# High-Performance Liquid Chromatography (HPLC)

# with UV-Vis (Diode-Array) and Fluorescence Detection

## PRELAB:

Questions to be answered before doing the experiment. **The answers are due at the beginning of each experiment without exception (the questions are for credit and some may appear on your final exam).**

1. In a few sentences, describe how compounds are separated by HPLC.
2. What is the difference between reversed-phase (RP) and normal-phase (NP) liquid chromatography in terms of stationary and mobile phases used?
3. In a few sentences, describe the meaning of diode array detection.
4. Why in this experiment are we using a fluorescent detector?

## Objectives

- To get acquainted with a general setup of an HPLC system.
- To develop a method for separating a mixture of caffeine and quinine by reversed-phase (RP) HPLC.
- To probe the advantages of a diode array detector.
- To investigate the limits of detection of quinine and caffeine reached and the sensitivity attained by absorbance and fluorescence modes of detection.
- To quantify caffeine and quinine in unknown samples using internal standard and external standard methods.

## Introduction

High-performance liquid chromatography (HPLC) is a chromatographic technique used for efficient separations and analyses of a mixture of components in a liquid phase. It falls under a broad category of liquid chromatography (LC). A sample in solution is introduced into a column packed with chromatographic media (stationary phase). Differential partitioning of the mixture components between a stationary phase and a mobile phase (the elution solvent) takes place, resulting in differential retention and elution of a mixture of components from the column. The polarity of the stationary phase is different depending on the length of the non polar components on the silicone backbone. A C18RP column is less polar for example than a C12RP column. The use of a high-pressure pump to move a sample through a tightly packed column is the distinguishing feature of HPLC and UPLC.

There are several modes of liquid chromatography. These are generally defined according to the specific types of interactions responsible for separation. In this experiment, we will employ reversed-phase (RP) liquid chromatography, where polar mobile and non-polar stationary phases are used. Here, the separation is based primarily on hydrophobic interactions between a sample

and a stationary phase. Mobile phase composition (pH, organic solvent used, % organic solvent) is the most important factor in optimizing reversed-phase LC separations, as it allows varying the degree of retention of a given analyte on a particular column.

Quinine and caffeine are both naturally occurring alkaloids. At one time, quinine was a common anti-malaria drug and today it is one of the main ingredients in tonic water (it is responsible for the bitter taste). Caffeine is the most widely used stimulant in the world and is found in a broad spectrum of commercial beverages. These two compounds therefore, have both historic significance, as well as current social relevance.

It was found that the standard mixture of caffeine and quinine at low pH fluoresces at 448 nm ($\lambda_{emission}$) when irradiated with light at 347 nm ($\lambda_{excitation}$). One initial outcome of this experiment is to determine which of the two compounds is responsible for the observed fluorescence of the mixture.

**WEEK 1:**

## Procedure:

Open "Galaxie" program. Enter the following:

- User Identification: "Students"
- Group: "Students 3590"
- Project: "Labs"
- Password: " Galaxie15"

When the program is loaded, click on the *Systems* tab at the lower left corner of the screen. Check *Both detectors* on top left of screen. Click on the *Both detectors* tab at the top of the right-hand section of the screen. You are now at the systems control screen displaying four data channels (top) and a scheme of the system setup (*Status Overview*) at the bottom.

**Instrument Startup.** Click on the *210-218* button which is the pump control screen. The bottle connected to pump A (top pump) contains TEA (triethylamine 0.25%) and phosphoric acid (to bring the ph to 3), dissolved in filtered (0.2 um) Milli-Q water. The bottle connected to pump B contains filtered (0.2 um) acetonitrile or methanol. With *Elution* option highlighted, click on the *Flow* button. Set *Flow* to 1.0 mL/min, leave *Ramp* at 1.0 min, and set *% B* to 10 (pump A will automatically read 90%). Click OK to activate the pumps. The column should be a C12 RP column. You will have to verify which RP-HPLC column is being used in the instrument.

Click on the *363 button*. Press *Lamp On* button to turn on the Xe lamp inside the detector. Click on the *335* button. Turn on the deuterium ($D_2$) lamp by pressing on the "*lit light bulb*" button. At this point, all necessary system components should be on. The system needs about 15 minutes to equilibrate (pump pressures, UV lamp, etc.), so move on to building a method for your first run.

## Materials

Methanol – in reagent bottle connected to the lower pump (channel B).
Triethylamine solution: (TEA made to pH 3 with phosphoric acid, connected to the upper pump, channel A).

**Stock standards (dissolved in M-Q water):**

Caffeine (1 mg/ml)
Quinine (5 ug/ml)
Theophylline (1mg/ml).

Make up the 2 mixtures below and run (after making your method and sequence) determine retention times and which detection method (fluorescence vs. Uv absorption) works for the three components. Use 0.25% TEA pH in 10% MeOH to prepare the dilutions. Prepare the mixtures in the 10 ml vials or tubes and then transfer to HPLC vials. **You need a volume of 1-1.5 mL in the HPLC vial. Get these samples running right away to see if everything is working. Then prepare your samples for overnight run.**

- **Mixture 1** (dissolved in 0.25%TEA,10% MeOH, ph=3.0)
  40 ug/ml caffeine (dilute stock 1 to 50, i.e. 0.1 to 5)
  0.05 ug/ml quinine (dilute stock 0.1 to 10)

- **Mixture 2** (dissolved in 0.25%TEA,10% MeOH, ph=3.0)
  20 ug/ ml caffeine
  20 ug/ml theophylline
  0 .05 ug/ml quinine

**<u>Standards for tonic water quinine determination</u>.**

Prepare a range of 0.01 to 0.1 ug/ml quinine standards in 0.25% TEA, 10% MeOH. It will be used to quantify quinine in tonic water. This standard curve range needs to cover the concentration of the unknowns. The range can be modified if necessary or sample dilution can be changed.

**<u>Standards for caffeine determination with theophylline as internal standard</u>.**

Prepare the following standard curve using the 0.25% TEA, ph 3 solution as diluent.

1. Caffeine 5ug/ml
   Theophylline 40 ug/ml
2. Caffeine: 10 ug/ml
   Theophylline 40 ug/ml
3. Caffeine: 20 ug/ml
   Theophylline 40 ug/ml

4. Caffeine: 40 ug/ml
   Theophylline 40 ug/ml
5. Caffeine: 80 ug/ml
   Theophylline 40 ug/ml

**Quinine Unknown:**

- Tonic Water diluted to 1 in 500 and 1 in 1000 with 0.25% TEA solution, pH 3. You will need to dilute at least twice, for such high dilutions.

**Caffeine Unknowns**:

- Cola, Diet Cola, coffee, diluted 1 to 5 and 1 in 10
- Energy drinks 1 in 10 and 1 in 20

All your unknowns should be diluted in 0.25% TEA, 10% MeOH. Add theophylline to your standards and each of the **caffeine** unknowns so that the final concentrations are 40 ug/ml. Theophylline is your internal standard.

**Build your Methods:**

You will need separate methods for your standards and unknowns. Everything will be the same in these methods aside from an extra "flushing step" in the unknown pump program to remove any compounds which do not elute from the column under gradient conditions. Make the standards method first, then just *save as* and change the pump program for the unknown method.

1. Click file, new method. Include "standards" and your group name somewhere in the title.

2. At the bottom left of the screen, click *control*. 5 icons will appear on the top right, each one represents a compartment of the instrument.

3. Click on the *210-218* button which is the pump control screen.
   a. Change %B on the prerun line to 10.
   b. On the next line, enter 20 min and change the %B to 40.
   c. On next line enter 21 min and %B to 10.
   d. On the next line enter 26 min and %B to10%
   e. This will equilibrate the column back to starting concentration of solvent.
   f. Enter 1 mL/min under the flow column for all lines.

4. Select the *335 icon* (which is the diode array detector).
   a. under *Analog option* for Wavelength 1 enter 204 nm, for Wavelength 2 enter 273 nm.
   b. Select yes for autozero, for both analogue channels.

5. Select the 363 icon (fluorescence detector)
   a. Set excitation to 347 and emission at 448

b. select AZ (autozero). Place mixture 1 and 2 in sample vials, place the vials in the autosampler.

6. File>Save Method

**Unknowns Method:**

7. File: Save Method As and create your unknown method.
8. The following are the changes to the pump program:
    a. Change %B on the prerun line to 10.
    b. On the next line, enter 20 min and change the %B to 40.
    c. On next line enter 21 min and %B to 90.
    d. On the next line enter 22 min and %B to 90%
    e. On the next line enter 23 and change % B to 10%
    f. On the next line enter 29 keep % B at 10%
    g. This will equilibrate the column back to starting concentration of solvent.
    h. Enter 1 mL/min under the flow column for all lines.

9. Save this method as your unknown method.

**Set up Your Sequence:** The sequence is used to communicate between the instrument and auto sampler and allows you to automate your run: For example, you can inject 20 samples sequentially using different methods if you need.

1. Click *File > new sequence* and name the sequence by group and date. Click next.
2. First enter your method(s) in the drag-down menu under the method column.
3. Enter a run name. This is the name of the file you will see when locating your chromatograms. Make sure it's clear to you what data is contained in that run.
4. Enter a suffix, 1 for run one. This is a required field.
5. Enter the injection volume, 10 uL. You can fill down for this field as well.
6. Place your vials in the auto sampler and enter the corresponding # into the vial column in the sequence. There is no rack number, just default value.
7. Save your sequence. Once you are ready to start your analysis press the green arrow. Observe for a few minutes to make sure the run starts. You will hear the auto sampler start moving, and *running* will appear across the top of the sequence.
8. Enter the OVERNIGHT method at end of sequence with a 10 min. run time, a vial number corresponding to your last vial and 0 volume injected. This will stop the pumps and turn off lights on instrument at the end of the run.

**Data Viewing:** To review data collected upon completion of an acquisition, go to *File*, and *Open*, select *Open Chromatogram*. In the Open File window, select your data file (year, month and day). In the left hand portion of the screen, your file name should appear with subsections for each data channel. Click on Data under a data channel of interest to view chromatograms. Clicking on *results* will display the integration data, with retention time and area.

Comparing mix 1 and 2 you should identify which of the three components of the mixtures fluoresce and/or absorb. The diode-array channel will show you the best absorption wavelengths. Comparing mix 1 and 2 should give you the identity and retention times of each component.

**WEEK 2:**

**Examine your data and see if it makes sense. Rerun** any samples that did not give good results. Collect all your data for areas and retention times. You should be aware of literature values and compare to your data.

**Limit of Detection.** Investigate the sensitivity of the two detectors and the limits of detection (LOD), and limit of quantitation (LOQ) based on the noise of the instrument using both UV absorbance and fluorescence detectors. To save solvent and time use the baseline from your solvent and standards where no peaks are found and use the baseline areas (found in the integration table) to calculate the noise. Discuss the LOD and LOQ in your write-up in detail.

After you have run your standard curves compare sensitivities of the two detectors. Determine how sensitive the detectors are for your particular compounds. Estimate sensitivity of the two compounds in UV absorbance and fluorescence detection based on your standard curves.

You will collect all of the data from week 1 from your automated run sequence. You ran a series of caffeine (plus theophylline) and quinine standards. The caffeine standards included the internal standard theophylline. You will use the standards to quantify caffeine and quinine in your respective unknown solutions. Quinine is to be quantified using External Standard method and caffeine is to be quantified using External Standard and Internal Standard methods.

**External Standard (direct comparison) method:** In the direct comparison method, the unknown is quantified by running a series of standards with varying concentrations. A calibration curve relating the detector response to the concentration of an analyte is then constructed. Analyte in the unknown may then be quantified using this calibration curve and the detector response to the analyte in the unknown.

You will quantify quinine in your Unknown Quinine Solution using this method. Open the chromatograms for quinine standards. View results for fluorescence channel only (Star 800 – 363). Write down the raw area counts (μV.min) for quinine peak in each chromatogram. Peak area is the response of the detector to a particular amount of analyte. Open the chromatogram for Quinine Unknown solution and obtain the area for quinine peak there. Using excel or any other graphing software, plot the detector response (y-axis) versus the concentrations of the standard solutions (x-axis). What kind of relationship do you observe? Obtain an equation for the fit and use this equation to calculate the concentration of quinine in the Unknown Quinine solution (Tonic water).

Calculate the amount of caffeine in your Unknown Caffeine solutions by applying the above procedure, the caffeine standards and the data from channel 1 (wavelength 204). Calculate the concentrations based on channel 2. (i.e. wavelength 273). The results should be similar. Report results +,- SD.

**Internal Standard method:**

In this method, an unknown solution is "spiked" with a known amount of some compound (internal standard), which has a known **response factor (RF)** to the analyte under a given set of experimental conditions. The response factor is used to determine the amount of the unknown compound in a sample without the need for a calibration plot.

Use the following equations to calculate the amount of caffeine in your Caffeine Unknown Solution using Internal Standard method:

$$\text{response}_{\text{analyte}} : \quad = \text{area}_{\text{analyte}} / \text{amount}_{\text{analyte}}$$

**(2)** $$RF_{\text{caffeine}} = \text{response}_{\text{internal standard}} / \text{response}_{\text{caffeine}}$$
$$= (\text{area}_{\text{internal standard}} / \text{amount}_{\text{internal standard}})/( \text{area}_{\text{caffeine}} / \text{amount}_{\text{caffeine}})$$
$$= (\text{area}_{\text{internal standard}} / \text{area}_{\text{caffeine}})/(\text{amount}_{\text{internal standard}} / \text{amount}_{\text{caffeine}})$$

(Equation line 2 is based on the data from analysis of the caffeine and theophylline standards)

**(3)** $\text{amount}_{\text{unknown caffeine}} = RF_{\text{caffeine (2)}} \square \text{amount}_{\text{internal standard}} \cdot \text{area}_{\text{unknown caffeine}} / \text{area}_{\text{interna standard}}$

(Equation line 3 is data from analysis of the unknown sample except RF which is from line 2)

Open the Caffeine Standard chromatograms and obtain areas for both theophylline and caffeine in each chromatogram. Calculate RF using data in each chromatogram. How well do these compare across the standard solutions? If the values are similar, use the average as your RF to calculate the amount of caffeine in your Caffeine Unknown Solution. The internal standard and external standard method should give similar results if you have done experiment well.

**Empty your vials and rinse for next week students. Do not leave full vials in auto sampler after your experiment is finished.**

**QUESTIONS:**

(1) If your mobile phase flow-rate was set to 0.5 mL/min and it took 2 minutes for the non- bound material to exit the column, what would be the volume of the column.

(2) HPLC is a non-destructive technique. What advantage is this compared to GC separations?

(3) What are some advantages to an internal standard method for the analysis of samples?

(4) What is an advantage in using a diode array detector compared to a fixed wavelength detector?