Chemistry 231 – Spring 2013
HPLC Experiment 1
Introduction to HPLC

Overview

This experiment is designed to introduce you to the Agilent 1100 HPLC instrument. You will separate a mixture of five compounds using reverse-phase HPLC, and then determine the identity and concentration of two of the compounds in an unknown sample. Be sure to read the entire lab before getting started.

Introduction

The Agilent 1100 HPLC instrument is one of the most commonly used HPLC instruments in academic and commercial laboratories today. Like many modern scientific instruments, the 1100 is controlled entirely through a computer interface. The advantage of this is: ease of use, high sample throughput, reproducible results across various users, and computer monitoring of critical system functions.

An HPLC system is made up of 4 parts. These are the: pump and eluent, the injector, the column, and the detector. With the 1100's ChemStation software, each of these components is represented on the instrument and run control screen. This makes controlling the 1100 relatively intuitive.

Up to four different eluents may be connected to the 1100's one high pressure pump at any time. According to the software settings, the pump will proportion the flow of the eluents such that any mixture of the four may be obtained for isocratic analyses. In addition, the software is capable of changing the ratios of the eluents during an analysis for gradient runs. The instrument has a built-in degassing module, so it is not necessary to purge dissolved air from eluents before attaching them to the system. If a salt is added to an eluent it is important to filter the eluent through a 0.5μm solvent filter before attaching the eluent to the system. Always be sure to use HPLC grade solvents. Since we will be using reverse-phase HPLC, one of the eluent bottles will always be filled with water for our weak eluent. To discourage the growth of bacteria, 1% methanol is added to the water. This should have a negligible and reproducible effect on retention times. For most of the work in this class, acetonitrile will be used as the strong eluent, so only two of the four eluent bottles will be utilized. The pump module allows pumping of the eluent mixture at accurately controlled flow rates between about 0.05 and 10 mL/min (although both high and low ends of this range will not be useful for specific columns).

An auto-sampler is used for all injections on the 1100 regardless of if you are analyzing 1 or 99 samples. Using the ChemStation software, the auto-sampler allows individual injections of any vial in the 99 vial sample tray, or, using a sequence file, injections of any combination of the samples in the tray. Multiple injections may also be made from the same vial. When preparing samples, be sure to always use HPLC grade solvents. Since solutes may contain particulate matter it is important to either filter your final sample, or filter the stock solute before making up your sample, with a 0.5μm syringe filter.

For reverse-phase HPLC we will be using a 25cm C_{18} column with 5μm spherical beads. With the 1100, the column is housed in a heated chamber. Eluent is pre-heated to the set temperature before entering the column. Increasing the column's temperature will have the effect of shortening retention times; however, this effect is much less pronounced for regular reversed-phase analyses than the effect of changing eluent composition. Regardless, having a

stable column temperature can improve reproducibility. The column chamber also houses a column switching valve that can select between two installed columns. Make sure that you are using the appropriate column before beginning your analysis.

Separated compounds are detected by a diode array detector (DAD) with a wavelength range of 200 – 900nm. The DAD contains both a UV and a visible lamp. One, or both, of these lamps may be turned on to detect compounds depending on the absorbance spectrum of analytes. The DAD has two major advantages over single wavelength detectors. First, several wavelengths may be monitored simultaneously for detecting peaks. Second, full spectra may be obtained during an analysis to confirm the identity of a compound, or to determine at what wavelength detection is most sensitive. For qualitative analysis, the lamp only needs a few minutes to warm up. For quantitative analysis, approximately one hour is needed for the lamp's intensity to become stable. Lamps are expensive and have a limited lifetime; it is important to turn the lamp off when the instrument is not in use.

Things to keep in mind when using the 1100:

Make sure that the eluent does not run out. The ChemStation software has a mechanism for stopping the instrument before eluent is completely used up. After filling an eluent bottle, be sure to reset the eluent volume in the ChemStation software.

Waste fluids are all directed to a large waste jug located under the 1100. Before starting a session on the HPLC, check the waste jug and make sure that it does not overflow during your session.

When finished using the 1100, make sure that the detector lamps, the column heating, and the pump are turned off. This is done solely through the ChemStation software. If you are running an unattended sequence, make sure that this happens at the end of the sequence by using the appropriate macro (It is a good idea to have the macro tested first). If you run an unattended sequence, you must be the first person to the instrument after the sequence is completed. This means that if you run a sequence overnight, you must be the first person to the instrument the following morning (to remove samples from the sample tray). Do not switch the main instrument power off.

Keep track of your method, sequence, and data files. These should be stored in your own directory on the computer. Be sure not to save changes you have made to somebody else's existing method or sequence file.

Experiment

You will develop a method for the separation of a mixture of the following six compounds:

Toluene
Benzene
Acetophenone
Uracil
N, N-diethyl-m-toluamide (DEET)
Phenol

Stock solutions containing each of the 6 compounds dissolved in MeOH will be provided. Concentrations of these solutions are in the range of 1000 μg/mL. You will also be given an

unknown that contains 2 of the compounds above. Each student will receive an unknown with various combinations of compounds and varying concentrations (also dissolved in MeOH). You will need to identify the two compounds and determine the concentrations in units of μg/mL. You will also need to determine the detection limits and the precision (confidence limits) for the two compounds in your unknown on the HPLC that you are using.

Separation

Use the systematic approach to HPLC method development that was talked about in class. Start with a gradient elution from 5% to 100% acetonitrile (ACN) in 30 minutes. Use a 10μL injection volume and monitor 254nm, 205nm, and 230nm with the diode array detector (DAD). If you identify 6 peaks and you are confident that they are the compounds listed, then you may stop the run early. From this chromatograph decide if an isocratic or gradient method is appropriate. Continue your method development until you have adequately separated the 6 peaks ($R_s > 1.5$) with the shortest run possible. Once you are confident in your separation method, you should collect spectra of the peaks in order to optimize detection of the compounds in future analyses. (Since file sizes increase when spectra are collected, only collect spectra when necessary.)

Peak Identification

Analyze your unknown sample using the method you developed. Based on the retention order, you may be able to make an educated guess as to what compounds are contained in your unknown. Make a standard for each compound from the stock solutions provided and analyze it on the HPLC until you positively identify your two compounds.

Compound Quantification

From the stock solutions, make four standards for each of your unknown compounds. Hopefully, at this point, you have a general idea of the concentration of your unknowns, and you can bracket your unknowns with one or two higher concentration standards, and two or three lower concentration standards. Before finishing with the HPLC, be sure that the peaks generated by your standards produce a linear relationship (area versus concentration), and that the area of your unknown is bracketed on each side by at least one of your standard peak areas. It is best if you analyze your standards and your unknown during the same session on the HPLC. Time permitting; analyze your unknown multiple times to increase the precision of the measurement. You will be using your low concentration standard to estimate the detection limit for this analysis. The shorter the peak height for this standard, the better your estimation of the detection limit will be.

Data Analysis

All data analysis should be done using a spreadsheet. If you are not familiar with any spreadsheet programs, then you need to become familiar quickly. The instructor can help you with the use of Excel. In your spreadsheet, be sure to show what equations you have used by entering the equation as text into another cell and indicating in which cell the actual equation is located.

Separation

For your final separation method, calculate the resolution (R_s) between the two least separated peaks in your chromatogram (critical pair) using one of the following equations:

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2}, \text{ or } R_s = \frac{1.18(t_2 - t_1)}{W_{0.5,1} + W_{0.5,2}}.$$

Where t is the retention time, W is the peak width at baseline, and $W_{0.5}$ is the peak width at half-height. Note that the integration generally gives peak widths as (peak area)/(peak height) which is equal to 2.51σ or $1.07W_{0.5}$. Also calculate the retention factor (k) of the first compound that elutes from the column and the last compound that elutes from the column using the following equation:

$$k = \frac{t_R - t_0}{t_0}.$$

Where t_R is the retention time of the peak of interest, and t_0 is the retention time of an unretained compound. This (t_0) can be found by either looking for the time at the first significant baseline disturbance during a run, or by injecting a sample using a very strong eluent (100% ACN).

Determination of unknown concentrations

To determine the concentration (μg/mL) of compound contained in your unknown, you will need to first derive an equation that fits the data derived from your four standard solutions. A good way to do this is to use the 'LINEST' function in Excel. In addition to providing the slope (m) and intercept (b), this function will also provide the standard deviations (s_m, s_b, s_y,) and the R^2 for your line. Then, to determine the concentration of an unknown (x), use the relationship:

$$x = \frac{y - b}{m}.$$

A little more tedious is determining the uncertainty in x. This can be calculated using the following equation:

$$S_x = \left(\frac{S_y}{|m|}\right)\sqrt{\frac{1}{k} + \frac{1}{n} + \frac{(y - \bar{y})^2}{m^2 \Sigma (x_i - \bar{x})^2}}.$$

Where k is the number of replicate measurements of the unknown, n is the number of calibration standards analyzed, x_i refers to the concentration of calibration standard i (not to be confused with x, which is the derived concentration of the unknown), and x and y with bars over them are mean values. The sum of the square of the differences term can be determined by a function in Excel called 'DEVSQ'.

Report your final value at the 95% confidence limit ($\mu_{95\%}$):

$\mu_{95\%} = \bar{x} \pm \frac{ts_x}{\sqrt{n}}$. $\mu_{95\%} = \bar{x} \pm ts_x$ (for k = 1, t determined for 95% confidence and n – 2 degrees of freedom)

Where *t* is the student *t* value, at a 95% confidence limit, obtained from a table of student's t values.

Detection limits

Use your lowest concentration standard to determine a detection limit for each of the two unknown compounds in your sample. Here, we will define the detection limit as a response that is three times the standard deviation noise of the baseline. You will need to measure the baseline noise (this can be done with software), and peak height of the peak corresponding to the compound of interest, then:

$$\frac{\textbf{Detection limit (DL)}}{\textbf{Standard Concentration}} = \frac{3 \times \textbf{Baseline noise}}{\textbf{Standard peak height}}.$$

Report

If you choose this experiment for your written report, your report should be double spaced, with 12 pt Times New Roman font and 1 inch margins. Include your name, a title, a 2-3-sentence abstract, a short introduction, experiment description, results and discussion, and a short conclusion. In the experiment description be detailed regarding the HPLC description and operating parameters, include how standard dilutions were made, and briefly describe your method development strategy. The results and discussion should include what you believe are key chromatograms that guided your method development, and chromatograms that show how you identified your unknown compounds. Chromatograms should have the key peaks labeled with identity and retention time. Remember that every chromatogram (figure) included in the report must be referred to and discussed in the text of your report. Discuss your method development and how each run led you to the final separation method. Include and discuss the results of the data analysis that you performed (you may want to include a table for this). I suggest including a sheet of calculations in the appendix.

Along with your report, hand in a printout of a well-organized spreadsheet that shows any significant calculations you made.

If you choose the GC experiment for your written report, you should write a 1 to 2 paragraph summary of how the HPLC experiment went. You need to turn in all of the raw data that you collected with the data labeled. This needs to include a chromatogram of the 6 component standard (with all peaks readily integrated and peak areas within the 15% variation expected). Also indicate the compounds in the unknown and complete the resolution, capacity factor, and detection limit calculations.