Analysis of Caffeine and Chlorogenic Acid in Green and Roast Coffee Beans by Extraction and High Pressure Liquid Chromatography

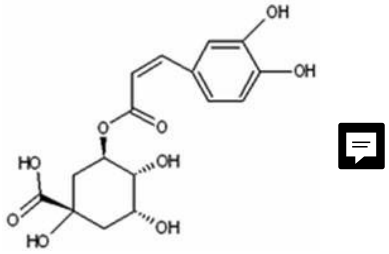
Introduction. When mixtures contain non-volatile or thermally unstable compounds, separation by gas chromatography becomes impractical. In these cases, the separation is often accomplished by high-pressure liquid chromatography (HPLC), a technique which is sometimes also called high performance liquid chromatography to distinguish it from older methods of liquid chromatography. Pharmaceutical analysis and analysis of natural products such as foods are the main areas of application of HPLC. Many natural product compounds and most pharmaceuticals are large molecules whose thermal stabilities are not well suited to thermal volatilization, so gas chromatographic separation is not possible. Yet, because natural products are complex mixtures of many chemical compounds, a very efficient chromatographic separation is essential. Sometimes, it is necessary to isolate a portion of the many components before attempting a separation. The most common form of preliminary isolation is by an extraction.

Any extraction involves equilibrium partitioning of a compound between two immiscible media: the extracting solvent and the sample matrix, as shown in equation 1 below

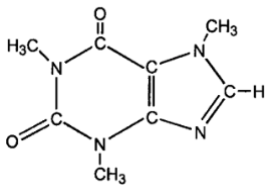
$$C_{solvent} / C_{matrix} = K_{compound} \quad (1)$$

The aim of an extraction is to selectively remove one or more compounds from a complex matrix by taking advantage of differential solubility of substances in the extracting solvent. When the differences in the K values of a mixture are small, many compounds in the matrix will behave similarly in the extraction, and it is sometimes necessary to exploit differences in the way their equilibrium constant K depends on the conditions of extraction. One way to do that is to find a solvent with favorable solubility for the target compound and unfavorable solubilities for the others. If that specific solvent cannot be found, the pH of the extraction can be adjusted or the temperature of the extraction can be changed to alter solubilities.

The aim of this experiment is to evaluate the effects of roasting on coffee composition. You will determine the amounts of two compounds, caffeine and chlorogenic acid, in green and roasted coffee beans. The structures of these compounds are



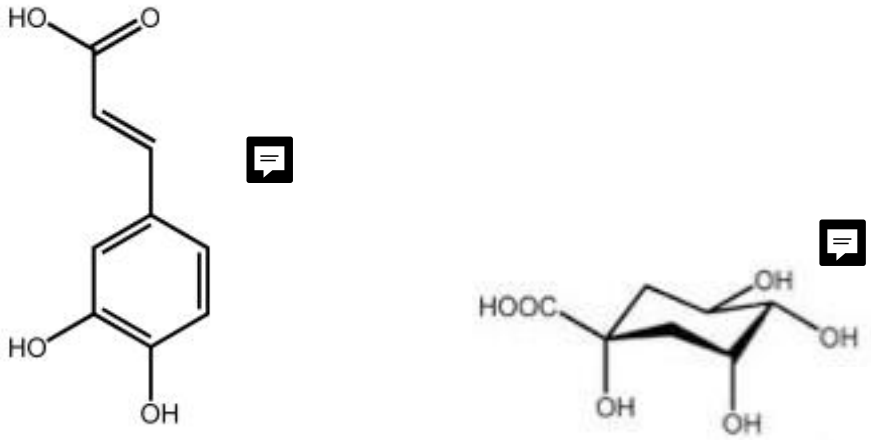
chlorogenic acid



caffeine

Figure 1. Structures of chlorogenic acid and caffeine analytes

Analysis of these compounds could be done directly in the bean matrix, but this approach requires a means of obtaining a representative sample and analysis of that sample using some highly selective method of detection to avoid contributions to the response from similar compounds in the mixture, such as those from caffeic and quinic acids.



caffeic acid quinic acid

Figure 2. Structures of caffeic acid and quinic acid

It is much simpler to take advantage of the chemical properties of the subset of the components in the coffee bean that have been selected for analysis, and to isolate the analytes in a simpler matrix by an extraction step. While many solvents could be used to extract the coffee beans, it is simplest and safest to use water, since both caffeine and chlorogenic acid are water-soluble compounds - as are the possible interferences, caffeic and quinic acid, among others. Because we seek a quantitative extraction of caffeine and chlorogenic acid over a short extraction time, it is necessary to provide heat, so that dissolution is favored strongly. The aqueous extract will be a simpler matrix with far fewer components than the coffee bean, but because interferences can also be present, some additional separation will be necessary before an analysis can be performed. We will separate the extracted aqueous mixture by reverse-phase liquid chromatography and quantitate the caffeine and chlorogenic acid in the extract sample with ultraviolet absorption spectrometry.

HPLC Theory. The theory of all chromatographic methods depends on one basic principle: The samples are separated using the differences in their tendencies to stay with either the mobile or the stationary phase. This tendency is a function of their molecular interaction(s) with the component(s) in those phases. If sample A has a partition coefficient ($K_A=[A]_{stationary}/[A]_{mobile}$) that is smaller than of sample B ($K_b=[B]_{stationary}/[B]_{mobile}$), then A will be more prevalent in the mobile phase, and thus move faster down the column. Better separation can be obtained by selecting the mobile and stationary phases so that maximum difference is achieved between the mobilities of samples. The separation principle is summarized in the following expression:

$$\text{If } K_A < K_B \Rightarrow \text{Retention time of A} < \text{Retention time of B} \quad (2)$$

As can be seen in the expression above, the quality of separation can be improved by adjusting the "attraction" of either the mobile or stationary (or both) phases to interact better with one of the samples. The polarity of mobile phase or stationary phase (or both) is the most commonly adjusted property. In such polarity adjustments for the mobile phase, different percentages of miscible polar/nonpolar (or less polar) solvents are used to achieve mixtures of optimum polarity (e.g., using a more polar solvent forces the nonpolar samples to interact with a nonpolar stationary phase (column), increasing its retention time, and decreasing its mobility).

The most commonly used solvent systems for the mobile phase in a reverse-phase separation are acetonitrile: water and methanol: water. As the mobile phase, you will use an acetonitrile: water/methanol (5%:95%) combination, with the water/methanol solution being an 80% H_2O : 20% CH_3OH mixture containing a small amount (< 1%) of trifluoroacetic acid. In most HPLC instruments, the polar and nonpolar components of the mobile phase can be mixed in specified ratios "on the fly", during pumping of these solvents. This is achieved by computer-aided adjustment of independent pumps (one for each solvent) and results in programmable solvent composition ramps as in temperature-based elution for GC. The change in solvent polarity achieved by the composition ramp decreases the interaction between stationary phase and the molecule of interest. In this experiment, we separate very distinct compounds with different affinity for stationary phase, and we will not need a polarity ramp; instead we will use an isocratic (constant mobile phase composition) separation.

While increasing selectivity though separation of the elution of components in time is a goal in chromatography, there is another way to increase selectivity: when the spectral responses of the components of a mixture are separated spectrally, it is possible to measure one component in the mixture with little to no contribution from other components. The structures of caffeine and chlorogenic acid differ a good deal, and so do their absorption spectra. Therefore, some additional separation of these components can be obtained from taking advantage of their spectral differences. A chromatographic run monitored at a wavelength where the molar absorptivity of one component is large may not detect a second component whose molar absorptivity at that wavelength is very small, even if the chromatographic resolution is low.

In this laboratory you will determine the effects of roasting on the caffeine and chlorogenic acid content of coffee beans. You will quantify the caffeine and chlorogenic acid content of samples of green and roasted coffee beans. To do this, you will extract ground samples of coffee beans with hot water, then you will determine components in the aqueous extract using liquid chromatographic separation with ultraviolet spectroscopic detection using calibration curves for caffeine and chlorogenic acid. Because the caffeine and chlorogenic acid absorb in different regions of the ultraviolet, it is necessary to run standards separately and to measure at different wavelengths.

Differences in the amounts obtained for green and roasted bean samples are indicative of chemical changes in the coffee bean that occur during roasting.

PRE-LAB ASSIGNMENT

Write the answers to these questions in your notebook, and be prepared to show them to your TA at the beginning of the lab period. Be ready to discuss these with your teammates and the TA.

1) Would extraction of the coffee with a slightly basic solution affect the extraction of chlorogenic acid and caffeine? Is using a basic extraction a way to reduce contamination by caffeic acid and quinic acid? What would happen if a slightly acid solution were used for the extraction?
2) As a rule of thumb, before loading the samples to HPLC, you usually must de-gas and filter the solutions you will be using. Explain the reason for this practice.
3) Why is a very small amount of trifluoroacetic acid included as an ingredient in the mobile phase for compounds that act as weak acids or bases?
4) What is the interaction between flow rate and quality of separation in liquid chromatography? If time were not a factor in the lab, would you prefer faster or slower flow for a better separation?

EXPERIMENTAL

Warning! This is a long experiment - probably the longest one of the semester, in fact. You can shorten it and improve your results by coming prepared to lab, by working efficiently and carefully to make your solutions, and by carefully following directions concerning the use of the instrument. If you take up lab time deciding how to make solutions or if you make mistakes in preparing solutions or in setting up the sequence used in the analysis, you may not complete the lab in the time allotted.

You will be given samples of a green and roasted coffee. Prepare all dilutions in the acetonitrile:water/methanol solvent described below, paying attention not to excessively aerate the solutions while mixing (ideally, they should be degassed after dilutions, which we are avoiding due to time constraints). The chromatograph is set to use a mobile phase made from 5% acetonitrile: 95% mixture of water/methanol (80:20 v/v) with a few added drops of trifluoroacetic acid as the mobile phase.

Your TA will instruct you on how to operate the LC instrument and the corresponding software. As has been the case throughout this course, we are not focusing on what buttons to push and how to operate specific instruments, but rather on understanding how the measurement is made and how to analyze and interpret the data that the instruments produce.

It is important to make the necessary solutions promptly, as the chromatographic runs take 10-15 minutes each. You will need to work efficiently to finish this laboratory in the allotted time.

Stock solutions containing caffeine (0.50 mg/mL in acetonitrile-water/methanol solvent) and chlorogenic acid (0.35 mg/mL in acetonitrile-water/methanol solvent) are provided in the hood.

Before you can perform the separations, you will need to find suitable detection wavelengths for the caffeine and the chlorogenic acid. To do that, you will take spectra of the compounds in the mobile phase used in this experiment. Using library spectra to decide on detection wavelengths will not work well here because these absorption spectra can change with solvent composition. Make sure that the UV-visible spectrometer is powered on, and then take a reference spectrum using the acetonitrile:water/methanol mobile phase solution.

Make separate diluted caffeine and chlorogenic acid standards, using 2.00 mL of the stock in each case and diluting each to 10.00 mL with acetonitrile-water/methanol solvent. Add 1 drop of dilute acetic acid to the caffeine solution to put all of the compound in its acidic form, then fill the cuvette with the diluted caffeine standard in the acetonitrile:water/methanol mixture, and record the spectrum. If the absorbances are well over 1.0, make a dilution as necessary. Keep in mind that these spectra are not used for quantitative analysis, so there is no need to get precise concentrations; only the wavelength(s) where the analyte absorbs best will be used later. Print out the spectrum of caffeine. Using the same reference spectrum, repeat this measurement for chlorogenic acid, and again be sure to add 1 drop of dilute acetic acid to the solution to put all of the compound in its acidic form. Print out this spectrum as well.

From the two spectra, choose two wavelengths for the analysis. You should choose one wavelength where the absorbance of caffeine is high and the chlorogenic acid is low. You should choose the second wavelength where absorbance of chlorogenic acid is high and the caffeine is low. This set of detection wavelengths gives maximum spectral resolution of the two compounds.

Because the HP 1090 chromatograph can only measure the response of a single detection channel over a run, it is necessary to make two runs on each standard and on each coffee sample, one using the detection channel you found for caffeine, and one using the detection channel you found for chlorogenic acid. For each measurement, you should set the detection channel at the selected wavelength, and select a 20 nm bandwidth to limit contributions from wavelengths removed from the selected wavelength. In all cases, the reference channel should be set at 550 nm, using a 100 nm bandwidth to reduce noise. One run is made using each detection wavelength by executing an analysis sequence. Because we require two separate detection wavelengths, we will run two different analysis sequences. Since the sequences are set to use samples in the analysis tray, it is necessary to have duplicate samples positioned in the tray, with one set of samples for each sequence. It is important to set these up correctly.

Prepare the standards for the two analysis sequences. Prepare **four** 10 ml volumetric flasks. Then add 0.50 ml, 1.00 ml, 2.00 ml and 3.00 ml of each of the stock solutions. Thus, you have one flask with 0.50 ml of each of the two standard solutions, one flask with 1.00 ml of each of the two standard solutions, and so forth. Label the flasks, and dilute each to the mark with acetonitrile-water/methanol solvent. *Mix all of these solutions well!* For the sequence, only 8 vials are needed.

Check with your Teaching Assistant to determine the correct settings for the pumps, detector and the order in which to load the automated sampler. Ensure that the flow rate is set to 1.0 ml/min, then start the solvent flow. The sampler should be set to inject 1.0 µl samples. You will need to load the auto-sampler in the correct way to perform the two sequences of analyses. Note the order of injections so that you can make sense of the data you collect during each sequence.

In each chromatogram, all detectable peaks should elute within about 5 minutes, but the run will take about 15 minutes to be sure that no carry-over of undetected compounds occurs.

While the set of standard samples is running at each detection wavelength, prepare to extract the coffee samples.

Weigh accurately by difference about 1.00 g each of the ground green and roasted coffee. For each ground coffee sample, do the following: place the weighed sample in a clean 250 mL beaker, and add about 70.0-75.0mL of distilled water and a clean stir-bar. While stirring slowly, heat the water-coffee mixture to boiling on the stirrer-hotplate, boil gently for about 10 minutes, then allow the sample to cool. Filter the cooled mixture through a glass wool plug into a clean 100 mL volumetric flask. Rinse the coffee on the filter with distilled water and add the filtrate to the volumetric flask, then dilute to the mark and mix well.

Prepare one vial for each of the coffee extracts. Take 2 mL of coffee extracts into a 10 mL volumetric flask and dissolved to the mark with acetonitrile-water/methanol solvent. Use 1 mL syringe to filter sample into vial. When the sequences for the standards are complete, run one each of the coffee samples at each detection wavelength using the analysis sequences run on the standards.

Print out and label all 12 chromatographs (4 standards x 2 wavelengths and 2 extracts x 2 wavelengths). Also print out and label the UV-vis spectra you obtained for the caffeine and chlorogenic acid standards.

SAFETY AND DISPOSAL:

Warning: ***Trifluoroacetic acid and acetic acid are corrosive and must be handled with gloves.***

The standards and mixture samples are to be disposed of in the organic waste jug in the instrumental analysis lab.

WRITTEN REPORT:

1. Using the computer software, obtain peak areas for all of the caffeine, and chlorogenic acid standards. **Do not use area percent values!** Do the same for the caffeine and chlorogenic acid peaks detected for the coffee samples. Note that some additional compounds may be present in the coffee samples – these may be the caffeic and quinic acids mentioned above. Then plot the ratio of standard area vs. the concentration of standard in mg/ml.

Obtain least-squares fits for the two calibration curves generated above. Calculate the slopes and intercepts, and also the error in the slopes and intercepts. Use these calibration curves to estimate the caffeine and chlorogenic acid concentrations in the extracts. From the errors in slope and intercept, propagate error to determine the error in the estimated unknown concentrations, and from this value, the number of significant figures.

From the results above calculate the amount (% w/w) of caffeine and chlorogenic acid in the green coffee and the roasted coffee.

Calculate the efficiency of the separation (N) based upon the peak width of caffeine. (See Skoog, Holler and Crouch, Chapter 26 or Harris, Chapter 22.)

7. Turn in your calibration curves, spectra and chromatograms with your result.

Answer the following questions in your report:

1. Why are there more peaks in the chromatogram of the roast coffee extract than in the green coffee extract? Use the structural information given above and the relative heights of the chromogenic acid peak in the green and roast coffee to propose a plausible explanation for what may happen during the roasting process. Can that be used to explain the extra peaks? Explain your reasoning.
2. Nicotinic acid (niacin) is also known to be present in small amounts in brewed coffee as the amide, nicotinamide, which is mostly bound up in the bean as NAD and $NADH^+$ but is partially released upon boiling. Given the structures (look them up if you can't remember them) of nicotinic acid and nicotinamide, do you expect that these compounds would be extracted into the aqueous phase under the conditions used here? At which detection wavelength would you expect to see these compounds in the coffee samples? Can release of nicotinic acid or nicotinamide explain the extra peaks in the roast coffee? Explain your reasoning.