CHEM 333: Lab Experiment 5:
INTRODUCTION TO CHROMATOGRAPHY;
Thin Layer and High Performance Liquid Chromatography:

PRE-LAB ASSIGNMENT!!! : The purpose of the first part of today's experiment is to determine the caffeine content of a beverage sample. Here is your chance to get involved and find out how much caffeine is in that stuff you drink. Which soda has the most caffeine? Red Bull™ really have more caffeine of normal cola? Tea have more caffeine than coffee? Which of your favorite coffee shops makes the most potent cup? Now is your chance to answer these questions and more that have plagued student-kind for centuries! (After all this information might come in handy as you study for your finals!) <u>YOU ARE ALL TO BRING SAMPLE OF THE CAFINATED BEVERAGE OF YOUR CHOICE TO THIS WEEKS LAB.</u>

<u>Pre-lab Reading</u> Chapters 7, 8 and 9

> *"This lab can actually be kind of fun (in a geeky sort of way). The techniques you will use this week are "analytical" and you will be able to make actual determinations of caffeine levels in beverages. Next week you will learn to perform chromatography on a preparative (though still small) scale."*

<u>Liquid Chromatography</u>

Liquid Chromatography is a powerful technique to separate and purify organic compounds. This week's laboratory deals with Thin Layer Chromatography (<u>TLC</u>) and High Pressure Liquid Chromatography (HPLC), which are used as <u>analytical</u> tools to analyze samples. In next week's laboratory you will use column chromatography. Column chromatography is a <u>preparative</u> method used to isolate or purify larger amounts of material (from milligrams to kilograms).

After reading the introductory information in the text, it is very important that you understand chromatography's relationship to the polarity of organic compounds. Be able to recognize polar functional groups, especially those that can form hydrogen bonds with the solid phase (*i.e.* 1° and 2° amines, alcohols, and especially carboxylic acids). Make note of Table 7.1 for its ordering of solvent polarities.

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (or High Pressure Liquid Chromatography) is a very sensitive analytical technique. It has the capacity to be employed as both an analytical (milligrams) and preparative (hundreds of milligrams) tool for separating mixtures of closely eluting compounds. The chromatogram of materials separated can also be integrated to give quantitative data on the amounts present in the original mixture (given proper calibration). Two types of detectors are widely used for monitoring separation. An absorbance detector measures the absorbance of a particular wavelength of ultraviolet light, usually tuned for aromatic compounds. The alternative, is a detector that monitors changes in the refractive index of the solvent being eluted. This allows for detection of compounds that may be transparent to UV radiation.

A variation of standard chromatography frequently appears with HPLC: the use of "reverse-phase" columns. The solid support in a reverse-phase column is coated with a greasy, non-polar substance (usually a long-chain hydrocarbon mix tethered to the support through a silicon linkage). In this way, the polarity rules are more or less reversed: non-polar compounds get bound to the column and move slowly, while polar compounds move more quickly with the solvent. More polar solvent mixtures (often aqueous) are usually employed.

Chromatography Part I. Extraction of Caffeine from Caffeinated

Beverages
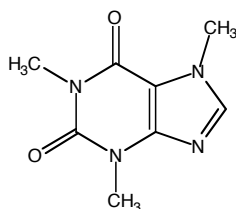
Caffeine

The purpose of this experiment is to determine the caffeine content of a beverage sample. You must bring your favorite beverage sample with you to lab. This lab uses reverse-phase chromatography: the stationary phase is non-polar and the eluting solvent is polar.

There are three steps: 1. Extraction, 2. Sample Preparation, 3. analysis by HPLC.

Extraction:

In a 125 mL separatory funnel decant 40 mL of your sample beverage, which
contains caffeine. (CAUTION! If you beverage is hot, you must first cool it
down to room temperature!) --YES WE ARE SERIOUS- Don't even
attempt to do this step with a hot beverage!!

Add 20 mL of methanol. Shake the funnel and release the pressure by turning
the stopcock.

Add 20 mL of dichloromethane and shake the funnel to release the pressure.
Repeat again with another 20 mL of dichloromethane and combined
both organic layers.

Dry the organic layer over sodium sulfate. Filter the solution and setup a plug
of normal phase silica in a column.

Sample Preparation:

Decant the solution to the column and apply gentle air pressure using an air
hose. [Note: this is to remove compounds from the beverage that could
damage the reverse-phase HPLC column.] Place the collected fractions
into a 100 mL RBF and concentrated *in vacuo*. Dissolve the solid with 1
mL of methanol. Filter the solution.

Analysis:

Transfer the solution into an HPLC vial. Analyze the sample by HPLC (the T.A.
will assist you in this portion of the experiment). If necessary, cap the
vial and save the sample to analyze the next lab period. Use standard
curve to calculate the amount of caffeine in the beverage you chose.

Safety Notes:

♦As you should know food or drink are **not** allowed in lab. Once the
beverages are in the lab, they are to be considered chemicals. The rest
of beverage must be properly dispose in the solvent waste container.
The student cannot drink the remainder of the beverage. Soda cans
should be dispose in the solid waste container and should not be place
in the normal trash

♦ When using the separatory funnel make certain to vent away from your
classmates. Release the pressure inside the hood.

♦ Caution should be taken when using dicholoromethane due its chemical
properties. Wear gloves.

♦ When applying gentle air pressure make certain that the column does not
become a closed system. The columns are not designed to take that sort
of pressure. The T.A.s should demonstrate proper technique to students.


Chromatography Part II. Thin Layer Chromatography (TLC) analysis of analgesics

Your text has a good discussion of thin layer chromatography. Note the
definition of the **R$_f$ value**. Note that R$_f$ is unit-less, unlike the retention *times*

used for GC or HPLC. The most common uses of TLC are monitoring reactions, *i.e.* the disappearance of starting material and appearance of product(s), and as a companion to column chromatography (where fractions eluted from a column are monitored for the presence of compounds. TLC will be employed for both these purposes in future labs!

Procedure:

You will be given plastic TLC plates with silica adsorbent (containing a fluorescent dye). Your T.A. will show how to prepare your spotters (the most difficult part of TLC for novices). Note that if you are careful to rinse your spotter between samples, it is not necessary to have a *new* one each time: two or three good spotters should be enough. The solvent system we will be using is butanol–ethyl acetate–methyl isobutyl ketone (2:9:9). You will be given an unknown sample, a chloroform solution containing two of the three analgesic compounds shown below. This sample will be analyzed by TLC.

| Acetaminophen | Aspirin | Caffeine |
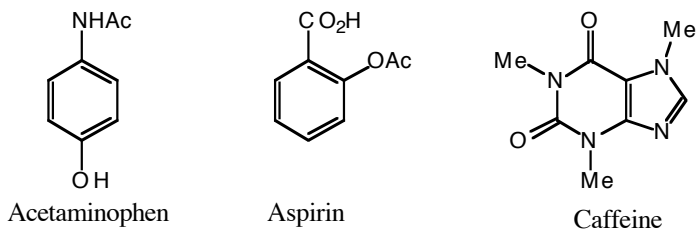
For these known compounds, share the materials with your bench mate. Place approximately 10 mg of each of the references in the wells of the depression plates provided, and dissolve the samples in a few drops of chloroform. Also, make up a small amount of a mixture of all three compounds. Be sure to keep your samples labeled!

Spot your first TLC plate as shown in Fig. 8.2 on page 74. These plates are easy to mark with a pencil. A light mark at either side about 1 cm from the bottom of the plate should do to mark the origin; if you wish you can make light scratches at the bottom to mark your "lanes." Fill your spotter with the desired solution, then carefully touch it to the TLC plate at the origin as shown. (You may wish to make a few practice spots on your plate: simply use chloroform solvent, and spot at the top of the plate where your marks will not interfere with the elution). Spot carefully to keep the spots as small as possible. Do <u>not</u> put down the entire contents of your spotter at once. Rather, spot, allow it to dry, and spot again at the same place. Repeat this several times. You may need to refill your spotter and spot again in the same place to ensure that you have sufficient sample on the plate. To prepare your spotter for the next solution, rinse the spotter with 2 or 3 dips in chloroform (spot on a paper towel to clear). Then continue with the next sample. If your spotter clogs, break off the end and try again, or use a new one.

Elute your plate in the jars provided. Put enough eluting solvent in the jar to cover the bottom to a depth of 5–6 mm. Make certain that it is not so deep as to cover the origin on your plate! To keep elution even, place a filter paper around the inside of the jar, dipped in the solvent, to help saturate the jar with solvent vapors. The plate will take a few minutes to develop. When the solvent is within 1 cm from the top, remove the plate and mark lightly to indicate the solvent boundary. Examine the chromatogram under a UV lamp and sketch the appearance of the plate in your notebook, indicating the location and approximate size of the spots and any distinctive colors. After this examination, place the plate in an iodine developing chamber for about 30 seconds, then remove and again record the appearance. Identify the spots in the chromatogram, including the spots in the lanes of your unknowns and measure the R_f of the major component in each sample.