Using HPLC and UVVIS for Determination of Caffeine

Samples

Do not bring your own coffee/tee samples. Samples will be given to you by the TA.

Required Reading

In addition to reading this project description and answering questions in the prelab section, you should read Chapter 32 on High-Performance Liquid Chromatography (HPLC) and Chapter 25 on Optical Instruments.

I. Background

Coffee is one of the most popular drinks in the world, especially so in Europe and North America. Tea is just as popular in China, Russia, Japan, and many other countries. Both drinks are prepared by an extraction of organic compounds from ground coffee beans or dried tea leaves with hot water. Different kinds of drinks can be prepared depending on the specifics of the extraction. What both coffee and tea have in common is relatively high concentration of caffeine and other alkaloids, molecules that are believed to give coffee and tea their unique flavor and stimulating properties. Some people prefer drinking decaffeinated coffee or tea to avoid intoxication by caffeine. In the past, decaffeinated coffee and tea were prepared by extraction of caffeine with rather nasty organic solvents (benzene, CH$_2$Cl$_2$, etc) but these days supercritical CO$_2$ extraction or water extraction are the preferred methods for health and environmental reasons.

Dr. Evil’s company Virtucon has been buying coffee and packaging it in beautiful boxes labeled “Evil’s Best Regular Coffee”, “Evilbucks Regular Coffee”, “Evilpeet’s Regular Coffee”, and last but not least “Mini-Me Delight”. Virtucon did not sell the coffee boxes to supermarkets but instead Dr. Evil personally showed up on UCI campus just before the finals week and gave all the coffee away to every student he bumped into. However, the chemistry students believe that something is up. They think that Dr. Evil’s cunning plan is to trick UCI students into drinking decaffeinated coffee instead of regular coffee during the finals week, fail the exams as a result of severe caffeine-deprivation, have to stay in college an extra year, thus triggering a collapse of the California economy. Your group’s mission is to analyze two coffee samples distributed by Dr. Evil and figure out whether they are decaffeinated or not. You must save the UCI students from failing the exams! The future of California depends on you!

II. Introduction

Chromatography is a useful technique for the separation and analysis of complex mixtures. Most chromatographic methods use a stationary phase held in a narrow tube and a mobile phase, or eluent, which carries the dissolved analyte through the column. The mobile phase is forced through the column using pressure. This particular lab uses High Performance Liquid Chromatography (HPLC), also known as High Pressure Liquid Chromatography. Typical pressures used in HPLC are between 30-300 atm.
Chromatograms show the peaks of each component vs. elapsed time traveling along the column. The migration rates of analytes down a column differ based on the degree of interaction with the mobile phase vs. stationary phase, allowing separation of the components in a mixture. The retention time (time for an analyte to pass through the column) allows for qualitative identification, and peak areas allow for quantitative analysis. Each molecule will have a unique retention time in a given column and for a given solvent.

The HPLC instrument in our lab uses reversed-phase partition chromatography, in which the liquid stationary phase (comprised of functionalized organic polymers) is less polar than the liquid mobile phase (mixture of water and methanol or acetonitrile). The more polar the analyte, the faster it is eluted due to its greater attraction to the mobile phase than to the non-polar stationary phase. Caffeine is a reasonably polar molecule (Figure 1), and we expect that it will elute faster when water is used as an eluent and slower when less polar acetonitrile is used. We have selected a mobile phase for this lab that provides a good compromise between the speed of analysis and the degree of separation between different compounds.

### III. HPLC Instrument

Depending on the year, students end up using different instruments for the analysis. Each instrument is briefly described below (your TA will tell you which specific instrument to use and provide further instructions). Each instrument has its own software and its own specifications but they all share a lot of common principles. You should read Chapter 32 of the text book so that you understand the role of the different commonly-used components of HPLC instruments.

#### Agilent HPLC Instrument

The HP (now Agilent) HPLC instrument is a fairly standard HPLC instrument used by hundreds analytical companies around the world. It has the following components.

- Solvent containers are used for storing eluents (A methanol; B: pH=3 phosphate buffer; C: acetonitrile; D: tetrahydrofuran (THF)).
- Degasser removes dissolved air from the solvents.
- Solvent mixer mixes two or more solvents in a user-defined proportion (e.g., 40% A, 30% B and 30% C). In gradient elution, this proportion can be programmed to change during the chromatogram in pre-programmed way.
- HPLC pump pressurizes the mixed solvents and pushes the eluent through the HPLC column.
- Manual injector is a complicated valve that makes it possible to inject several microliters of the analyzed mixture into the flow.
- HPLC column separates the analyte compounds by their polarity.
- UV-VIS diode array detector continuously detects optical absorption by molecules eluting from the column. The chromatogram in this case is the measured absorbance as a function of the elution time. This particular detector has 5 independent channels, which means that it can produce up to 5 separate signals corresponding to 5 different absorption wavelengths in parallel. The user usually picks one or more wavelengths corresponding to the peaks in the absorption spectra of the compounds of interest. Caffeine absorbs strongly at 273 nm, and we will use this wavelength for detection.
- Fluorescence detector (not used in this lab)
The instrument is controlled by a computer. Familiarize yourself with the instructions (next to the instrument) so that you know how to run the instrument.

**Shiseido Instrument**

This instrument has the following parts (see description of different parts above):

- One solvent container (isocratic elusion only!)
- Degasser
- HPLC pump
- Automatic injector. In contrast to the Agilent instrument, where the injection is done manually, the automatic injector in the Shiseido instrument is programmed to automatically withdraw the sample from vials loaded in the autosampler.
- HPLC column
- UV-VIS diode array detector. This detector provides absorbance at one selected wavelength (which is typically set at 270 nm for caffeine experiments).

**IV. Preparation of Caffeine Stock Solutions**

Weigh about **0.2000 g** of caffeine (to 0.1 mg precision), dissolve it in about 200 mL of nanopure water, and qualitatively transfer it to 500 mL volumetric flask. Fill it to the mark. This will result in a 0.400 mg/mL = 400 µg/mL stock solution (label it as C0). It is OK if it is not exactly 400 µg/mL as long as you know the precise concentration.

Use appropriate pipettes and volumetric flasks to prepare the following dilutions. Use this table as a guide for the range of concentrations that you want. You may need to share or borrow volumetric flasks to prepare these solutions.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (µg/mL)</th>
<th>mL of stock:</th>
<th>Dilute to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0 (stock)</td>
<td>400 µg/mL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C1</td>
<td>20 µg/mL</td>
<td>5 mL of C0</td>
<td>100 mL</td>
</tr>
<tr>
<td>C2</td>
<td>16 µg/mL</td>
<td>10 mL of C0</td>
<td>250 mL</td>
</tr>
<tr>
<td>C3</td>
<td>8 µg/mL</td>
<td>5 mL of C0</td>
<td>250 mL</td>
</tr>
<tr>
<td>C4</td>
<td>4 µg/mL</td>
<td>1 mL of C0</td>
<td>100 mL</td>
</tr>
<tr>
<td>C5</td>
<td>1.6 µg/mL</td>
<td>1 mL of C0</td>
<td>250 mL</td>
</tr>
</tbody>
</table>

**V. Preparation of Coffee/Tea Extracts**

You will have two identically looking vials, one containing regular coffee (or tea) and the other containing decaffeinated coffee (or tea). The samples will look identical to the eye but your HPLC and UVVIS analysis will tell you for sure which is which. Our expectation is that the sample prepared from regular coffee (or tea) will have at least an order of magnitude more caffeine in it compared to the decaffeinated sample.

Regular coffee contains roughly 1% of caffeine by weight. Our aim is to prepare an extract with the concentration of caffeine that is roughly similar to that in stock solution C4. You will also prepare an extract from decaffeinated coffee (or tea) in exactly the same way. It is convenient to prepare both extracts in parallel in order to save time.

Weigh about 0.2000 g of your sample (ground coffee or tea leaves) to 0.1 mg precision. Transfer in into a 250 mL Erlenmeyer flask and add 200 mL nanopure water. Bring it to boiling and keep it boiling for a couple of minutes. Filter the solution into a dry clean beaker to get rid of solid residues (use gravity filtration or vacuum filtration if available). If you notice any residues
flowing in the solution, filter it again. Transfer the solution into a 500 mL volumetric flask and fill it to the mark. The expected concentration of caffeine in this solution is \((0.2 \text{g} \times 0.01)/500 \text{ mL} = 4 \mu \text{g/mL}\).

**Even though your extract is now filtered you will need to filter a small portion of it using a syringe filter before injecting it into HPLC.** To do so, fill a plastic syringe with 10 mL or so of your filtered extract. Screw on a syringe filter onto your syringe. Slowly push the content of the syringe through the filter into a clean vial. Label this vial as “filtered coffee extract”. Note that the syringe filters are not reusable. The syringe itself can be used multiple times.

Repeat the procedure with both samples you have.

**VI. Sample Analysis by the UV-VIS method**

Turn the instrument on (Jasco instrument located in RH481). Start the Spectra Manager and choose “Spectrum Measurement”. Insert two identical 10 mm quartz cuvettes filled with nanopure water into the sample and reference compartments of a UV-VIS spectrometer. Run a baseline (baseline → measure → start). Leave the reference cuvette in the spectrometer after that.

**WARNING:** Quartz cuvettes are very expensive ($250 each). Treat them with care. Unfortunately, much cheaper plastic cells are NOT suitable for this experiment because they absorb below 300 nm.

In order to calibrate the UV-VIS instrument and measure the molar extinction coefficient of caffeine it will be necessary to record absorption spectra of several caffeine solutions of known concentrations. Use the UV-VIS spectrometer to take spectra of solutions C5, C4, C3, C2, and C1 (in this order) in the range of 230-600 nm. In each case, rinse the sample absorption cuvette with your solution, fill it, insert it in the sample compartment, and record the spectrum. Save each spectrum as a text file – you are going to need these spectra for your report. (USB port for the data transfer is located on the front of the computer case).

Now record the spectra of both of your coffee/tea extracts. In all likelihood, the extracts will be too concentrated, and your absorbance at the wavelengths of interest (230-300 nm) will be too high (absorbance > 1). If this is the case, dilute the extracts by well-defined amounts using appropriate pipettes and volumetric flasks (e.g., you may want to use 10 mL pipette and 100 mL volumetric flask in order to dilute it by a factor of 10). Record the spectra of your appropriately diluted extracts. If absorbance values in the wavelength range of interest (230-300 nm) is below 1, save your spectra as text files.

**VII. Sample Analysis by the HPLC method**

During this part of the lab you will be injecting different samples and keep your solvent composition constant (for example, you TA may have set it to 10% water; 0% methanol; 90% acetonitrile). Do not change the solvent composition. You will perform the following injections either manually (if you use the Agilent instrument) or with an autosampler (is you use the Shiseido instrument).

<table>
<thead>
<tr>
<th>Injection</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C1</td>
</tr>
<tr>
<td>2</td>
<td>C2</td>
</tr>
</tbody>
</table>
WARNING:  Remember to filter your coffee extracts using a syringe filter. Injecting a poorly filtered sample will ruin the HPLC column. If we catch you carelessly injecting unfiltered solutions your group will lose ALL points for this lab!

Method

• Load an appropriate method. Method is a file that stores all information about different instrument parameters. The following steps will transfer the method parameters to the instrument and start the pump(s).
  o If you use the Agilent instrument load the method called 151L_2011.m. Then execute the “System On” command. This will transfer the method parameters to the instrument. The pumps should start running at that time.
  o If you use the Shiseido instrument open method 2010F_151L_caffeine. Then go to “Control” and chose “Download method”. This will transfer the method parameters to the instrument.

Injection with the Agilent instrument:

• Before every injection, rinse the syringe with your sample solution by filling it and disposing of its content.
• Ignore the “Instrument not ready” message. As long as the pumps are running normally and you see the absorbance being measured, the system should be ready to inject.
• Verify that the injector is in the LOAD position. Inject 2-3 syringes of your sample in the injector in order to first condition and then fill the 20 µL injection loop with your sample. Make sure you are not injecting any air bubbles. Filling the syringe slowly will minimize air bubbles in the syringe.
• When you are ready to run a chromatogram, select “Run Control → Run Method” in the control program. Specify the file name (see below) and subfolder name and press “Run Method”. The system will be waiting for you to inject your sample. (It can wait indefinitely).
• Please give the informative names to each of your data files. We suggest YEARMMDDtXX where YEAR is year, MM = month; DD = today’s date; td = am or pm depending on whether you are in the morning or afternoon section, and XX is the index of your file 01, 02, 03, 04, …. Example: 20111102pm01 – for the first file recorded in the afternoon of November 2, 2011.
• Rotate the valve to INJECT position. Do this in a smooth, rapid motion so you get injection of the sample as a plug onto the column. This will automatically start the data acquisition. WARNING: Always make sure that the manual injector handle is never left HALF WAY BETWEEN THE LOAD AND INJECT POSITIONS!!! This may ruin the HPLC pump.
• Use “View...Online Signals" to observe your diode array detector response.
• Wait for the caffeine peak to come out. Depending on your solvent and column it may take between 1 min and 10 min. Two minutes after the caffeine peak is out or if nothing happened in 10 minutes, stop the run use “Run Control → Stop Run/Inject” sequence.
• Return the lever to the LOAD position only when the run has finished.

**Injection with the Shiseido instrument:**

**WARNING:** If you use the Shiseido instrument with auto injector never open the injector tray during data acquisition. This may irreparably damage the injector needle ($500 item)!

• When you are ready to run a chromatogram, go to “Control → Single Run”. Write down the file name in your lab notebook. The file names are generated automatically by the program.
• For solutions C1, C2, C3, wait for the caffeine peak to come out, then wait 2-3 additional minutes, and stop the run using “Control → Stop Run”. For the unknowns, wait for all peaks to come out; it should take about 10-15 minutes. You may stop the chromatogram after that.

**VIII. Integration of peaks**

After the HPLC instrument is off, you should open the files and integrate your chromatographic peaks corresponding to the elution of caffeine. The integration procedure is instrument/software dependent, and therefore it is not described here. Refer to the usage notes next to the instrument. You should write down the areas for the caffeine peaks in the chromatograms of all of your standard solutions and unknowns. You will need them for analysis later.

**IX. Clean up / Shut down**

**Waste:** All the stock solutions can be poured into the drain without any treatment. HPLC waste cannot be poured down the drain. Notify the TA if the waste bottle is getting full.

**UVVIS:** Rinse the UV-VIS cuvettes with water and put them in storage. Turn off the UV-VIS instrument.

**HPLC:** If you use the Agilent instrument: Leave the injector in the load position. Inject several syringes of nanopure water in it. Put the syringe in the storage box. Turn off the HPLC instrument as described in the instructions; be sure to leave the degasser on. If you use the Shiseido instrument: you do not have to do anything.
X. What to Submit in Your Report

Specific sections, tables, and graphs that need to be included in your report are described below. As usual, turn in your prelab at the beginning (5 pts), and your duplicate copies of all the notebook pages at the end of the laboratory session (5 pts). Remember: your lab will count for nothing if your lab notebook pages are not submitted or contain meaningless information.

1. (1 pts) The **Title Page** should include the title of the project, your name, date, your UCI ID number, your TA’s name, your lab section, names of your lab partners, and the **codes of your unknowns**. State on the title page which of the two unknowns is caffeinated in your opinion.

2. (17 pts) In 1-2 pages of the **Introduction** section, explain the principles of high pressure liquid chromatography (HPLC). Describe the purpose of the basic components of an HPLC instrument: pump, degasser, injector, mixer, column guard, column, detector. Discuss the significance of the nature of the mobile and stationary phases for separating mixtures. Why is it advantageous to do LC at high pressure?

3. (3 pts) In the **Experimental** section, describe only steps that deviated from the above procedure and reasons for the deviations. Do not exceed half-a-page.

4. (5 pts) For the **Results and Discussion** section (not that we are merging results and their discussion together for this lab, which is sometimes done in shorter papers), prepare a graph showing UV-VIS spectra of caffeine stock solutions that you recorded. All the spectra of the stock solutions you recorded should be in the same graph. Label all traces clearly.

5. (10 pts) Measure the value of absorbance at 273 nm from each spectrum. Make a plot of the absorbance at 273 nm as a function of caffeine concentration in mol/L (you will have to convert your concentrations in µg/mL into mol/L). Confirm you’re your data follow Beer’s law. Use a linear fit to get the molar extinction coefficient ε; report ε in L mol⁻¹ cm⁻¹. Also calculate and report the uncertainties in ε estimated from your fit.

6. (5 pts) Make a plot showing absorption spectra of both coffee extracts (appropriately diluted so that absorbance is <1 at the wavelength range of interest). Both spectra should be in the same graph. Label the traces clearly.

7. (15 pts) Use your value of ε to estimate the concentration of caffeine in your extracts. Note that you will need to account for the fact that caffeine is **not the only absorber** in coffee at 273 nm. The figure below shows you how you can estimate absorbance due to caffeine. Our strategy is to estimate the height of the caffeine bump on top of the much larger background absorption by other compounds in coffee. Convert the measured absorbance due to caffeine into the weight fraction of caffeine in coffee (i.e., mg of extractable caffeine per mg of coffee). Do it for both of your extracts.
8. (10 pts) Make a calibration plot of the caffeine HPLC peak areas as a function of caffeine concentration in µg/mL (for your solutions C1, C2, C3). Do a linear fit of your 3 points if they appear to fall on a straight line. Report the slope and intercept, as well as their uncertainties in the correct units.

9. (14 pts) Integrate the peaks corresponding to caffeine in the HPLC spectra of your extracts. Use your calibration to calculate the concentrations of caffeine in your extracts (in µg/mL). Calculate the uncertainties in the concentrations. Convert the results in the weight fraction of caffeine in coffee (i.e., mg of extractable caffeine per mg of coffee).

10. (10 pts) Make a table of your final results that looks like this. Based on your results, state, which one in your opinion is regular and which one if decaffeinated. You will likely get different answers with the UV-VIS and HPLC methods. Explain the difference, and discuss which method is likely to provide more reliable answers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Caffeine weight fraction from UV-VIS</th>
<th>Caffeine weight fraction from HPLC</th>
<th>Decaffeinated or regular?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>XXX±XXX</td>
<td>XXX±XXX</td>
<td>XXXXX</td>
</tr>
<tr>
<td>2</td>
<td>XXX±XXX</td>
<td>XXX±XXX</td>
<td>XXXXX</td>
</tr>
</tbody>
</table>

Your file name must be “HPLC_secXX.yyy” before the upload, where XX is your section index (01, 02, 03, etc.), and yyy is your file extension (doc for Microsoft Word files; pdf for Adobe Acrobat files, etc.). Using other name formats will lead to a loss of 10 points.