Introduction:

Chromatography is a group of processes that are used in analytical chemistry to separate a mixture into its individual components. There are several types of chromatography the two most commonly used are probably gas chromatography (GC) and high performance liquid chromatography (HPLC). In general, chromatographic systems have four main components: a column or capillary, a stationary phase (non-moving) in the column or capillary, a mobile phase that flows through the column or capillary and a way to detect compounds at the end of the column or capillary. The basic principle of chromatography is that different compounds in a mixture spend different amounts of time in the stationary phase and therefore, take different amounts of time to reach the end of the column or capillary. The amount of time a compound spends in the stationary phase depends on one or more chemical or physical properties of the compound including, but not limited to: size, molecular weight, polarity and shape.

HPLC can be used to separate compounds or “analytes” that are in solution. HPLC has many different subcategories, but the most common category, reversed phase, uses a hydrophobic (greasy, water-hating) stationary phase and a hydrophilic (water-loving) mobile phase. This tends to separate compounds in the mixture based on polarity where highly polar compounds elute before highly non-polar compounds. When a compound leaves the column or “elutes” it then passes through a detector that senses the presence of the compound and registers a response. The detection could be based on many properties of the solution including pH, refractive index, conductivity, fluorescence and most commonly, UV-vis absorbance. A plot of the detector response versus time is called a chromatogram. The time that a compound elutes (shows a peak on the chromatogram) from the column can be used to identify the compound for qualitative
analyses. The area of each peak can be used to determine the amount of compound present in the original mixture (quantitative analysis) as long as a calibration curve is prepared first relating the peak area to the concentration of the analyte.

**Purpose:**

There are very stringent labeling and testing parameters for over the counter (OTC) pharmaceuticals to ensure that each pill or capsule contains exactly the amount of active ingredients that it claims. There are several ways to accomplish these tests, but a very useful procedure is to utilize HPLC with a UV absorbance detector. The HPLC can be used to separate the various chemicals in the pill and the UV detector can be used to quantify each component with a calibration curve. The UV detector can “see” the chemicals shown below due the “conjugated” double bonds in the rings. The purpose of this lab is to determine the amount of each active/inactive ingredient in OTC pain relieve medications.

![Chemical Structures]

- Acetaminophen
- Acetylsalicylic Acid
- Caffeine
**Instrumentation:**
- Buck Model BLC-10 Isocratic High Performance Liquid Chromatograph (HPLC)

![Image of HPLC](image)

**Supplies:**
- Caffeine, acetylsalicylic acid and acetaminophen (to prepare standards)
- HPLC Grade Methanol and Water (for mobile phase and sample dilutions)
- Glass Vials for sample preparation
- Volumetric pipet (1 ml)
- Volumetric flasks (100 ml)
- 50 μl syringe

**Samples:**
- Obtain samples of several OTC medications that contain acetylsalicylic acid and/or acetaminophen (many of them may also contain caffeine).

**Safety:**
- Avoid exposure to methanol (it could be irritating to the skin and eyes). Wear gloves when preparing samples and handling the mobile phase. Wear goggles at all times in the lab.

**Procedure:**

**Mobil Phase Preparation:**
- Make at least 1 L for each time this experiment is to be run all the way through.
- In volumetric flask add methanol to make 30% total volume (i.e. to make 100 ml’s mobile phase add 30 ml’s of methanol to the volumetric flask)
- Add water to bring the solution to volume in the flask.
- Place the cap on the flask and shake well.
- Remove cap (When methanol and water combine the total volume is less than the addition of the original volumes)
- Add water again to mark and repeat shaking process (This is done to improve reproducibility of the mobile phase preparation.)
- Filter mobile phase to remove and particulate matter that might be present.
- Degas the mobile phase (sonicate or sparge with helium until all bubbles have been removed)
**Instrument Setup:**
- Follow the specific instrument procedures in the manual for initial instrument setup
- Turn on HPLC
- Place solvent line with frit on end into mobile phase (30:70 methanol/water)
- Turn on pump and run mobile phase for at least 20 minutes at 1 ml/min and monitor baseline to make sure there is no “drift”.

**Standard Preparation:**
- Mass 40 mg of acetylsalicylic acid into 100 ml volumetric flask (record exact mass) and add distilled water to the mark. This will produce a 400 ppm acetylsalicylic acid standard.
- From the 400 ppm standard produce 100 ml’s each of 50 ppm, 75 ppm, 100 ppm and 125 ppm standards.
- Repeat for all chemicals of interest (caffeine and acetaminophen)

**Sample Preparation:**
- Mass each pill that is going to be used as a sample and record the value.
- Mass approximately 0.020 grams of each sample separately.
- Transfer to 150 ml beaker and dissolve in 100 ml’s of mobile phase.
- Place stir bar in solution and place beaker on stir/heat plate.
- Stir and heat gently (no more than 80° C) for at least an hour.
- Filter all samples

**Chromatographic Data Collection:**
- When system is ready, use syringe to obtain ~ 50μl of sample or standard making sure no bubbles are present in the barrel of the syringe. Make sure the injector knob is pointing to “Load”, place needle in injection port and depress plunger on the syringe. Repeat twice
- Turn injection port to “Inject” and then quickly back to “Load” (this should automatically send a signal to the Peak Simple software to begin data collection). Remove syringe and rinse at least 3 times with methanol after each injection.
- Allow separation to run for 15 min and record and save results. (The time may be reduced if all peaks are eluting well before 15 minutes.)
- Note the elution time (time at the center of a chromatographic peak) of the major components.
- Repeat each sample/standard three times.
- Dispose of all waste in appropriately labeled waste containers.
- Use Peak Simple to integrate the area of the peaks for all of the standards and the samples.
- Save and record all integration data.

**Calculations:**
- Use the area of all standard peaks to produce calibration curves for each chemical of interest.
- Using the calibration curve, determine the concentration of the chemicals (acetylsalicylic acid, acetaminophen and/or caffeine) in each of the diluted samples.
- After determining the concentrations of chemicals in the diluted samples, calculate the concentration in the original sample.

**Questions:**

- Are you certain that the peaks from the samples are only due to one component? Could there be other molecules that are eluting from the column at the same time? What would be a way of testing to see if this was the case?
- If you weren’t able to separate the peaks that you were interested in (poor resolution), what could you do to improve the separation?