LC/UVS Determination of Caffeine in Beverages

Introduction:

Caffeine $C_8H_{10}N_4O_2$ is one of the most widely used drugs in our society (it is a stimulant). A lot of people (especially here in the Pacific Northwest) find it hard to make it through the day without that little jolt it supplies in the morning. This experiment will give you the ability to measure the concentration of caffeine in your favorite beverage and will also acquaint you with the use of liquid chromatography (LC) in quantitative analytical chemistry.

We also use gas chromatography (GC) in this class to measure relatively volatile compounds. LC makes a nice complementary technique to GC because it can be used on compounds that are not volatile enough to be brought into the gas phase (or that decompose on heating to the gaseous state) including larger, biologically relevant molecules or ionic compounds. The separation in LC is almost always based on polarity and the method we will use in this experiment is referred to as *reversed phase* LC, meaning that the stationary phase is non-polar, the mobile phase is polar, and the more non-polar compounds will be increasingly retained, lengthening their retention time. (*Normal phase* is the opposite with polar stationary and non-polar mobile phases.) We will use an *isocratic* elution in this experiment, meaning that there will only be one multicomponent solvent used as an eluent. You will also note that the physical nature of the column (and the rest of the experiment) is different in LC vs. GC – the column is a relatively short, wider bore piece of stainless steel tubing filled with very fine (~ 5 µm) particles on which the stationary phase is chemically bonded, there are pumps to move the eluent through the column at a few mL/minute (requiring quite high pressures) and the detector is a UV absorbance spectrophotometer where the cuvette is placed immediately after the column. All of this should be apparent by looking carefully at the instrument, which is not very complicated.

We will be basing the quantitation of the unknowns on a regular external calibration and an *internal standard* based calibration and will compare the reliability of the working curves established by the two methods. The internal standard will be one of the methoxybenzoic acids (ortho, meta, or para) all of which are presumed to be absent from most beverages. The unknowns will need to be degassed and filtered before they are injected into the LC and will probably contain significantly more eluates than the standards, resulting in more complex chromatograms.

Apparatus:

50 mL volumetric flasks (10)

0.5 mL, 1 mL, and 5 mL volumetric pipets (1 each)

Instrumentation (See Appendix for Operating Instructions)

Hitachi Model L-6200A Intelligent Pump (with a column, the LC)

Hitachi Model L-4000 UV detector

PC with Analog to Digital (A/D) converter (Measurement Computing, PMD-1208FS) and

LabView Virtual Instrumentation Software.

Solutions Available:

The eluent – 50% methanol in water with 0.5% phosphoric acid (in large eluent bottle)

Caffeine stock solution (~ 200ppm – TA will prepare and provide concentration to ±0.001 ppm)

o-methoxybenzoic acid (a.k.a., o-anisic acid, ~ 200ppm, as above)

m-methoxybenzoic acid (m-anisic acid, ~ 200ppm, as above)

p-methoxybenzoic acid (p-anisic acid, ~ 100ppm, as above)

Experimental Procedure:

(1) Prepare the unknowns: For carbonated beverages, you must first remove the carbonation. If you read this early enough, you can leave the cap off for a couple of days. If not, you can put a small amount of the beverage in a beaker and carefully bring it to a boil on a hotplate. Bring the unknown back to room temperature before you proceed with the filtering.

For all unknowns, you must remove all suspended particulates (solids) before you inject into the LC. You can vacuum filter your solutions using Gooch crucibles and an aspirator/vacuum flask setup.

If the caffeine content is given for your unknown, calculate an appropriate dilution to give a caffeine concentration near the middle of your calibration range (~ 10 – 15 ppm). If the caffeine content is not given, find a good approximation and calculate an appropriate dilution factor. Prepare this dilute solution without internal standard and run a chromatogram, then adjust the dilution factor if needed.

{Note: you must compensate for the dilution to achieve high accuracy and 10% of the points for this report are based on the accuracy and precision (5 points per) of the unknown caffeine determination. If you choose an unknown that has variable caffeine content (coffee, tea, etc.) instead of one with a given content (most sodas and energy drinks) all 10 points will be based on your precision.}

(2) Determine which of the internal standards is most appropriate for this experiment. Prepare 3 - 50mL volumetric flasks: one with 5.0mL of the caffeine stock and 10.0mL of *o*-methoxybenzoic acid , one with 5.0mL of the caffeine stock and 10.0mL of *m*-methoxybenzoic acid, one with 5.0mL of caffeine stock and 2.0mL of *p*-methoxybenzoic acid. Fill to the mark with deionized water. Run a chromatogram of each (see Appendix and notes below) and choose the "best" standard to prepare the rest of your working solutions. (Ask your TA before making this choice if you are unsure of what constitutes the best internal standard. You will be asked to justify this choice in your report.)

(3) Liquid Chromatographic Analysis: Follow the directions in the Appendix for setting up the instrument and the data collection program. Verify that the UVS detector is on and zeroed (detector settings:

wavelength = 274 nm, Recorder range = 0.005, Response = Fast) and that there is eluent coming out into the waste container. Verify that the LC Control.exe program is running and is prepared to capture a chromatogram (if not, fill in a Group and Sample name and click the run arrow button in the upper left hand corner). Move the injector into the "load" position (the injector should be left in the "inject" position, except when you are loading the loop). {If this is your first injection, it's a good idea to run about 5 mL of clean methanol through the loop as if you were injecting it.} Draw some liquid (~1 mL) standard or unknown sample into the injection syringe, insert the syringe all the way into the port on the front of the injector valve and press the plunger until you see liquid coming out the other side of the injection loop. Switch the valve to the "inject" position and press the green Start button on the LC Control program. Default run length is set to 25 min (one of the internal standards will take this long), but you can stop the collection earlier by clicking the red Stop button after all peaks are eluted and a good baseline has been collected. During collection of calibration standards, you can adjust the default run length accordingly.

(4) Preparing the standard solutions: Use the 1.0 mL pipet to place 0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the caffeine stock solution into a series of six 50 mL volumetric flasks. Add your chosen internal standard (2.0mL of *p*-anisic acid or 10.0mL of *m*-methoxy- or *o*-methoxybenzoic acid). Then fill each flask to the mark with deionized water and mix thoroughly. Run a chromatogram of each standard (preferably <u>not</u> in order of concentration) and collect replicates if possible, but you will need to be efficient to obtain data for the unknown, three internal standards, and the full range of calibration standards.

(4) LC Analysis of unknown(s): Prepare an unknown solution with the chosen internal standard using the same dilution factor as you used in step (1) above. Run the LC separation as you did with the standard solutions. If you cannot tell easily which of the peaks is caffeine due to the complexity of the chromatogram, spike the sample using the caffeine stock solution and rerun the chromatogram to verify the peak assignment. If you are careful about the spike, you can also use this run as a "single spike" standard addition procedure as an additional verification of your determination of caffeine in the unknown.

(5) Shutdown: When you are finished running your unknowns and standards, put the valve in the "load" position and run a full 5 mL of clean methanol through the loop to clean it out for the next group. Then put it back on "inject" and turn off the pumping and detector units and empty the waste container. If the eluent is less than half-full, notify the TA. You can leave the data system computer running for the next lab.

Report: A minor report is required for this experiment (50 points); in preparing it, you should consider/complete/discuss the following:

1. Identify the internal standard that you chose and the reasoning behind your choice.

2. Plot the full set of calibration chromatograms (0 – 5.0mL caffeine stock solutions) on a single figure (try to line up the peaks in time). Rationalize the observed peak area behavior. Tabulate the relevant raw peak areas, ratios to the internal standard, and quantitative results for caffeine for both calibration procedures (direct and internal standard).

3. Graph both the direct external calibration results (not ratios, just caffeine peak areas) and the internal standard external calibration (ratios of peak areas vs. concentrations of standards). Comment on the linearity and standard errors of the slope, intercept, and regression for the two procedures.

4. Present the results and uncertainty on the results for the unknown samples for the two calibration procedures.

5. Show the chromatogram for the unknown beverage sample, indicating the peaks for caffeine and the internal standard.

6. If you ran multiple unknowns, comment on the relative concentrations of caffeine in the various beverages.

References:

http://www.forumsci.co.il/HPLC/topics.html

2012-2-6 – JGR, DBA