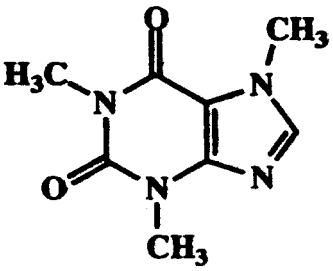
High Performance Liquid Chromatography

I. Introduction

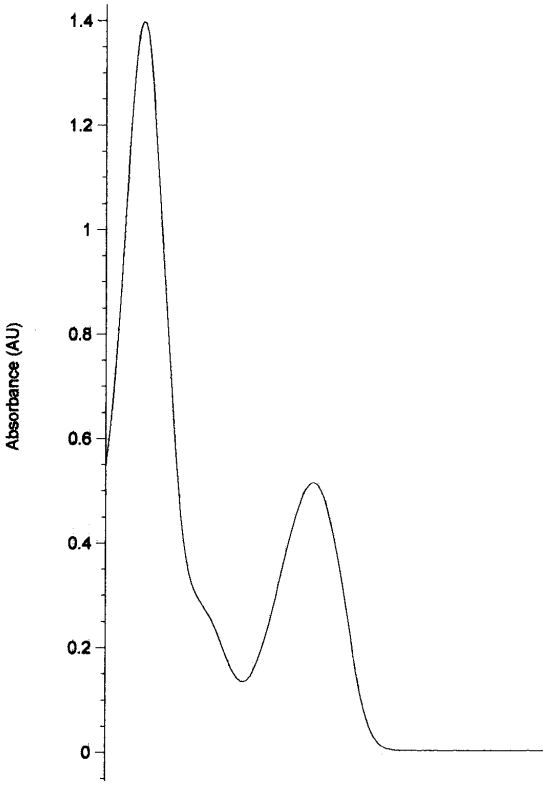
Many beverages such as soft drinks, coffee and tea contain the mild stimulant caffeine ($C_8H_{10}N_4O_2$). The caffeine content varies widely from about 100 µg/mL (100 ppm) in sodas to over 1000 µg/mL in certain types of coffee. In this experiment the caffeine content of a diluted soft drink will be determined using high performance liquid chromatography (HPLC).

The chemical structure of caffeine is shown here. Spectrophotometry provides a sensitive method for the detection and measurement of caffeine. The UV absorption spectrum (see figure below) of caffeine exhibits a pair of absorption bands peaking at 205 nm and 273 nm with a characteristic absorption shoulder between them. Typically, caffeine content is determined by measuring the absorbance at 275 nm. Soft drinks contain a wide variety of substances, many of which absorb UV light at 275 nm. Consequently, the direct measurement of the caffeine absorbance in soft drinks is not possible and one must first separate the caffeine from other components before making the absorbance measurement.



Caffeine

The caffeine separation is easily achieved using HPLC. Liquid chromatography can be divided into several types, including normal-phase (e.g., a silica or alumina column), reversed-phase and ion exchange. Reversed-phase partition chromatography uses a non-polar organic coating on a silica structure for the stationary phase. The non-polar coating is commonly formed by reacting an organochlorosilane with the OH groups on the silica surface. With normal-phase HPLC, the most commonly used solvents are hexane, isopropanol or THF, whereas a more polar mobile phase such as methanol/water or acetonitrile/water is commonly used for reversed-phase partition chromatography. In this experiment, the caffeine separation will be done using a non-polar C_{18} column and a methanol/water mobile phase. A series of caffeine standards that bracket the unknown sample concentration will be measured to construct a calibration curve. A comparison of the caffeine peak area in the soft drink sample compared to those for the standards permits a quantitative determination of the caffeine content.



II. Equipment and Materials

A. Equipment

1. Shimadzu Model LC-10AT liquid chromatograph with Model SPD-10A UV-VIS detector.

2. Tandem reversed-phase cartridge columns (C-18); Pecosphere, 4.6 x 33 mm, 3 micron particles coated with octadecylsilane groups.

3. 25-µL **flat nose** syringe.

B. Solutions (obtain unknown and standard from instructor)

1. Methanol/water mobile phase for elution (filtered)

2. Caffeine standard solution (1000 µg/mL, filtered)

3. Unknown caffeinated beverage (de-gassed and filtered)

4. Caffeinated beverage of choice

III. Procedure

A. Calibration Standards

1. Devise a plan for making a series of dilutions to prepare 4 calibration standards with concentrations from 20 to 100 µg/mL starting with the 1000 µg/mL caffeine standard. Use only your 10-mL and 25-mL volumetric flasks and HPLC grade water for the dilutions. Store each solution tightly capped in a labeled 15-mL vial.

B. Preparation of Caffeinated Beverage

1. Obtain a sample for which you wish to measure the caffeine content. This might be your favorite coffee or tea. If you choose a carbonated beverage, your sample must be degassed before injection. (Discuss how to do this with your lab instructor.)

2. Discuss with your lab instructor how to dilute your sample so that its concentration is in the range of the standards. Filter a few milliliters of your diluted sample using a syringe and submicron filter disk.

B. Analysis of Standards

1. Your instructor will demonstrate how to inject samples into the liquid chromatograph and how to collect, manipulate and print data using the computer interface.

2. Make sure that the method **321L_caff** is loaded. This method will run the system with the methanol/water solvent at a flow rate of 0.80 mL/min and a total run time of 2.5 minutes. **Note: Do NOT save any changes to this method if prompted to do so by**

3. Click on the blue arrow on the Tool Bar and enter the sample ID, then click START. When the "waiting for trigger" message starts flashing at the bottom of the window, load the injection port with at least 3-4 syringe volumes of the highest concentration caffeine standard. When the injection loop (20 µL) is properly flushed and filled, the sample can be injected. Leave the inject port control in the INJECT position until the run is over. When the run is finished, you must confirm that the baseline drawn for the caffeine peak is appropriate, and, if necessary, draw a manual baseline (your instructor will explain how this is done) for the caffeine peak before printing out the results. Print out a copy of the Area % Report by selecting CUSTOM REPORT under the METHOD menu, then with the cursor in the chromatogram window, RIGHT click and select PRINT. Note the retention time (t_R) and peak area for caffeine.

4. Repeat step B.3 for each of the other standards.

C. Analysis of Unknown and Beverage

1. Repeat step B.3 for the unknown solution beverage. On the chromatogram display locate the caffeine peak (How will you tell which one is caffeine?). Verify that is it adequately resolved from the other peaks and that its peak area is in the range of the values found for your standards. The presence of other peaks in the sample chromatogram affects the baseline of the caffeine peak. Draw a manual baseline, as you did for the standard peaks, for the caffeine peak in each chromatogram of the unknown solution and beverage before printing out the results.

2. Make two additional injections of the unknown solution and print out the chromatogram and report for each run.

IV. Calculations

A. Calibration Curve

Prepare a plot of peak area versus caffeine concentration using the data obtained for your standard solutions and fit the data with a least-squares line (remember 0,0 is a data point). Take into account the actual volume delivered by your pipet when determining the standard concentrations.

B. Unknown and Caffeinated Beverage

From the equation for the least-squares line and the peak area for each unknown and beverage analysis, calculate the caffeine concentration (µg/mL) in your samples. Calculate the mean and RMD for your results.