

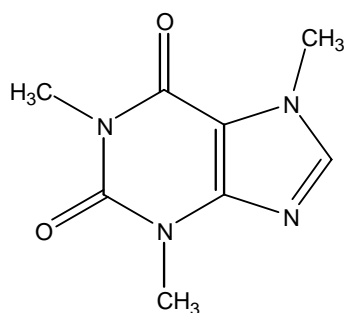
Method Development in HPLC. Determination of Additives in Cola Drinks

Purpose

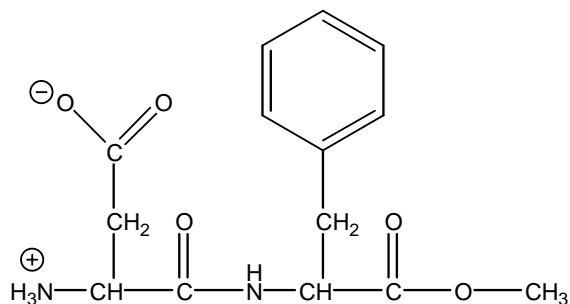
This is a multipart experiment designed to provide experience with some of the basic aspects of method development in HPLC, including optimization of mobile phase composition and pH, flow rate, construction of a calibration curve, and determination of common additives in diet cola drinks.

Background and General Approach

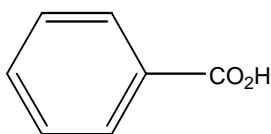
Most diet cola drinks contain caffeine and a sweetener, either aspartame or saccharin. Aspartame is the preferred sweetener, replacing the past use of saccharin. In addition, sodium benzoate is usually added as a preservative. In addition to these additives, standards for HPLC analysis may contain uracil, which is used as a t_o marker.



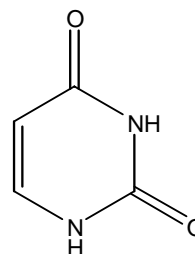
Caffeine
 $pK_a = 3.6 (HA^+)$



Aspartame
 $pK_1(H_2A^+) = 3.1, pK_2(HA) = 7.9$



Benzoic acid
 $pK_a = 4.19 (HA)$



Uracil
 $pK_a = 9.45 (HA^+)$

These are polar organic compounds with ionizable functional groups. Their separation by HPLC is affected by the relative polarities of the stationary and mobile phases, and the pH of the mobile phase. A convenient “secondary” source of information about aspartame can be found at <http://www.chm.bris.ac.uk/motm/aspartame/aspartameh.html>

Development of a successful HPLC method for determination of these additives requires selection of a number of separation parameters. This experiment is designed as a multi-

part exercise that requires up to four 3-hour laboratory periods. The entire method development may be done by a single individual or group of students, or each part may be completed by a different student or group. In the latter case, results obtained for one part must be written up and provided to the groups performing the subsequent parts.

Equipment and Chemicals

Apparatus

- HPLC System
 - Alltech 301 HPLC Pump
 - Rheodyne 7725 injector with 20 uL loop
 - HPLC column, Higgins Haisil 100 C18 3- μ m packing, 3.0 x 100 mm (Manufacturer and details may vary. Student should record details on column used.)
 - Linear UV-Visible detector, model UVIS 200 (variable wavelength, adjustable range and rise time, 1 V/AU integrator output). Flow cell is 316 stainless steel, 6-mm path length, 9 uL volume.
- Data System: LabSystems Chromatography Server and Atlas Chromatography software
- pH meter and standard buffers, pH 4 and 7.
- Magnetic stirrer and stir bar

Chemicals

- Mobile phase solvents
 - Deionized water. This will be placed in one of the mobile phase reservoirs for flushing mobile phase from the column at the end of the period.
 - Methanol (HPLC grade)
 - Acetonitrile (HPLC grade)
- Buffer chemicals
 - Ammonium acetate, solid
 - Ammonia (1 M), acetic acid (1 M), filtered through 0.45- μ m filter
- Standards
 - Uracil, aspartame, benzoic acid or sodium benzoate, caffeine

Supplies

- HPLC mobile phase filtration system
 - Glass filter with clamp, 1-L filter flask, 0.45- μ m filters, tweezers
 - 1L beaker for preparation of mobile phase
- Mobile phase bottles (2) with purging system
- Helium purge gas
- HPLC syringe, 100- μ L, blunt needle

Theory

The goal of any analytical separation method is to provide adequate resolution and quantitation of all analytes of interest within a reasonable time. For determination of

small organic molecules in aqueous mixtures, reversed phase liquid chromatography (RPLC) is widely used because of its high resolving power and wide range of applicability. No single set of conditions is suitable for all types of samples. A particular analysis by RPLC generally requires the identification and optimization of a number of parameters, including column configuration, stationary phase, mobile phase, detection method, sample injection solvent concentration, and calibration method. Each of these is discussed in the following paragraphs.

- **Column configuration.** For much of the 1990s, the standard analytical column was a 4.6-mm i.d. x 250-mm long stainless steel column, packed with 5- μ m porous silica particles with a covalently bonded stationary phase. Modern trends include the use of shorter columns packed with 3- μ m or smaller particles to provide shorter analysis times. While other column formats are in use, the column must be compatible with existing HPLC system hardware, including the pump, injector, detector, and component connections. Potential advantages of high speed, sensitivity, and resolution offered by small format columns packed with small particles may be lost if the other system components are designed for standard analytical columns.
- **Stationary phase.** A C18 stationary phase, or octadecylsilica, is the most widely used in RPLC, offering satisfactory retention for a wide range of organic solutes. Similar phases offering weaker retention include C8, C4 and C1.
- **Mobile phase.** In RPLC, the mobile phase is typically an aqueous buffer with an organic modifier such as methanol or acetonitrile. The aqueous buffer provides solubility and ionization control of polar solutes. Increasing the concentration of organic modifier in the mobile phase generally causes a decrease in retention, especially for less polar solutes. The polarity index P_{AB} for a mixed mobile phase is given by

$$P_{AB} = \phi_A P_A + \phi_B P_B$$

where ϕ is the volume fraction and P is the polarity index for solvent A or B. A change in the mobile phase polarity yields a change in retention in RPLC according to the approximate relation

$$\frac{k_2}{k_1} = 10^{(P_2 - P_1)/2}$$

- **Detection.** The typical UV detector for HPLC is based on measuring the absorbance of eluting solutes, usually at 254 nm. Most UV detectors have an adjustable time constant, or rise time, which is either preset for a given HPLC system, or must be adjusted depending on the system configuration. The time constant is adjusted to produce a chromatogram with a baseline that is relatively free from noise, which producing solute peaks that accurately reflect the change in concentration in the detector flow cell with time. A time constant that is too great (long rise time) may result in distorted peaks, causing loss of resolution and unsatisfactory quantitation.
- **Sample injection.**
 - For the “standard analytical” column, a 20- μ L injection loop is usually used. Larger sample sizes may result in loss of efficiency and resolution due to extra-column band broadening. Smaller diameter columns require the use of smaller injection volumes. For quantitative HPLC, the full-loop

loading technique is preferred, in which total volume introduced from the sample syringe is at least 5 times the volume of the sample loop. Partial-loop loading is an alternative but less precise technique in which a volume not exceeding 50% of the capacity of the sample loop is introduced from the syringe.

- The sample injection solvent ideally should be the same as the mobile phase. In any case, it is best to avoid using a solvent that is stronger than the mobile phase. A strong solvent is one in which the sample is very soluble and for which the sample is weakly retained on the column. In RPLC the strong solvent is the organic modifier
- **Calibration.** A calibration curve usually employs external standards, in which a series of solutions containing the solutes at known concentrations is prepared and injected using the same conditions as those for the sample.

Procedures

Chromatographic Conditions and Procedures

Use the following general procedures as appropriate for each part.

- Mobile phases. All mobile phases should be filtered through a 0.45- μ m filter. Place the mobile phase in the mobile phase reservoir and purge with helium for a minimum of 10 minutes before pumping.
- Flow rate. For the 3- μ m column, use a flow rate of 1.0 mL/min unless directed otherwise.
- Injection. Use the full-loop loading procedure. If the sample injection loop has a capacity of 20 μ L, inject a minimum of 100 μ L to ensure that all residual sample and mobile phase from the previous operation is flushed out.
- Detector settings
 - Wavelength: 254 nm
 - Time constant or rise time: 1 second
 - Span: The span setting is inactive if computer data acquisition is used.

Part A. Composition of Mobile Phase. Effect of pH and Amount of Organic Modifier Using Methanol

The most common mobile phases for RPLC separations are buffered aqueous solutions containing either methanol or acetonitrile as organic modifier. Nonpolar solutes are most strongly retained on the nonpolar C18 stationary phase, while polar solutes may be weakly retained and ionic solutes not at all.

In this part of the experiment you will determine the overall retention for mobile phases buffered at pH 3 and 6, and containing different amounts of methanol as the organic modifier. The information obtained from these experiments is then used to optimize the concentration of methanol and pH.

Preparation of the Mobile Phase

Prepare approximately 1 liter of aqueous buffer containing 10 mM ammonium acetate. Filter the solution through a 0.45 μ m filter using the filtration apparatus provided. Use this to prepare approximately 200 mL of each of the following solutions.

- 20% methanol, pH 3.0 to 4.0
- 80% methanol, pH 3.0 to 4.0 (but same as above)
- 20% methanol, pH 7.0
- 80% methanol, pH 7.0

To prepare these solutions, first adjust the pH by addition of acetic acid or ammonia, then mix the aqueous buffer with methanol in the appropriate proportions.

Obtain the Chromatograms

Standard Test Solutions

Prepare individual standard solutions for uracil, aspartame, benzoic acid and caffeine in 20% methanol at 0.40 mg/mL each.

Prepare a mixed standard containing all four solutes, each at 0.40 mg/mL, in 20% methanol.

Obtain the Initial Chromatograms

For each of the four mobile phases,

1. Flush the column with mobile phase for approximately 10 minutes to equilibrate the column.
2. Inject 20 μ L of the mixed standard and record the chromatogram.

Data Analysis

For each chromatogram, use the chromatography software to integrate and assign retention time for all of the peaks of interest, including uracil. If the software routine identifies too many or too few peaks, consult the instructor for assistance in adjusting the integration parameters.

For each separation condition used, print a report showing the chromatogram and a table of separation parameters, including retention time, width at half height, and efficiency for each peak.

Optimize the Organic Modifier and pH

Based on the results for the initial chromatograms, prepare and test one or more additional mobile phases that will yield an improved or optimum separation. For best results, the range of retention factors for all solutes should be $1 < k < 5$. Baseline resolution of all solutes is ideal, but $R \geq 0.8$ is acceptable for quantitative work if the areas of the overlapping peaks are similar. To predict the appropriate concentration of organic modifier, the equations discussed in the theory section may be employed. Inject individual standards to confirm the identity of each peak.

Report

Prepare a written report that includes detailed information on the conditions used for each separation. Include a sample chromatogram for each separation condition used, and a table listing for each separation condition:

- the retention time for each solute
- the retention factor for each solute
- the resolution for each solute pair
- the efficiency for each solute

Give clear and specific instructions for preparation of the optimized mobile phase. Your report should be completed and given to the individual or group scheduled to perform the next part of this experiment in a timely fashion.

Part B. Composition of Mobile Phase. Effect of pH and Amount of Organic Modifier Using Acetonitrile

Repeat the appropriate procedures described in Part A, to determine the optimum separation conditions using acetonitrile as the organic modifier. As a starting point, use the composition of the optimized methanol/water mixture to predict the percentage of acetonitrile required to yield the same overall polarity. Adjust the polarity if needed to yield a range of retention factors between 1 and 5. Vary the pH to obtain the optimum pH. Prepare a report as described above.

Part C. Analysis of Diet Colas

Preparation of Samples

Obtain samples of three diet colas or similar beverages. With the samples at room temperature, pour each between two beakers several times to eliminate most of the carbonation. Transfer to a labeled screw-cap bottle. Mark the original beverage container with the words "Lab Sample" and keep on hand for reference.

Calibration and Analysis

Prepare a mixed standard containing 0.400 mg/mL each of uracil, caffeine, benzoic acid (or sodium benzoate), and aspartame. Using the optimized mobile phase determined above, inject 20 μ L of each sample and record the chromatogram. Note the response for each standard.

Perform at least one injection of each individual standard to confirm the identities of the peaks.

Prepare a series of standard solutions (a minimum of three) that will bracket the concentrations of each of the solutes in the samples. Do three injections for each standard. Also perform three injections for each of the samples.

For each chromatogram, integrate each of the peaks using the tools available in the chromatography software. Print a report that includes information for each peak, including retention time, peak area, and peak width at half height. Also print a sample chromatogram of the standards, and a sample chromatogram for each of the samples.

Construct a calibration curve and report the concentration of each of the identified components in mg/mL, along with the 95% confidence limits.

Report

Prepare a report that includes the following information

- A summary of what you did
- A description of the details of the separation conditions
- Detailed information on the preparation of the standard solutions.
- A table of data, listing for each injection the elution time and peak area for each solute.
- A calibration curve for each solute that you determined.
- A brief table of results showing the concentration levels of each component with confidence limits.