Turning Over a New Leaf-
The search for a New Caffeinated Tea

Learning Objectives:

- Introduction to organic chemistry
- Isolation and purification of an organic compound from a natural product using chemical and physical properties
- Identification of extracted substance
- Filtration
- Liquid-liquid extraction technique
- Drying an organic solvent
- Use of a Rotary Evaporator (RotoVap)
- Sublimation
- Melting Point (238 0C)
- HPLC

The Problem

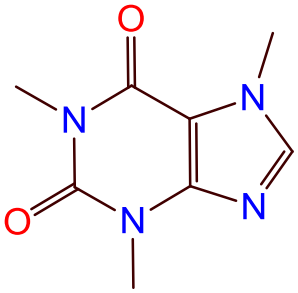
In China, the most enduring legend dates back over 4,000 years. Emperor Nun Shen, a scholar and herbalist, was kneeling beside a fire, boiling water. With the water at a tempest, a breeze blew the topmost leaves of a nearby tree into the pot. The aroma drew Shen to taste the beguiling beverage. Immediately delighted, Shen claimed that this liquid was both delicious and invigorating.

India's legend originates with a saintly priest named Bodhidharma. About 1,900 years ago, the eventual founder of Zen Buddhism was in the fifth year of a seven-year sleepless contemplation of Buddha. Finding himself dangerously close to falling asleep, Bodhidharma snatched some leaves from a nearby bush and chewed them. He was immediately revived. Bodhidharma turned to these leaves—the leaves of a wild tea tree—whenever he again felt drowsy, and was thus, according to legend, able to complete his seven years of meditation.

 Designed and written by: Alan B. Cooper, Ph.D.

So the search for tea leaves continues. Tetley tea® corporation recently discovered four new tea leafs found in the forests of Papua New Guinea. The objective is to find tea that has the highest concentration of caffeine. Before this is used for human consumption, we need to confirm that the caffeine content is higher than presently sold tea to compete with their presently marketed caffeinated tea products. We also need to confirm that the caffeine molecule is indeed caffeine. Therefore, you must isolate the caffeine from this new tea leaf to determine its caffeine content and confirm that the caffeine isolated is indeed caffeine. Purification will be by liquid-liquid extraction followed by sublimation. Methods used for the determination and characterization of the amount of caffeine will be HPLC and melting point.

Caffeine
1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione



Chemical Formula: $C_8H_{10}N_4O_2$
M.P.= 238 ^{O}C
Molecular Weight: 194.19
Elemental Analysis: C, 49.48%; H, 5.19%; N, 28.85%; O, 16.48%

Experimental Background:

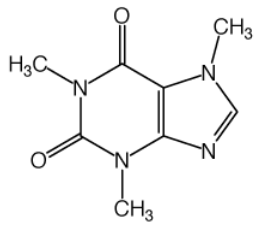
Purpose:
The purpose of this lab is to determine the Caffeine content, by mass, from a new tea leaf. This new tea leaf was discovered in the forests of Papua New Guinea. During the lab, a cup of tea was made and then strained. For each cup, straining will be followed by the addition of sodium carbonate to solubilize the tannins followed by three washings of isopropyl acetate taking place inside a separatory funnel. The isopropyl acetate layers will be combined, dried over sodium sulfate, filtered and the isopropyl acetate evaporated to obtain a tan solid.

General background and overview of the experiment:
Humankind has historically used advantageous compounds derived from plants and animals. For instance, various extracts from plants have been used as teas, potions, medicines and poisons. Though these extracts can contain a mixture of many different chemicals, often only one or few are responsible for the activity of the extract. For decades such extracts were

used directly from their natural sources. This practice has many disadvantages, as the composition of the extract can vary from time to time and it is highly dependent on the availability of the natural source. At the beginning of the 19th century chemists made the first attempts to isolate the active components within these natural mixtures. The first compound to be isolated and purified was morphine from opium. Sertürner accomplished this by extracting opium with hot water and precipitating morphine with ammonia. He obtained colorless crystals that were poorly soluble in water but soluble in acids and alcohol. To make sure that the effect of the compound was identical to that of raw opium he tested the crystals on himself…Following this initial experimentation, many natural products were isolated and their structures were determined. Once a structure was elucidated, chemists were able to devise synthetic methods to synthesize these compounds. This was the beginning of modern pharmaceutical chemistry. Today, researches are still looking for new compounds from natural sources. Potential drugs are often isolated from sea creatures, like sponges or slugs, parts of plants that were used in traditional medicine, or new a species discovered in the rain forest.

In this lab we will extract and purify caffeine from tea leaves. First water soluble compounds will be extracted from dry tea leaves with boiling water. Then, caffeine will be preferentially extracted from the water into organic solvent. The solvent will be removed and the crude material will be purified by sublimation or re-crystallization.

Caffeine (Figure 1) belongs to a group of compounds known as alkaloids. Alkaloids are nitrogen-containing ring compounds of plant origin that usually have a bitter taste and some biological activity. Other well-known alkaloids include morphine, strychnine, quinine, ephedrine, and nicotine. Caffeine is a central nervous system stimulant. Because its ingestion results in wakefulness, it is the principal ingredient of No-Doz tablets. It is also used to counteract the hypnotic effect of other drugs and is found in a number of analgesics.



Brewed tea is a solution of a variety of compounds that impart color, flavor, and pharmacological activity. These compounds mostly fall into four groups: tannins and their hydrolysis products, xanthines like caffeine and theophylline, flavonoids like catechin, and chlorophylls. The structures of some of these compounds are shown in below. It is important to know that OH groups are fairly acidic when the oxygen is attached to a benzene ring as it is in gallic acid and catechin.

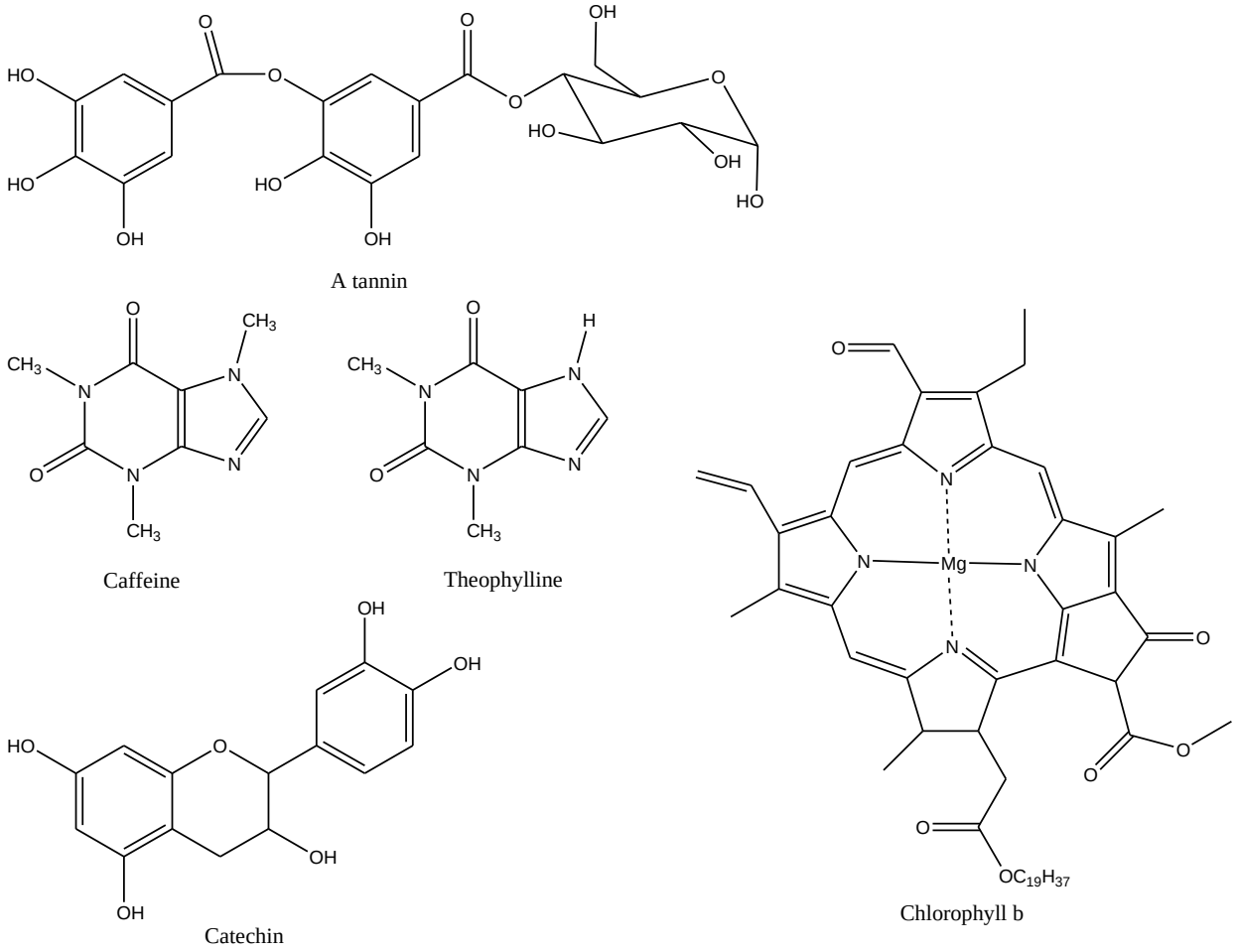


Figure 1. Compounds found in tea.

Caffeine is more soluble in dichloromethane or isopropyl acetate than it is in water but most of the compounds in tea are (or can be made to be) more soluble in water than in an organic solvent. This difference in solubilities can be used to extract nearly pure caffeine from tea.

Experimental Background

Liquid-liquid extraction is a technique used to selectively remove one component from a solution containing several dissolved components. Typically, the solution is extracted with another solvent capable of dissolving only the desired component *and* the desired component will be *more* soluble in the extraction solvent. The two solvents must be immiscible when performing a liquid-liquid extraction.

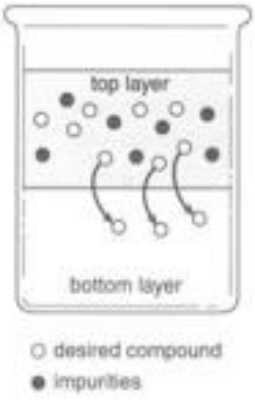


Figure 1 Extraction occurs when the desired compound changes layers, leaving impurities behind

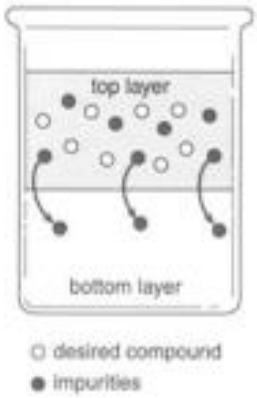


Figure 2 Washing occurs when impurities change layers, leaving the desired compound behind

The figures above demonstrate that extraction solvents (left) remove the desired component. On the right, a washing solvent would selectively remove impurities. The density of the solvent pair is not particularly important. That is, the extracting solvent may be more or less dense than the impure solution. It is crucially important though that the two solvents be immiscible.

A separatory funnel is the most commonly used piece of glassware to achieve the extraction. The aqueous layer is added to the separatory funnel and then the extraction solvent is added. The mixture is shaken and the separatory funnel is "burped" to release any pressure in the funnel. The funnel is then placed in an iron ring to allow the layers to separate from each other. The stopcock at the bottom of the separatory funnel is then opened to remove the bottom layer into a flask. One extraction is usually not enough to remove all of the organic compound, so more organic solvent is added to the aqueous layer and shaken. This is allowed to separate out again and then the bottom layer is removed once more. This procedure can be repeated as necessary. Fresh solvent is added each time an extraction is done to increase the removal of the organic product from the aqueous layer.

The organic solvent layers can also be extracted with fresh water to remove any contamination by inorganic compounds such as acids, bases or salts. The organic layer can also be dried with a solid drying agent to remove any traces of water. The drying agent is then filtered out of the organic layer. The organic solvent is then evaporated or distilled leaving the organic product.

For an extraction to be successful, the two solvents used must be immiscible in each other. That is they must be insoluble in one another. Water is immiscible in most organic solvents. Some of these solvents are butanol, chloroform, cyclohexane, methylene chloride, ethyl acetate, hexanes, toluene, diethyl ether and pentane. Water is miscible in methanol, ethanol, isopropyl alcohol and acetone, so these solvents would not be used in aqueous extraction procedures. Acetone and ethanol are miscible in most every solvent so they are not used at all in extraction procedures.

Some solvent pairs are slightly solvent in each other, so when they are used in extractions some of the compound will remain in the water layer and some water will remain in the organic solvent. Water can be removed with a drying agent, but the compound left in the water will not be able to be recovered.

When an extraction is done, the compound that is being extracted will be dissolved in each of the solvents to some extent. How soluble is it in each of the layers depends on the solubility of the solute in each of the solvents. The ratio of the concentration of the solute in each of the solvents at a particular temperature is a constant called the distribution coefficient or partition coefficient (K).

$$K = \frac{\text{concentration in solvent}_2}{\text{concentration in solvent}_1}$$

where $solvent_1$ and $solvent_2$ are immiscible liquids

$Solvent_2$ is the solvent in which the solute is more soluble. The solute is assumed to not react with the solvent. This is a ratio, so concentration can be in any units as long as both concentrations are in the same unit. Distribution coefficients can be used to calculate how much of a solute will be extracted from a solvent. Increased extraction of a solute can be achieved by dividing up the total amount of solvent into smaller portions. Then adding only one portion at a time and repeating the extraction with all of the individual portions making sure to remove the bottom layer each time.

When choosing a solvent for extraction, there are some things to consider. One is that the extraction solvent is immiscible in the first solvent. The compound being extracted needs to be soluble in the extraction solvent and unreactive with the solvent. Major impurities should not be soluble in the extraction solvent so that they will remain in the first solvent. The extraction solvent should be fairly volatile so that it can be removed from the solute and nontoxic and nonflammable. Most organic solvents are not nontoxic or nonflammable so caution must be used with extraction solvents.

The following table includes some of the common extraction solvents along with their formulas, densities, boiling points and comments about flammability and toxicity.

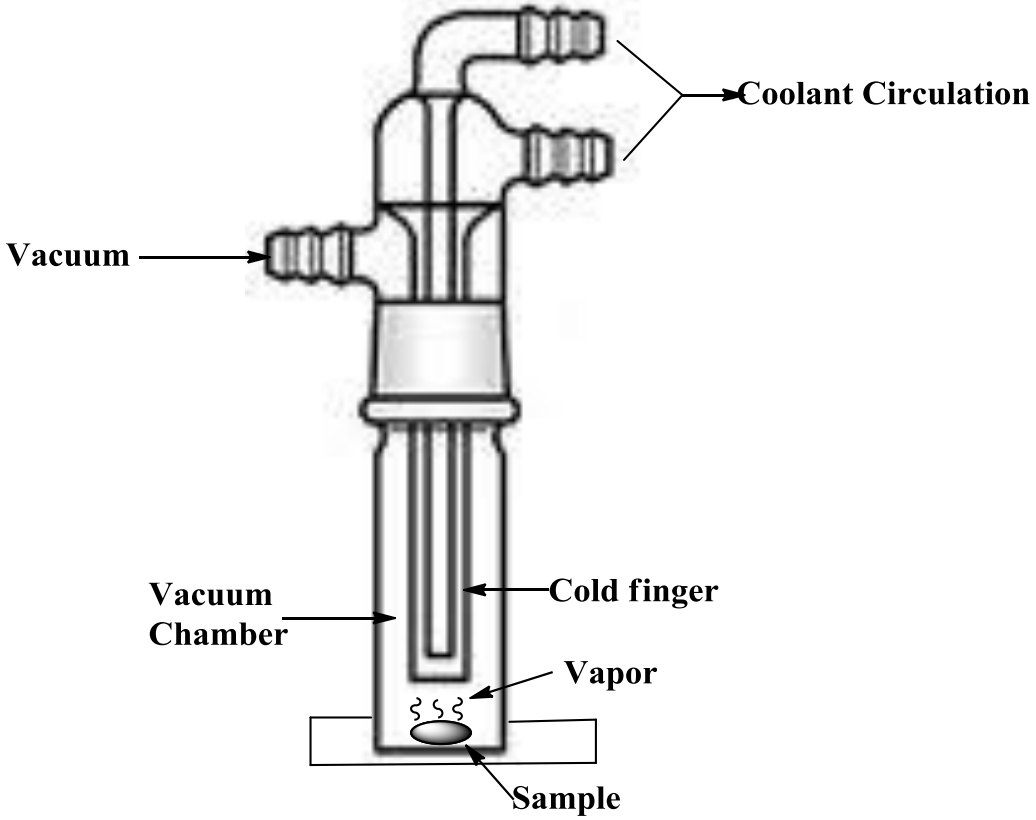
Some common extraction solvents

Name	Formula	Density (g/mL)	Boiling point (°C)	Comments
Diethyl ether	$(CH_3CH_2)_2O$	0.7	35	Highly flammable
Petroleum ether	C_nH_{2n+2}	~0.7	~30-60	Flammable
Hexanes	C_6H_{14}	~0.7	67-69	Flammable
Benzene	C_6H_6	0.9	80	Flammable, toxic, carcinogenic
Ethyl acetate	$CH_3COOC_2H_5$	0.9	77	Flammable
Toluene	$C_6H_5CH_3$	0.9	111	Flammable
Methylene chloride	CH_2Cl_2	1.3	40	Toxic
Chloroform	$CHCl_3$	1.5	61	Toxic
Carbon tetrachloride	CCl_4	1.6	77	Toxic, carcinogenic

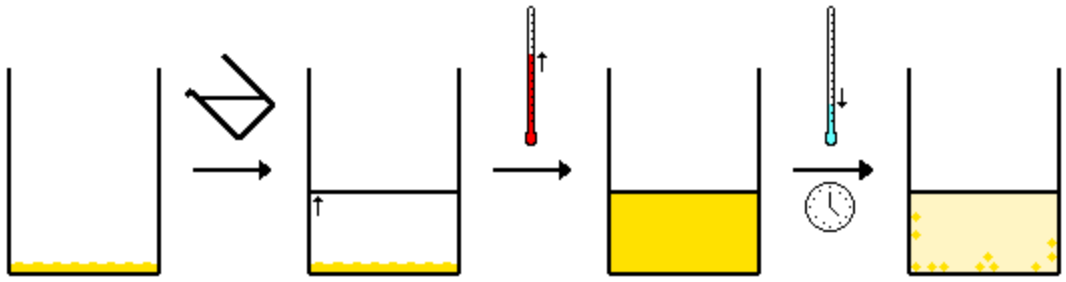
If there is any doubt as to which layer is the organic layer and which is the aqueous layer, then testing the two layers in a test tube is recommended. Simple add water to two test tubes and then drop a few drops of one of the layers into one of the test tubes and watch to see if it is immiscible or not. Repeat with the other layer. When performing an extraction, it is wise to save all the layers until the experiment is finished.

Purification:

Sublimation: Sublimation is the process of changing from a solid to a gas without passing through an intermediate liquid phase. Carbon dioxide, at a pressure of one atmosphere, sublimates at about -78 degrees Celsius. Ice and snow on the Earth's surface also sublimate at temperatures below the freezing point of water. The sublimation procedure that you will be carrying out depends upon a solid changing to the gas phase and then condensing on a cold finger back to the solid phase. The end result of this process is the purification of the original solid.



Recrystalization: Recrystallization is a procedure for purifying compounds. The most typical situation is that a desired "compound A" is contaminated by a small amount of "impurity B". There are different recrystallization techniques that can be used. We will be using single solvent recrystallization. Typically, the mixture of "compound A" and "impurity B" are dissolved in the smallest amount of hot solvent to fully dissolve the mixture, thus making a saturated solution. The solution is then allowed to cool. As the solution cools the solubility of compounds in solution drops. This results in the desired compound dropping (recrystallizing) from solution. The slower the rate of cooling, the bigger the crystals form. The crystallization process requires an initiation step, such as the addition of a "seed" crystal. In the laboratory a minuscule fragment of glass, produced by scratching the side of the glass recrystallization vessel, may provide the nucleus on which crystals may grow. Successful recrystallization depends on finding the right solvent. This is usually a combination of prediction/experience and trial/error. The compounds must be more soluble at the higher temperature than at the lower temperatures. The desired major compound A will then crystallize leaving the undesired compound B in solution.



Equipment and Materials:

Chemicals

Tea bags
Caffeine
Na_2CO_3
Ethyl acetate or isopropyl acetate
Iso-Propanol
Acetone
Anhydrous Na_2SO_4
Saturated NaCl solution
Distilled (de-ionized) water
HPLC solvents:
Methanol
water
phosphoric acid

Equipment

Dispenser for 30 mL of IPrOAc (iPrOAc)
2-4 Ice bath
4- 250 mL separatory funnel
20- 125 mL Erlenmeyer flask
4- 100 mL round bottom flask
4- cork rings
4- Plastic Funnels
Fluted Filter paper
4- 100 mL graduated cylinder
4- 1mL pipetters
Pipette tips
Weigh paper
2- RotoVap fitted to a vacuum pump and chiller
2-Vacuum pumps for rotovaps
4 Stirrers and 4- stir bar
Small fritted filters with small rings

50 mL Side arm flasks
Balance
4- Spatula
4- 100 mL Heating mantle
Rheostat
Thermocouple temperature monitor
Sublimation apparatus
Chiller
Vacuum pump
4 Ring stand/ clamps for sublimation
4 ring stands for extraction
4-TLC plates
4 TLC tanks
12-HPLC vials and caps
12- Syringe filters
12- Syringes
2- HPLC Column: Phenonenex Luna C8 (2) 3 um, 4.6 x 30 mm

For Each group Set-Ups:

250 mL separatory funnel
5- 125 mL Erlenmeyer flask
100 mL round bottom flask
Cork ring
Plastic Funnel
Fluted Filter paper
100 mL graduated cylinder
1 mL pipetter
Pipette tips
stir bar
Spatula
1- 25 mL volumetric flask
TLC plate
TLC tank
TLC spotter
Cold bath

For Group/ shared areas:
Balance
Anhydrous Sodium Sulfate
Sodium Carbonate
TLC solvent (1:1 Acetone: IPA)
Brine (saturated NaCl)
Isopropyl acetate bottle with pipettor (30 mL)

Weigh paper
Pencil
Plastic pipets

Sublimation:
- 2 Ring stand/ clamps for sublimation
- 2- Heating blocks
- Thermocouple temperature monitor
- Sublimation apparatus
- Chiller
- Vacuum pump

Vacuum manifold

Recrystalization:
4-50 mL side arm filter flasks
4- 2 mL filter funnel
4 vacuum adaptors
Heptane
Plastic pipets
Vacuum pump with trap

HPLC Station Set up:

HPLC Column = Phenonenex Gemini C18 3 um, 4.6 x 50 mm
Guard Column = Guard Column = Phenomenex Security Guard C18
Mobile phase = 32/68/0.1 methanol/water/phosphoric acid
Syringe wash = 30/70 methanol/water
Caffeine standard: 0.04 mg/mL
3- HPLC vials and caps per group
3- Syringe filters per group
3- Syringes per group
2-100 mL volumetric flask per group

Rotovap station Set-ups:
2- RotoVap fitted to a vacuum pump and chiller
Vacuum pump for rotovaps
Waste solvent containers
For Clean-Up:
acetone wash bottles (in hood)
2 x 1-Liter Waste Collection Beakers (one for each hood)
Paper towels

Pre- Lab Procedure:

1. Boil 2000 mL of distilled water
2. Add 40 tea bags and continue boiling for 10 minutes
3. Let cool to room temperature.
4. Divide into 4- 500 mL bottles.
5. Add appropriate amount of pure caffeine (100, 200, 300, or 400 mg) to the bottles (A, B, C, D) of tea extract. One of the bottles (A, B, C or D) will be given to each group
6. HPLC set up (see below)
7. Caffeine standard - prepare a standard of approximately 0.04 mg/mL. Suggested dilution scheme is as follows:

    a. Weigh ~100 mg caffeine into a 100 mL vol flask
    b. Add ~5 mL of methanol to wet and ~50 mL 30/70 methanol/water
    c. Place in an ultrasonic bath and sonicate until dissolved
    d. Dilute to volume with 30/70 methanol/water and mix well
    e. Transfer 2.0 mL of this solution to a 50 mL vol flask
    f. Dilute to volume with 30/70 methanol/water and mix well

Experimental Procedure:
Isolation/ Purification:

1. Turn on rotory evaporator heating bath to 55 degrees C and start chiller circulating.
2. Turn on chiller and heating block for sublimation
3. Measure 100 mL of tea extract in graduated cylinder and transfer to 125 mL Erlenmeyer flask. Add stir bar to flask.
4. Use pipettor to transfer 1 mL of tea extract to a 10 mL volumetric flask labeled (**sample 1**)
5. Spot tlc plate with tea extract
6. Weigh 1.6 grams of sodium carbonate (Na_2CO_3) on balance. Add to tea extract and stir for 3 minutes. (Note: the mixture becomes clear)
7. Transfer the mixture to a 250 mL separatory funnel
8. Add 30 mL of isopropyl acetate (iPrOAc) from dispenser, stopper the funnel and rock the mixture for 20 times. (do not shake violently since this will result in an intractable emulsion). Vent funnel after 5 rocks.
9. Let the layers separate (~ 5 min). There will be some emulsion. Open the stopcock to let the bottom water layer separate out until the point of the emulsion is at the

bottom. Add the upper layer to a 125 mL erlenmyer flask. It's OK if the upper layer contains some emulsion. (Note: label the lower layer Erlenmeyer and upper layer Erlenmeyer so as not to confuse them.)

10. Repeat steps 8 and 9 two more times collecting all the isopropyl acetate layers in the same Erlenmeyer flask.
11. Return the isopropyl acetate fraction to the seperatory funnel and add 50 mL of saturated NaCl and shake.
12. Drain the lower aqueous layer and add the upper layer to the 125 mL Erlenmeyer flask.
13. Add dry sodium sulfate with a spatula to the combined isopropyl acetate layers and swirl. If the solid clumps up, add a little more sodium sulfate to dry the mixture. Filter through a funnel with fluted filter paper into a 125 mL Erlenmeyer flask. Rinse the funnel with filter paper with ~10 mL of isopropyl acetate.
14. Weigh your 100 mL round bottom flask and record the weight.
15. Add ~ 50 mL of the filtrate to your 100 mL round bottom flask and evaporate the isopropyl acetate on a rotory evaporator with water bath set to 55 degrees C to obtain a solid film on the flask.
16. Repeat until the entire iPrOAc layer is evaporated. Take a tlc sample of the crude caffeine after adding your second filtrate to the round bottom flask.
17. Dry further under vacuum.
18. Re-Weigh the flask.
19. Subtract the weight of flask from step 14 to obtain the weight of the crude caffeine.
20. Weigh out a 4 mg sample of the solid for HPLC analysis and set aside **(sample 2)**
21. You will now purify the sample further by sublimation (step 22) or recrystallization (go to step 26):
22. SUBLIMATION:
    Transfer the crude caffeine to a sublimation apparatus that is connected to vacuum and circulating 5 ^{o}C cooling bath.
23. Put the sublimation apparatus, under vacuum, while coolant circulating into a 160 ^{o}C heating mantle. Monitor the temperature with a thermocouple thermometer.
24. Watch the caffeine sublime onto the cold finger (~ 30-45 min).
25. Release the vacuum slowly, let cool and remove a sample of the solid from the cold finger for HPLC analysis. **(sample 3)**

26. RECRYSTALIZATION:
    Add 5mL isopropanol to round bottom flask containing crude caffeine. Place in warm bath if sample does not dissolve. Place flask in ice water bath and leave over lunch. If sample does not crystalize, add seed crystal of caffeine.
27. Use plastic pipet to transfer material to glass filter funnel attached to vacuum flask and filter crystalized caffeine. Wash with a little heptane.
28. Take sample of purified caffeine for HPLC analysis

CLEAN-UP

Identification and Characterization:

1. Run an HPLC analysis of the crude and purified caffeine products.
2. Take a melting point of reference caffeine, and the final sublimed/ recrystallized product.
3. Run TLC of collected samples and a caffeine standard.

Thin Layer Chromatography

1. Add a few drops of isopropanol to a caffeine standard. This will not dissolve all of it, but enough will dissolve to allow you to do TLC on the solution.
2. Spot the TLC plate with the tea extract, isolated caffeine and with authentic caffeine from a solution of caffeine in isopropanol. Label the samples on your plate.
3. Develop the chromatogram using a mixture of 1:1 isopropyl acetate: acetone (5 mL of each solvent) as mobile phase.
4. After the development, air dry the plate and examine the plate under UV light to observe the spots. Outline the spots with a pencil and confirm the purity of caffeine from Rf value (It is the ratio of distance traveled by sample spot from the origin to the solvent front from the origin) of both the spots.

Melting Point

1. Scrape crystal into a small pile
2. Place the crystals into capillary by crushing tube (open end down) on top of pile of crystals
3. Place samples in the Melting Point Apparatus
4. Record melting range

HPLC

1. Prepare Caffeine standard - prepare a standard of approximately 0.04 mg/mL.
    a. Weigh ~100 mg caffeine into a 100 mL vol flask

b. Add ~5 mL of methanol to wet and ~50 mL 30/70 methanol/water
c. Place in an ultrasonic bath and sonicate until dissolved
d. Dilute to volume with 30/70 methanol/water and mix well
e. Transfer 2.0 mL of this solution to a 50 mL vol flask
f. Dilute to volume with 30/70 methanol/water and mix well
g. Filter into HPLC vial with syringe filter **NOTE: Autosampler vials can only be filled between ½ and ¾ of total volume**

2. Dilute **sample 1** 25 fold
   a. Dilute 1 mL of tea extract to 25 mL in volumetric flask with HPLC solvent
   b. filter into HPLC vial **NOTE: Autosampler vials can only be filled between ½ and ¾ of total volume**
3. **Samples 2 and 3**: prepare ~ 0.04 mg/ mL solutions and filter
   a. Weigh out ~4 mg and dilute to 100 mL
   b. Filter into HPLC vials **NOTE: Autosampler vials can only be filled between ½ and ¾ of total volume**
4. Run your 3 samples on HPLC

Record amount caffeine isolated and purity of compound

IF YOU HAVE EXTRA TIME, YOU CAN RUN THE FOLLOWING:

IR

Running a Background IR Spectrum

1. Place one salt plate in the sample holder and lay the holder flat on the bench top.
2. Place a second salt plate over the first.
3. Place the cover over the plates and insert the holder into the IR spectrophotometer.
4. Record the background spectrum.

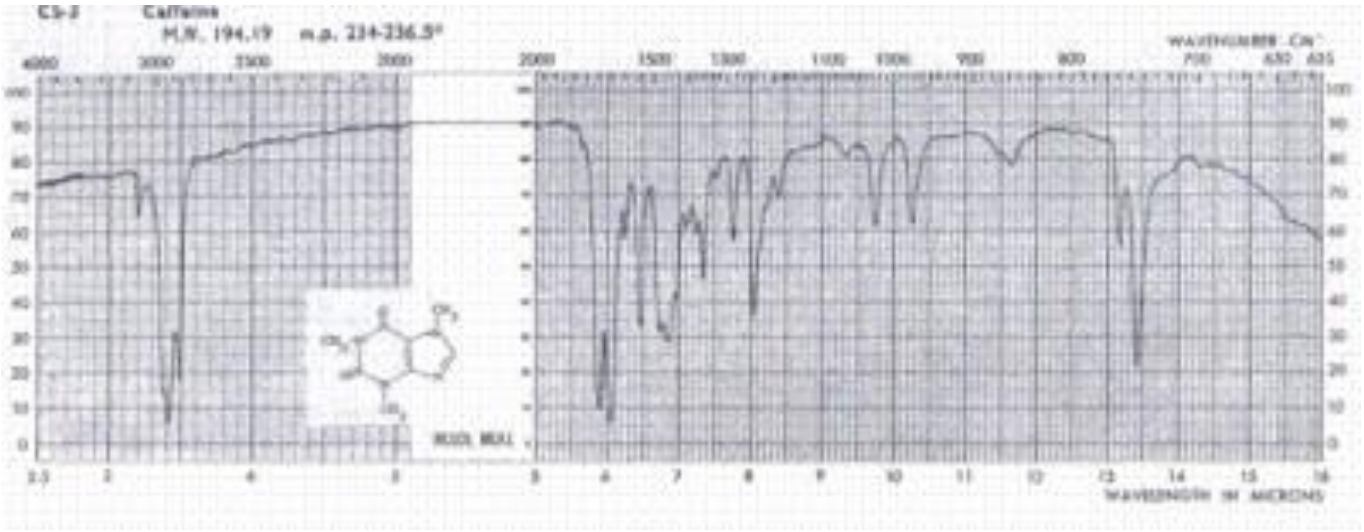
Running a Nujol IR Spectrum

1. Place one salt plate flat on the bench top.
2. Place 1 drop of Nujol on one the face of the salt plate.
3. Place a second salt plate over the first to spread the Nujol.
4. Carefully place the salt plate sandwich into the sample holder.
5. Place the cover over the plates and insert the holder into the IR spectrophotometer.

6. Record the Nujol spectrum.
7. After obtaining the spectrum, disassemble the holder and wipe both plates clean with a Kimwipe.

Preparing a Nujol Mull

1. Using the spatula, transfer a very small amount of your sample to the agate mortar. Note each spatula is marked with line to provide guidance on the amount of sample required.
2. Grind the sample to a very fine powder using the agate pestle.
3. Add 4 drops of Nujol (mineral oil) and grind to disperse the sample (forms a Nujol mull).
4. With pestle push the mull into the center of the mortar.
5. Place one salt plate flat on the bench top.
6. Place one drop of the Nujol mull on the face of the salt plate.
7. Place a second salt plate over the first to spread the Nujol mull.
8. Carefully place the salt plate sandwich into the sample holder.
9. Carefully place the cover over the plates and insert the holder into the IR spectrophotometer.
10. Record the sample spectrum.
11. After obtaining the spectrum, disassemble the holder and wipe both plates clean with a Kimwipe.
12. Place a few drops of isopropanol on each plate and wipe clean with a new Kimwipe.



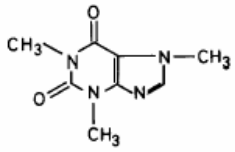
UV

Below is a UV absorbance spectrum of caffeine in several solvents. We will measure our

extracted caffeine sample (in water) and compare.

1. Clean the quartz cells by rinsing them with anhydrous ethanol
2. Fill both cells with anhydrous ethanol and dry the outsides with a Kimwipe
3. Place the cells in the reference and sample cell holders within the spectrometer with the clear sides of each cell facing the open slots of the cell holder
4. Click the grey start button on the computer monitor
5. When the dialog box comes up for the blank, click OK. A background correction is carried out from the maximum to the minimum wavelength range (400 nm – 200 nm).
6. Wait for the next dialog box to come up
7. Take out the sample cell (front cell) and place its contents into the waste beaker
8. Fill the sample cell until approximately ¾ full with the sample solution and wipe the outside of the cell with a Kimwipe. Place the sample cell back into the spectrometer and click OK to record the sample spectrum
9. Repeat steps 6 – 8 for the reference standard solution

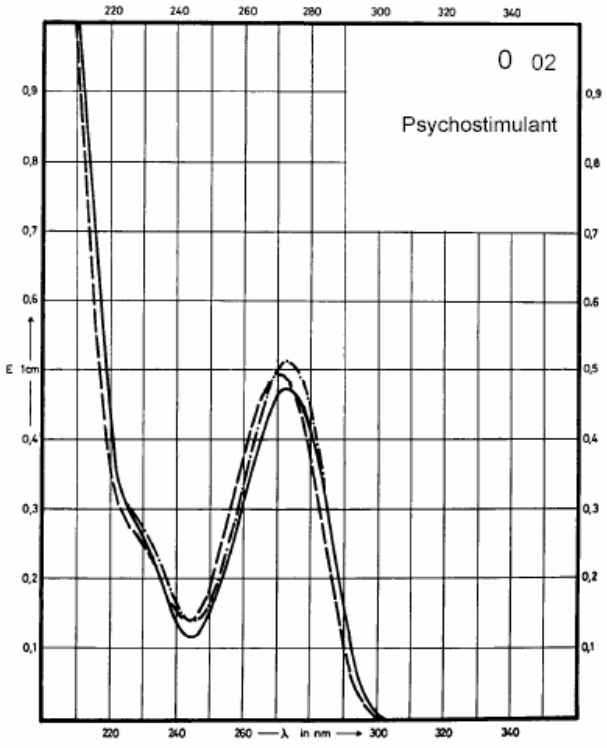
Name CAFFEINE



M_r 194.2

Concentration 1 mg / 100 ml

Solvent Symbol	Methanol	Water	0.1 M HCl	0.1 M NaOH
Maximum of absorption	273 nm	273 nm	270 nm	273 nm
$E^{1\%}_{1cm}$	475	515	495	510
ε	9220	10000	9610	9900



Instructor Notes:

Assignments:

Break the group up into 4 sets of 2 and assign an instructor to each set.
-Primary instructor goes over procedures, safety, and assignments.
-Primary instructor assists secondary as needed.
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Introducing the experiment:

The instructor will bring all the students to the back wall monitor and present the caffeine PowerPoint and the web link chromatography separation animation. Once this is complete (10-15 min), the team partners are positioned at the four workstation

Running the experiment:

- Set up with one assistant covering two separate team partners, each preparing one sample.
- Briefly run through the experimental details.
- Demonstrate how to carry out the separatory extraction by rocking the separatory funnel 20 times.
- Explain the two layers that will be seen (upper layer is the lighter isopropylacetate layer and lower layer is the water layer).
- Let the students do the three extractions.
- Explain how sodium sulfate dries the isopropylacetate layer.
- Show how to form a fluted filter paper and set up the filtration.
- Tare a 100 mL round bottom flask.
- After filling the rb flask half way explain how a rotory evaporator works. Explain why we use vacuum.
- Dry the flask under vacuum for 10 minutes. Explain why this is done (residual solvent).
- Take a 2-3 mg sample and save for melting point and HPLC.
- Show them the sublimation apparatus and explain the theory behind it (solid to vapor to solid). Let the sublimation run through lunch
- After lunch collect the sublimed product and weigh a sample for HPLC and a sample for melting point.
- Explain how the melting point will decrease if impure

Setting up the HPLC System:

The following are the HPLC conditions using HPLC 2:

- **Detector** (switch on left rear of unit) at 225 nm, 0.05 RSP, Range 0.500 AU

- **HPLC pump** (switch on right side front) using Method #04 (1.3 mL/min, 100% mobile phase A). If not on Method #04, press F6 key (DIR) and arrow up or down to Method #04. Press F4 key (RCL) and recall Method #04. Press Enter. (NOTE: Method #06 is a copy of Method #04)
- **Autosampler** using Method #04 (vials 1-4, 15 uL, 5.0 min run time). If not Method #04, use similar procedure as described for the pump. (NOTE: Method #06 is a copy of Method #04)
- **Vacuum degasser** (switch on ride side – the rear of the unit)
- Mobile phase A = 32/68/0.1 methanol/water/phosphoric acid
- Syringe wash = 30/70 methanol/water
- HPLC Column = Phenonenex Gemini C18 3 um, 4.6 x 50 mm
- Guard Column = Phenomenex Security Guard C18
- Caffeine standard - prepare a standard of approximately 0.04 mg/mL. Suggested dilution scheme is as follows:
    - Weigh ~100 mg caffeine into a 100 mL vol flask
    - Add ~5 mL of methanol to wet and ~50 mL 30/70 methanol/water
    - Place in an ultrasonic bath and sonicate until dissolved
    - Dilute to volume with 30/70 methanol/water and mix well
    - Transfer 2.0 mL of this solution to a 50 mL vol flask
    - Dilute to volume with 30/70 methanol/water and mix well

HPLC pump

Before starting up the pump, open the front left door on the pump, place a syringe on the fitting, open the valve by turning CCW. Use the Purge key and set flow rate to 3 mL/min for Mobile Phase bottle A. Purge for 1-2 min. Press STOP. Close valve, remove and empty syringe and press key F8 (STRT). Watch to see that the pressure only varies by ~50 – 70 psi after a minute or so. If the fluctuation is higher, purge the pump again to remove trapped air. This start up procedure only needs to be done at the beginning of the day.

Pressure should not exceed 3000 psi. If it does, change the guard column.

NOTE: if the screen becomes unfamiliar, the QUIT key will bring it back to the beginning condition.

Autosampler

At the beginning of the day, press the FLUSH key on the autosampler to remove air from the syringes. If air bubbles are present, repeat.

The autosampler is set for running 5 injections. If you are running more or less, then the autosampler must be reset for the new value. Press the F2 key (METH). Using the arrow keys, move to the "LAST" column and enter the number for the last injection. Then press the yellow ENTER key. Once the value is accepted, press the F6 key (STOR) and the unit will give you a new method number. Input 4 (Method 4) and when the unit asks if you want to

overwrite the method press yes (the #1 key) and when it asks for a new name press no (the #0 key).

If you want to start at a vial number other than 1, press the F2 key (METH) and move to the "FIRST" column and enter the location of the first injection. Follow storing the method as above.

Detector
Once the pump has run for ~10 min, press the "autozero" button on the detector.

Setting up TurboChrom Data Acquisition:

The computer should be set up and running. If so, pressing the admin key gets you to the TurboChrom program screen. If the computer has shut off, follow the procedure below:

- Turn on computer
- Go to the TCNav icon
- Login with a "admin" logon and "admin" password
- Status may start with "Port Comm Error" in red, will become "No Method" in red after 2 -5 min. If not, shut off computer and start again

The data acquisition system uses "Sequences" and "Methods". The naming convention for sequences is as follows:

"Today's date" as in Mar16

To find the sequences (and to save them correctly), open the Sequence Editor (Build Sequence) and go up one directory level. It is in the folder marked "Tea". The entire hierarchy from the C-drive is as follows:

C:PenExe/TcCS/Ver6.3.2/Tea

Copies of all the caffeine files (tea.seq, tea.mth, and tea.rpt) are found in the Templates directory:

C:PenExe/TcCS/Ver6.3.2/Templates

All the working caffeine files must reside in the Tea directory.

Once all the samples and correct data files are entered into the sequence file, it is saved and the Sequence Editor MUST be closed prior to loading the sequence by initiating Instrument Setup on the main screen. (This also goes if either the Method Editor or the Report Editor is opened. They must be closed for the data system to initiate setup.)

Click Instrument Setup and browse the Tea directory for today's date. If it exists, load it by pressing "select" and be sure that lines 1 through 5. While there are 13 lines in the sequence (a standard, 4 crude caffeine, 4 caffeine recovery, and 4 recrystallized caffeine) only the first group are listed for loading. Approximately 30 sec or less after the Instrument Setup is closed, the "status" screen will change color to grey-blue and say System Ready. If this does not happen, confirm that the sequence, method, and report editors are closed. Once closed, the Instrument Setup must be re-initiated as above.

When other samples arrive, confirm the flask used for their prep and only load those lines during setup (e.g. starting with line 6 and ending with line 9).

NOTE: Crude caffeine is set up for a 25 mL flask, which is entered as a Dilution Factor of 25 in the sequence (column farthest to the right) and both caffeine recovery and recrystallized caffeine for a 100 mL flask. If different volume flasks are used, that info must be entered into the sequence before it is loaded

If today's data does not exist, you must generate today's sequence.

Generating Today's Sequence

If there is no sequence with today's date, load a previous day's sequence and proceed as follows:

- Find the column marked "Data" and click on the first line
- On the toolbar go to Change and select Smart Fill
- Start on row 1
- End on row 13
- Make Base File Name today's date with no spaces (e.g. Mar29)
- Make starting number "1"
- Save As today's date

Running Samples on the HPLC

Once the system is ready, press the autozero button on the detector and press the green start button on the autosampler to begin the run.

Click on Real Time Plot to open a screen that allows viewing of the chromatogram as it is generated.

Open the Sequence Editor and the current file as "Read Only" to be able to see what the upcoming sample is.

NOTE: If you miss inputting the correct number of injections during Instrument Setup, after the last autosampler injection, go to "Run" on the toolbar and "Clear Setup". Go to the instrument "PE-LC" under "Lab" to clear and return to the red No Method status.

A printout of the injection will occur after each injection.

During the HPLC run you can view the developing chromatogram using Real Time Plot. F5 key makes the chromatogram bigger (mV scale is smaller). The F6 key makes the chromatogram smaller (mV scale is larger). The F7 key set the baseline back to the normal position. NOTE: If the F7 key is pressed while the chromatogram is above baseline, the peak will track below the visible area. Continuing to press F7 will put the chromatogram back on scale as the peak returns to baseline.

