High performance liquid chromatography (HPLC) makes use of a high pressure pump to deliver a mobile phase solvent at a uniform rate at pressures that are typically from 500 to 5000 psi. The most obvious advantage of HPLC over gravity liquid chromatography is that samples can be separated much more quickly. In addition, samples that are not volatile or that would thermally decompose in gas chromatography can be rapidly and routinely separated in HPLC. Consequently, this powerful analytical method is a great complementary instrument to gas chromatography.

Introduction

HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (termed the mobile phase) that passes through a column packed with particles of a stationary phase. As in gas chromatography, separation of a mixture into its components depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phases. A variety of HPLC separation techniques that utilize different stationary and mobile phases have been developed.

In absorption chromatography, the stationary phase is a solid of a polar nature such as particles of hydrated silica or alumina. The mobile phase and the solute (components in the sample) are in competition for active adsorption sites on the stationary phase particles. Thus, more strongly adsorbed components are retained longer than weakly adsorbed components. Because more polar compounds adsorb on a polar surface to greater degree than do less polar compounds, retention in the column is related to sample polarity. A generalized polarity scale for various classes of compounds is shown in Table 1.

Table 1: Polarity Scale for Various Classes of Compounds in order of increasing polarity and retention.

- Fluorocarbons
- Saturated hydrocarbons
  - Olefins
  - Aromatics
- Halogenated compounds
- Ethers
- Nitro compounds
- Esters ≈ ketones ≈ aldehydes
- Alcohols ≈ amines,
  - Amides
- Carboxylic acids
Retention also can be controlled by the polarity of the mobile phase, which competes with sample components for adsorption sites. Thus, a more polar mobile phase will more effectively displace adsorbed solute molecules and cause the retention time to decrease. Table 2 ranks solvents in the order of their strength of adsorption on the adsorbent alumina. Such a scale is called an eluotropic series. A solvent of higher polarity will displace one lower in the polarity scale.

**Table 2: Eluotropic Series for Alumina and Silica**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Relative Polarity</th>
<th>Solvent</th>
<th>Relative Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Pentane</td>
<td>0.00</td>
<td>Tetrahydrofuran</td>
<td>0.45</td>
</tr>
<tr>
<td>Isooctane</td>
<td>0.01</td>
<td>Acetone</td>
<td>0.56</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>0.04</td>
<td>Ethyl acetate</td>
<td>0.58</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>0.18</td>
<td>Aniline</td>
<td>0.62</td>
</tr>
<tr>
<td>Xylene</td>
<td>0.26</td>
<td>Acetonitrile</td>
<td>0.65</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.29</td>
<td>Propanol</td>
<td>0.82</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.32</td>
<td>Ethanol</td>
<td>0.88</td>
</tr>
<tr>
<td>Ethyl ether</td>
<td>0.38</td>
<td>Methanol</td>
<td>0.95</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.40</td>
<td>Acetic acid</td>
<td>large</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In partition chromatography, the solute is distributed between the liquid mobile phase and a second, immiscible liquid that is coated on or bonded to solid particles as the stationary phase. Compounds that partition more strongly into the stationary liquid phase are retained longer in the column. This type of chromatography is termed normal phase if the stationary phase is more polar than the mobile phase and reverse phase if the mobile phase is more polar than the stationary phase. The stationary phase can be a liquid coated on solid support particles. Bonded phase columns have the stationary phase chemically bonded to the solid support and are the most popular column for partition chromatography. For example, *n*-octadecane can be bonded directly to silica by attachment to surface hydroxyl groups to form what is termed a C18 column. The extent of partitioning of a solute into the stationary phase can be controlled by varying the solvent polarity.

Ion exchange HPLC is based on the partition of ions between a polar liquid phase and a stationary phase with ion exchange sites. The ion exchange sites are typically immobilized in small beads of resin that are formed by a cross-linked polymer. Bonded phase columns in which the ion exchanger is bonded to small particles of silica also are available. Cations are separated on cation exchange resins which contain negatively charged functional groups such as $\text{SO}_3^-$ and $-\text{COO}^-$. Anions are separated on anion exchange resins which contain positively charged functional groups such as $\text{CH}_2\text{N}^+ (\text{CH}_3)_3$, a quaternary ammonium ion. Separation is based on ions partitioning into the ion exchange phase to varying degrees. The selectivity of a resin for an ion is determined primarily by the charge on the ion and its hydrated radius. Resin affinity increases with increasing charge density. A schematic drawing out a typical high performance liquid
The chromatograph is shown in Figure 2.2.1.

The apparatus consists of a container of mobile phase, a pump capable of pressures up to 4000 psi or greater, a valve for injecting the sample (usually 10 to 500 μL volumes), the column (sometimes thermostatted), a detector, electronics associated with the detector, and a recorder.

**Figure 2.2.1:** Schematic Diagram of a High Performance Liquid Chromatograph. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column or guard column, (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector. Created by Yassne Mrabet.

UV-visible absorbance is the most commonly used mode of detection. Such detectors enable the effluent from the column to flow through an 8 to 10 μL spectrophotometric cell for detection of compounds at a particular wavelength (often in the ultraviolet, < 400nm, where essentially all organic molecules absorb). Electrochemical and fluorescence detectors often are used to achieve lower detection limits. The other commonly used detector is based on measurement of the differential refractive index.

A typical liquid chromatogram is shown in Figure 2.2. Each component in a mixture can be qualitatively identified by its retention time $t_R$ which is the time between injection and detection.

**Figure 2.2.** Reverse Phase (C18) Separation of Amino Acids.
As with gas chromatography the retention time of a particular compound is constant for a fixed set of chromatographic conditions (flow rate, temperature, column condition). Qualitative identification is made by comparing \( t_R \) of the unknown with retention times of standards that have been injected into the chromatograph. This strategy works so long as components have unique retention times.

The area under each peak is proportional to the concentration of that component in the original mixture. If the peaks are reasonably sharp and the flow rate is carefully controlled, the peak heights are approximately proportional to concentration. Thus a calibration curve can be prepared by plotting either peak height or peak area as a function of concentration for a series of standards.

### A. Separating a Mixture of Parabens

In this analysis, the number of components in the parabens mixture is determined. Also, the results of gradient elution are compared with isocratic elution. The components of the mixture are 4-hydroxy benzoic acid, methyl-4-hydroxy benzoate, ethyl-4-hydroxy benzoate and propyl-4-hydroxy benzoate. You will separate the parabens by reverse phase HPLC using a C18 column.

![General paraben structure](Figure 2.3: General paraben structure)

Parabens are a class of chemicals widely used as preservatives in the cosmetic and pharmaceutical industries. Parabens are effective preservatives in many types of formulas. These compounds, and their salts, are used primarily for their bacteriocidal and fungicidal properties. They can be found in shampoos, commercial moisturizers, shaving gels, personal lubricants, topical/parenteral pharmaceuticals, spray tanning solution and toothpaste. They are also used as food additives.

#### Procedure

In **reversed phase HPLC** water is considered a "weak" chromatographic solvent and organic solvents such as methanol are considered "strong" chromatographic solvents (refer to discussion in Chapter 28, Skoog, Holler and Nieman). A typical methods development procedure for HPLC starts with elution using a strong solvent to elute all components in a mixture quickly. In a series of isocratic runs, weaker solvents are blended in with the strong solvent to achieve resolution of the components of interest. The series of isocratic experiments is as follows:

<table>
<thead>
<tr>
<th>Experimental Run</th>
<th>Mobile Phase Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 % methanol</td>
</tr>
</tbody>
</table>


Often salts and buffers (as well as pH adjustment) are required and commonly added to the water in order to suppress ionization of polar compounds. Use the following procedure to analyze the parabens mixture.

**PROCEDURE FOR ISOCRATIC AND GRADIENT EXPERIMENTS FOR PARABENS MIXTURE ANALYSIS**

First ready the HPLC. Make sure the solvent reservoirs are filled with HPLC grade solvent. Make sure the waste bottles have enough space to accommodate at least 1.0 L of additional solvent.

Start the Open Lab program, if it is not already running, by clicking on the "HPLC1 (online)" blue LC icon on the left side of the desktop (Figure 2.4).

![Figure 2.4: HPLC1 (online) LC Open Lab icon](image)

This will launch the Open Lab program. This software is very similar to that used for the CE experiment. Make sure you are using the "Instrument Control" tab inside the program window (Figure 2.5.) All the components should be green and indicate "ready" status; if they are not, hit the "ON" button. The DAD may require a few minutes to warm up. If there is a lightening bolt through the purple lamp image in the DAD window then wait until it has gone away and the ready bar has turned green. We will not be using the fraction collector.
These instruments are designed to test multiple samples automatically. To do this "methods" and "sequences" need to be created. A "method" is the parameters such as pressure, flow rate, solvent mixtures, etc. that are to be run on a single sample. A sequence is the order samples should be run and what method should be run on each sample. So for the parabens the sample is the sample for all 5 isocratic runs and the gradient run. However, the pump parameters are different for each run. So 6 methods needed to be created, which can be seen labeled accordingly in the method tree on the left of the window or by selecting the drop down for "method" on the menu bar. If you want to run a single run you can click the single sample icon, of an image of a single vial, in the upper left and it will run whatever method selected in the "method" menu bar. To run the sequence such that you do not need to inject the sample nor change the samples manually select "PARABEN.S" in the "sequence" menu bar. Go to "sequence" then "sequence template" to see the order of the samples. Since your sample is always the same, each run uses vial slot 1. As you can see though, the method is different for each run. To observe the methods select a "method" in the menu bar then go to "method" and select "edit entire method." When everything looks good filter the parabens solution into a vial filling the vial. Since the sample is the same for each run it will take sample from the same vial for each run, so you do not want it to run out during the sequence. Put the vial in slot 1. Click run sequence.

You can print out the reports after each run or print them out at anytime by selecting "data analysis" in the bottom left.

QUESTIONS AND DATA TREATMENT

Include your data, plots, calculations, and answers to the following questions in your written report for Exp. #2:

1. How many compounds are in the parabens mixture? Identify each peak on the gradient chromatogram.
2. Compare your results from the series of isocratic runs to the results from your gradient run.
3. What advantages does gradient elution offer?
4. What disadvantages does gradient elution have compared to isocratic elution?
5. Plot your results of retention time of the last component (longest retention time in isocratic runs) versus % MeOH for the series of isocratic runs (plot log r.t. vs % MeOH). Do you obtain a linear plot?

MAKE SURE TO INCLUDE YOUR ANSWERS TO THESE QUESTIONS IN YOUR REPORT WRITE-UP.

B. Analysis of Caffeine in Beverages

Reverse phase HPLC is used to determine the concentration of caffeine in coffee, tea, and soft drinks. The traditional method for the determination of caffeine is via extraction with spectrophotometric quantitation. Use of the liquid chromatography system permits a fast and easy separation of caffeine from other substances such as tannic acid, caffeic acid, and sucrose found in these beverages. Five standard solutions of caffeine are prepared and injected into the HPLC. In addition, the beverages coffee, decaffeinated coffee, tea, and caffeine containing soft drinks are prepared as indicated in the following section and injected into the HPLC. From the resulting chromatograms, measurements of retention time, tR, and peak areas are made. If the flow rate and pump pressure are held constant throughout the entire experiment, tR may be used as a qualitative measure and the peak area as a quantitative measure. A calibration curve for peak area against concentration of the caffeine standards can then be employed to determine the concentration of caffeine in the
four beverages. The solvent (mobile phase) in this experiment is 47% methanol / 53% water.

Preparation of Caffeine Standards

Accurately weigh out 10.0 mg of caffeine then transfer it to a clean 100 mL volumetric flask. Dilute to the mark with HPLC grade water. Carry out a series of dilutions of the stock 0.1 g/L solution to obtain standards of 0.01 g/L, 0.025 g/L, 0.05 g/L, and 0.075 g/L. Make 10 mLs of each of the dilutions. Use HPLC grade water to make the dilutions. Shake the five caffeine solutions to insure adequate mixing.

Sample Preparation

- **Coffee**: Pipette 5 mL of coffee into a CLEAN and DRY 50 mL volumetric flask and then dilute to the mark with HPLC grade water. Be sure to filter the sample into a clean vial. Rinse the filter before collecting the sample by filtering the first 1-2 mLs to waste. Record three chromatograms for each coffee sample.

- **Decaffeinated Coffee Samples**: Pipette 25 mL of the decaffeinated coffee into a CLEAN and DRY 50 mL volumetric flask and then dilute to the mark with HPLC grade water. Be sure to filter the sample into a clean vial. Rinse the filter before collecting the sample by filtering the first 1-2 mLs to waste. Record three chromatograms for each decaffeinated coffee sample.

- **Tea Samples**: Pipette 10 mL of your tea sample into a clean and dry 50 mL volumetric flask. Dilute to the mark with HPLC grade water. Be sure that you filter your sample into a clean vial. Rinse the filter before collecting the sample by filtering the first 1-2 mLs to waste. Record three chromatograms for each tea sample.

- **Soft Drink Beverages**: If your beverage is carbonated, you must decarbonate it by pouring it back and forth between two beakers until the bubbling ceases. Now pipette 25 mL of your beverage into a clean and dry 50 mL volumetric flask and dilute to the mark with HPLC grade water. Be sure to degas this sample for 5 minutes. Also, be sure to filter the sample into a clean vial. Rinse the filter prior to collecting the sample by filtering the first 1-2 mLs to waste. Record three chromatograms for each beverage.

Warning

If your samples are off scale on the detector, you must make an additional dilution of your sample and run it again. Do not change the sensitivity of the detector.

Save standards and samples for the capillary electrophoresis experiment.

Procedure

Filter each solution through the syringe filter into a clean vial. Filter the least concentrated sample first. Wash the filter between samples by filtering 1 mL into waste before you collect the rest. Do not use tape to label vials, it can get the vials stuck. Use a pen.

Determination of the UV Absorbance Spectrum of Caffeine

You must determine at which wavelength you will monitor the elution of your compound from the column. Caffeine is a colorless solution so the absorbance, if any, will be in the UV range. You should remember from Chem 105 how to acquire a UV spectrum. Obtain quartz cuvettes from the stockroom. Use the HPLC grade water as your blank and then measure
the spectrum of your 0.01 g/L solution from 190 nm to 400 nm. What wavelength will you use to monitor the elution of caffeine from the HPLC column? Recall that many solvents absorb below 220 nm.

**Operation of the Instrument**

Consult with the T.A. before operating the instrument. Injection procedure and solvent purity are important in this experiment. The T.A. will advise you how to get the best results and avoid contamination of the sample.

You will do this experiment in isocratic mode with 53% water and 47% methanol.

Put your standards in slots 1-5 with the least concentrated standard in slot 1 to decrease the chance of previous runs affecting the data of later runs. Then in slots 6-9 put your samples.

For this experiment all the standards and samples are being run under the same conditions, so the method will be the same for all the runs. Select "CHE 115 CAFFEINE.M" in the "method" menu bar. Go to "method" then "edit entire method" and make sure the parameters such as the solvent mixture are correct. Then select "CAFFEINE_LC.S" in the "sequence" menu bar. Go to "sequence" then "sequence template" to see the order of the samples and make sure the method is the correct one for each run. Then click run sequence.

Run the sequence two more times if you have time. Make sure that the peak heights of the repeated runs are within 5% of each other. If they are not, repeat the runs until they are reproducible.

**C. Treatment of Data**

Plot the area of the caffeine peak against the concentration in g/L or mg/ml, and the height of the caffeine peak against the concentration, for the caffeine standards. Comment on the relative merits of these plots.

Make a calibration curve in Chemstation. Since you have to wait awhile while the data is being acquired it is good to do this during data acquisition. You can begin to do this even if you have not finished all your runs. From the "Method and Run Control" view click the "Data Analysis" tab in the bottom left corner. It will open up another window of "HPLC1" but it will be "Offline." Under the "Data Analysis" window on the left there will be a file tree. Go to the "Chem_115" folder and select your data file. The sequence runs you have done will appear in the main window. Double click on your first standard run in the "Sequence" window at the top of the screen. The line for the first standard run should now appear in bold font. On the menus bar go to "Calibration" and select "New Calibration Table." The window "Calibrate: HPLC1" will appear. Select "Automatic Setup" set the "Level" to "1" and put in the concentration of your first run in "Default Amount." Click "OK." Then double click on the second run. Go to "Calibration" on the menu bar and select "Add Level." Set the "Level" to "2" and enter the concentration in the "Default Amount." Click "OK." Repeat for the rest of the standards. The "Calibration Table" and the "Calibration Curve" windows are at the bottom of the view.

Make sure all reports have been printed. Using the peak areas of the standards plot a calibration curve in either Chemstation, Matlab or other data analysis software. From the calibration curve, calculate the concentrations of caffeine in each of the unknowns.
Treatment of Data

Determine and report the caffeine concentration in g/L (for each sample) using the calibration curve. This may be done by either taking the area of the peak or the height of the peak. (Or both. What are the advantages of each method?) Explain why you chose either the peak height or the peak area for your determination. A proper estimation of errors is expected in the report. Be sure you take into account the dilution of your samples. In your report, discuss how retention time depends on the methanol content and pH of the mobile phase. What factors determine the choice of mobile phase composition and pH in the present analysis?

References


Old HPLC procedure:

**PROCEDURE FOR ISOCRATIC AND GRADIENT EXPERIMENTS FOR PARABEN MIX ANALYSIS**

Turn on the power to the Varian HPLC pump and the diode array UV/Vis detector (power switches are on the front panels). If the computer is not already turned on, do that as well. Pump settings and the detector are controlled by the computer. The detector software should be started by clicking the leftmost icon on the Star Toolbar:

![System Control icon](image)

This starts "System Control," which is used to control the detector. If a popup window appears for configuring communications, cancel it.

Two windows will appear, one for the Varian ProStar 230 LC Pump and one for the Varian ProStar 310 UV-Vis.

Click the "Method" button on the 310 UV-Vis window. For analysis of the parabens, enter a display wavelength of 254 nm. After entering the desired values, close the window.

**Isocratic Pump Setup**

Make sure the pump is on. Click Pump on the 230 LC Pump window to start pumping. After clicking pump there should be no red lights on the pump face and the window for the Varian ProStar 230 LC pump should show that the "Pump Operation" is running.

To set the solvent composition for isocratic runs, click the "Method" button on the window for the Varian ProStar 230 LC pump.

By convention, the weak solvent is A, and the strong solvent is B. You can change the description of each solvent at the bottom of the window. The C solvent entry on this pump should always be 0%, since this course only uses binary solvent
systems. Also, make sure that the flow rate on this screen is set to 2.0 mL/min. First program the pump to run 100% methanol. Close the window and click yes save changes.

Press “Reset” to allow the pump to equilibrate. After four minutes or so, the pressure should stabilize.

After the system has equilibrated as described above, place the injector valve in the "Load" position. Using the syringe with the flat-tipped needle (never use a sharp needled syringe - you could ruin the injection valve!), rinse the syringe with methanol three times then inject at least 25 uL into the sample loop, and leave the syringe in place.

On the computer screen, all "Run Status" lights should be green. On the menu bar, select "Inject - Inject Single Sample." Enter your name or group name in the Popup window and click "OK." When the second popup window appears, click "Inject." The program will now go through a sequence of steps, and you will see "Not Ready," "Waiting," and "Running" in the status display. Don't do anything until the system displays "Running" and then immediately turn the injector valve to the Inject position. You can then sit back and watch the chromatogram appear. Do not press the "Start" key on the pump.

When the run is complete, you can view or print out the report. The default run time is 20 minutes, but you can press "Reset" on both window screens if you are sure that all components have eluted. To view or print out the report, click once on the second icon from the right on the Star tool bar, and choose from the drop-down menu:

You will need printed reports for your lab report.

Repeat these steps for each isocratic elution listed above. Always allow 3-5 minutes for the column to equilibrate with the new conditions.

For the gradient run:

1. In isocratic mode set the conditions to 50% A and 50% B. Allow the system to equilibrate for 5 minutes.
2. In the next row in the method table, enter Time as 10 minutes, and %B as 100%. Make sure the flow rate is still 0.8 mL/min.
3. Inject the mixture of parabens as before, but this time you must press "Start" on the pump window when you turn
the valve. This tells the pump to run a gradient over the next 10 minutes from initial condition (50% methanol) to final conditions (100% methanol).

B. Analysis of Caffeine in Beverages

Operation of the Instrument

Consult with the T.A. before operating the instrument. Injection techniques and solvent purity are important in this experiment. The T.A. will advise you how to get the best results and avoid contamination of the sample.

1. Sample Preparation

Filter each solution through the syringe filter into a clean vial. Filter the least concentrated sample first. Wash the filter between samples by filtering 1 mL into waste before you collect the rest.

You will do this experiment in isocratic mode with 53% water and 47% methanol. The flow rate should be 0.8 mL/min. The procedure for setting the mobile phase composition is identical to that described for the paraben analysis.

2. Injection Procedure

1. Fill the syringe with at least 20 μL of the most concentrated caffeine standard.
2. Insert the syringe into the injection port, turn the valve to the LOAD position (counter clockwise), and then inject some of the standard solution until you see a few drops come out of the overflow port.
3. Next, start detector data collection as you did with the parabens. Remember, this is an isocratic run, so do not press “Start” on the pump.
4. Allow the peak due to the caffeine to be recorded (approximately 4 minutes). You may press “Reset” to terminate data collection without waiting the full 20 minutes. Do the same procedure (steps 1 through 4) again for the most concentrated sample. Continue until you have recorded three chromatograms for the most concentrated sample.
5. Repeat the above steps (1 through 4) for each of the remaining standard samples, going from the most concentrated to the least concentrated. Note that it is good practice to rinse the syringe out a few times with the methanol/water mixture when changing to a caffeine sample of different concentration. A blank can be run to be sure that the syringe is free of caffeine. Do three injections of each standard. Make sure that the peak heights are within 5% of each other for each standard. If they are not, repeat the injection until it is reproducible.

D. DETERMINATION OF CAFFEINE IN VARIOUS BEVERAGES

Analyze the four different caffeine-containing beverages that have been approved by the instructor. Follow the general instructions given below for each type of beverage.