory funnel. Due to the likelihood of solid tea residues present in the mixture, it is recommended to pour the aqueous phase out by the top (wide) opening of the separatory funnel.

12. Add a scoop of Na_2SO_4 to the organic phase. The Na_2SO_4 is a drying agent and will remove any trace amounts of water present in the organic phase. You will know that your drying agent has worked by the presence of clumps of hydrated salt in the beaker.
13. Gravity filter the organic solution into the Erlenmeyer using filter paper and a funnel.
14. Set 1 – 2 mL of solution aside for TLC analysis.
15. Using a steam bath, gently boil the solvent in the fume hood to evaporation.
16. Scrape out the crude caffeine product and obtain the mass.

Sublimation

17. Heat a hotplate on medium (level 4 - 5).
18. Place the crude caffeine into a 250 mL side-arm flask.
19. Ensure that the cold finger (test tube + stopper) is clean and dry on the outside.
20. Take the mass of the test tube + stopper in a 50 mL beaker
21. Place the cold finger into the mouth of the side-arm flask, ensuring that there is a tight seal and ~ 1 cm between the flask bottom and the cold finger.
22. Place the side-arm flask on the hot plate and secure it with a clamp.
23. Fill the cold finger with crushed ice. You will likely need to refill the cold finger trap to maintain the condensation.
24. Allow the caffeine to sublime onto the cold finger until there is negligible product remaining in the base of the flask.
25. When sublimation is complete, carefully remove the cold finger from the flask so as not to drop any purified crystals back into the crude mixture. Tap any loose crystals into the 50 mL beaker used in step 20, and remove any residual dry ice. Weigh the coated test tube + stopper in the 50 mL beaker, and determine the mass of purified caffeine. Compare your yield of the crude to the purified. How much were you able to isolate via sublimation?
26. Determine the melting point of the purified crystals. Consult Appendix I for instructions on using a melting point apparatus.

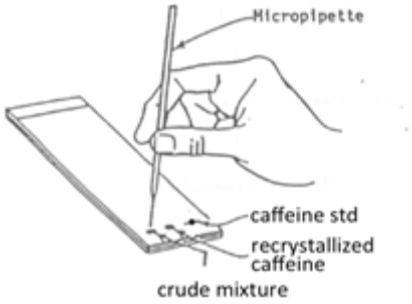
In your report, indicate the mass of the isolated product and determine the percent yield of caffeine. You can assume that the tea contained 4% caffeine by weight as a theoretical value.

Thin Layer Chromatography

Take the time to read the appendix on chromatography before starting this part. Before spotting on TLC plates, practice your technique using a piece of brown paper towel. Your TA will demonstrate proper technique.

27. Obtain a TLC plate and lightly pencil a line ~1 cm from the bottom of the plate.
28. Dissolve a few crystals of recrystallized caffeine in about ~2 mL of dichloromethane in a test tube.
29. Repeat the above step with the crude product and the authentic caffeine sample in 2 separate test tubes.

30. Spot a small amount of crude caffeine in the left most lane, a small amount of recrystallized caffeine in the centre lane, and a small amount of authentic caffeine in the right most lane of the TLC plate (see diagram). Label each lane appropriately.



31. Pour 10 mL of 99:1 acetone:acetic acid mixture in a developing jar to use as the developing solvent system. Make sure that the solvent level is below the starting line of your plate.
32. Carefully place the TLC plate into the jar, avoiding any splashing, and ensuring that the plate is as upright as possible. Cap the jar.
33. Allow the solvent to develop to about 1 cm from the top of the plate and mark the finish line with a pencil.
34. Allow the solvent to evaporate off the TLC plate and then visualize the developed plate with a UV lamp. **NEVER LOOK DIRECTLY AT A UV LAMP.**
35. Using a pencil, mark the spots on the plate.
36. If your spots are streaky or blotchy, then you over-spotted your TLC plate. If you have time, repeat this section spotting *less* on your next TLC plate. If your spots are faint, repeat the TLC by precisely double spotting for each necessary spot.
37. Record the results in your lab book. Calculate the R_f value of each spot before leaving. Including a digital picture of your TLC in your report is recommended.

Part B: Extraction of Carotenoids and Chlorophylls from spinach

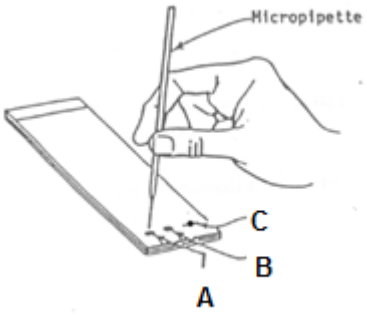
1. Obtain 1 – 2 spinach leaves and remove the large stems and veins. Tear the leaves into small pieces. Weigh out 1.0 g of the leaves and place them in a 15 mL centrifuge tube.
2. Add 1.0 g of anhydrous Na_2SO_4 and 1.0 g of sand to the tube. Carefully grind the leaves using a Teflon-coated stirring rod until you obtain a fine pulp.
3. Add 2.0 mL of acetone to the tube and then cap it and shake it. Allow the solids to settle for 5 minutes, then stir (or shake) and let settle for another 5 minutes. Note: A more concentrated spinach sample will result in better TLC results.
4. Using a pipette, carefully transfer the liquid into a clean test tube **labelled A**. To maximize the transfer of compounds, wash the centrifuge tube with an additional 2 mL acetone and pipette-transfer this liquid to the same test tube A. Rinse out the centrifuge tube with soap and water and return it to your TA.
5. Transfer ~2 mL (half) of solution from test tube A to a new test tube **labelled B.** Add 3.0 mL of hexanes to test tube B, followed by 3.0 mL of water. Place a rubber stopper on test tube B

and shake gently to perform a mini-extraction. Identify which layer is aqueous and which layer is organic. Take note of any changes you observe.

6. Pipette ~1 mL (half) of the organic phase of test tube B into a new, clean test tube, **labelled C**. Add 3.0 mL of 1.0 M HCl solution to test tube C. Stopper the test tube and shake gently, venting occasionally. Take note of any changes you observe.

Pigment separation by TLC

7. Obtain three TLC plates and, using a pencil, draw a line ~1 cm from the bottom of each plate. Obtain a capillary spotter from your TA and spot a <u>small</u> amount from test tube A in the left lane, B in the centre lane, and C in the right lane (see diagram) of each plate. Label each lane and each plate appropriately. Depending on the brightness (or lack thereof) your solutions, you may want to double-spot.



8. Obtain 10 mL of 3:7 hexanes:acetone developing solvent and transfer it to a clean developing jar. Carefully place the first plate in the jar and seal the jar. Develop the plate until the solvent is ~1 cm from the top of the plate. If the solvent reaches the top of the plate, you must redo your TLC.
9. Using tweezers, remove the plate from the developing jar and mark down all observations. Note the Rf values and colour of each spot. Sketch a copy of the TLC in your notebook or take a picture if you like, but a digital picture is recommended. Note that compounds are sensitive to light and will degrade over time.
10. Repeat steps 7 – 9 with two other mobile phases: 70:30 hexanes:acetone and 50:25:25 cyclohexane:hexane:acetone.

The following table may help you organize your results. You may need more rows than are present. It is recommended to organize data based on similar colours and R_f values.

Colour	Rf values								
	30:70 Hex:Ace.			**70:30 Hex: Ace.**			**50:25:25 cy.hex:hex**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**

Identify the spots on the TLC plate with the natural products found in spinach leaves (B-carotene, lutein, violaxanthin, chlorophyll a and b, pheophytin a ad b, xanthophyll). Justify your assignment of each spot using the principles of chromatography. You are encouraged to speculate on the assignment of additional spots. Your TLC should show between 4 – 7 different pigments in each lane, and may show more than those listed in the table above.

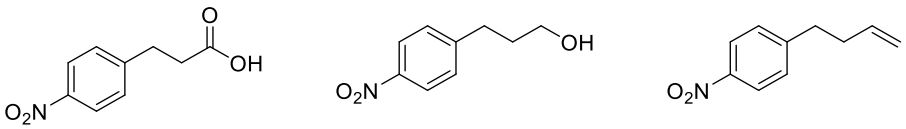
REPORT (53 POINTS)

Your report must include the following sections

- Title page (1 point)
- Introduction (6 points)
- Experimental procedure (1 point)
- Observations and Results (4 points)
- TLC plates (4 points)
- Flow chart for part A only (10 points)
- Discussion (12 points)
- Questions (15 points)

QUESTIONS

1. How does a NaCl solution function to remedy an emulsion? (1 point)
2. Supposing that the goal of Part A was to isolate catechin instead, describe how you could isolate this polyphenol from brewed tea using a liquid-liquid extraction to obtain the crude product (2 points).
3. Using the principles of TLC, rank the following compounds based upon the distance that they will travel along a TLC plate. Justify your ranking with relevant theory. (5 points)



4. Using a flow chart and reactive extraction, explain how you would isolate benzyl, benzoic acid, and p-methyl phenol from a solution of dichloromethane containing all three? (4 points)
5. A student is running behind in the caffeine purification, and doesn't read the note about ensuring the cold finger rests ~1 cm from the base of the flask. He places it about 3 cm from the base. Will this have any effect on his yield? Why? (2 points)

Locker Check-Out

Please circle your course number. (2123 : 2523 : 1321 : 1721)
Print your name and student number.
Student (1) _____
Student (2) _____
Locker # _____
Demonstrator _____

TOP SHELF	IN	OUT
RUBBER TUBING	4	
WASH BOTTLE 250 ml	1	
UNIVERSAL CLAMP	4	
EXTENSION CLAMP	3	
CLAMP HOLDER	7	
TEST TUBE HOLDER	1	
CRUCIBLE TONGS	1	
SUPPORT RING 3"	1	
WIRE GAUZE	1	
CORK RING MEDIUM.	2	
CORK RING SMALL	2	

BOTTOM SHELF	IN	OUT	STORAGE BOX (ORANGE)	IN	OUT
BEAKER 50 ml	2		FILTRATION FUNNEL	1	
BEAKER 150 ml	1		POWDER FUNNEL	1	
BEAKER 250 ml	2		WATCH GLASS 90mm	2	
BEAKER 400 ml	1		ERLENMEYER 50 ml	3	
BEAKER 600 ml	1		TEFLON STIRRER	2	
BEAKER 1000 ml	1		DRYING TUBE (plastic)	1	
TEST TUBE 16 x 150	12		SCOOPULA	1	
TEST TUBE RACK	2		SPATULA	1	
DEVELOPMENT JARS 8 oz.	2		GRADUATED CYLINDER 10 ml	2	
ERLENMEYER 125 ml	2		GRADUATED CYLINDER 50 ml	1	
ERLENMEYER 250 ml	1		GRADUATED CYLINDER 100 ml	1	
SUCTION FLASK 500 ml	2		« C » CLAMP (NALGEN JAR)	5	
BUCHNER FUNNEL	1				
SUCTION ADAPTER	1				
FUNNEL SUPPORT (WOOD)	1				

Note : Each locker contains a full organic glassware kit on the bottom shelf. You must show your locker to the demonstrator before leaving.