

HPLC: High Pressure Liquid Chromatography

2013 Chem 413

Introduction

Chromatography can be described as a mass transfer process involving adsorption using a nonpolar stationary phase and a mobile polar phase titrating through the column. The active component of the column, the sorbent or the stationary phase, is typically a granular material made of solid particles (e.g. silica, polymers, etc.), 2-50 μm in size. The components of the sample mixture are separated from each other by means of mobile phase and different degrees of interaction with the sorbent particles based on their relative polarity. The pressurized liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol). Its composition and temperature plays a major role in the separation process by influencing the interactions taking place between sample components and sorbent. These interactions are physical in nature, such as hydrophobic, dipole-dipole or ionic.

High performance liquid chromatography (HPLC) is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying or purifying the individual components of the mixture. Before the invention of HPLC, chemists had column chromatography at their disposal, and column chromatography was time consuming.

To speed up a classic column chromatography, chemists would have to use a short column for separation, however this lead to poor separation of molecular components held within solution. The basic setup of a classic column chromatography would include the column that varied in I.D. from 10 to 50mm and column lengths of 50-500cm. The column was then packed with the stationary phase ranging in particle size from 150 to 200 μm thick. Chemists, wanting to speed the separation process up, first experimented with the introduction of a vacuum source or a high pressure source. However, they found with the increased negative or positive pressure, the column length would have to be increase linearly in order to acquire a valid separation that could be used for analytical data with a high confidence level. Chemists realized that with the development of pressurized systems, reducing the particle size would increase the efficiency. It was not until the late 60's that chemists and industrial engineering process acquired adequate technology and manufacturing techniques to develop a smaller grained stationary phase that would be cohesive with a pressurized system. Today, HPLC has many uses including medical (e.g. detecting vitamin D levels in blood serum), legal (e.g. detecting performance enhancement drugs in urine), research (e.g. separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and

manufacturing (e.g. during the production process of pharmaceutical and biological products), (Kealey, 1987).

Block Diagram and Explanation

A basic block diagram of an HPLC is shown in Figure 1.

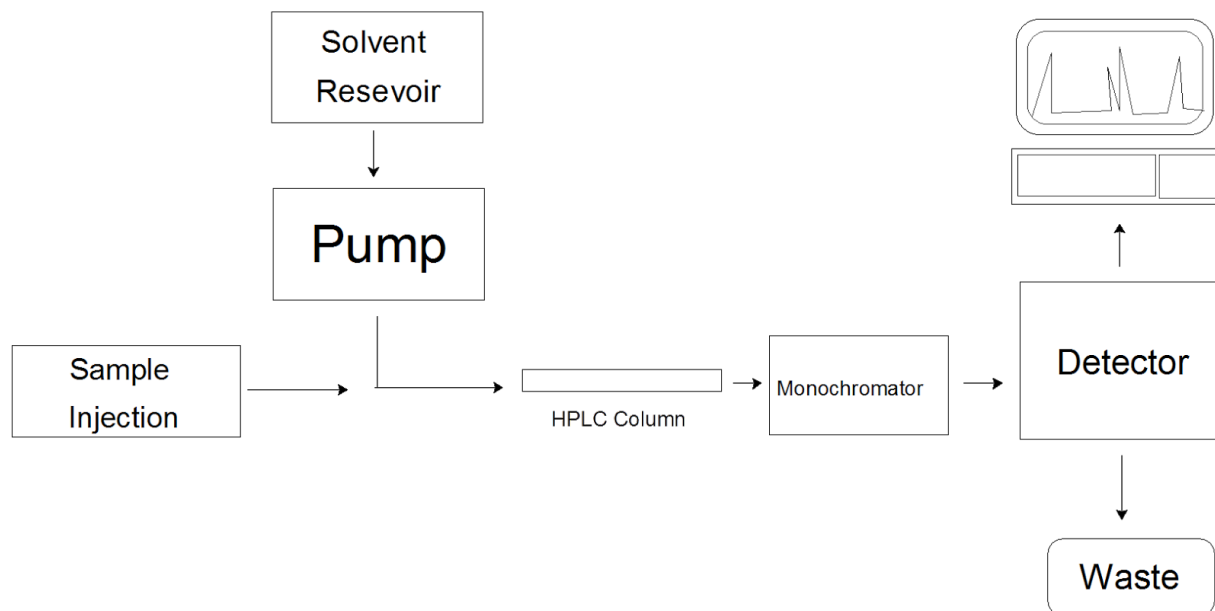


Figure 1: Block Diagram of an HPLC

Your desired solvent mixture travels through capillary tubes, from the solvent reservoir to the pump, where it becomes highly pressurized. The pump is also used to control the flow rate of the mobile phase substance, which is typically measured in mL/minute. The prepared sample is then injected into the line, where it travels with the solvent into the HPLC column. There are many different columns you can choose from, depending on the sample you want analyzed. As the solvent moves through the column, molecules from your sample will stick to the silica in the column and detach at different times, making them distinguishable from one another. The detector detects when these molecules detach from the silica and reports the data in the form of a chromatogram. Various types of detectors can be used such as a UV-VIS, fluorescence, or an evaporative-light scattering detector (ELSD). Once the solvent has traveled through the column it goes into a waste container, or can be collected if desired.

The parameters of the HPLC, like any instrument, are important and are dependant on your sample. The solvent mixture, containing a strong solvent and a weak solvent, will depend on whether your sample is polar in nature or not. Common solvents include water, methanol and acetonitrile. Two different solvent methods can be used, isocratic or gradient. With isocratic, the solvent mixture stays the same, 50:50 for example. With a gradient, the solvent will start with a 100:0 ratio of weak solvent:strong solvent and increase in increments over time to the final

mixture ratio. It's important to flush the system before running your samples in order to insure that the solvents used for the previous sample does not interfere with your samples. The mobile phase flow rate is important and can range from 1-10 mL/min, though 1 mL/min is a good place to start with most experiments. It's important to monitor pressure when adjusting the flow rate, as the pressure should not exceed 400 bar. The injection can also vary in volume, anywhere from 0.1-100.0 μ L. For concentrated samples, 3-5 μ L is appropriate and 25 μ L for dilute samples. Starting with an injection of 10 μ L is typical. The temperature can be adjusted but for most samples 25 $^{\circ}$ C is adequate. The temperature setting should never exceed 50-60 $^{\circ}$ C. You also need to know what wavelengths in the UV-VIS spectrum you want to monitor. A diode array detector has a range of 210-400 nm and for samples, the default program setting are fine. The HPLC at UAF is an Agilent 1100 Series and is located in downstairs instrument room. (Figure 2).

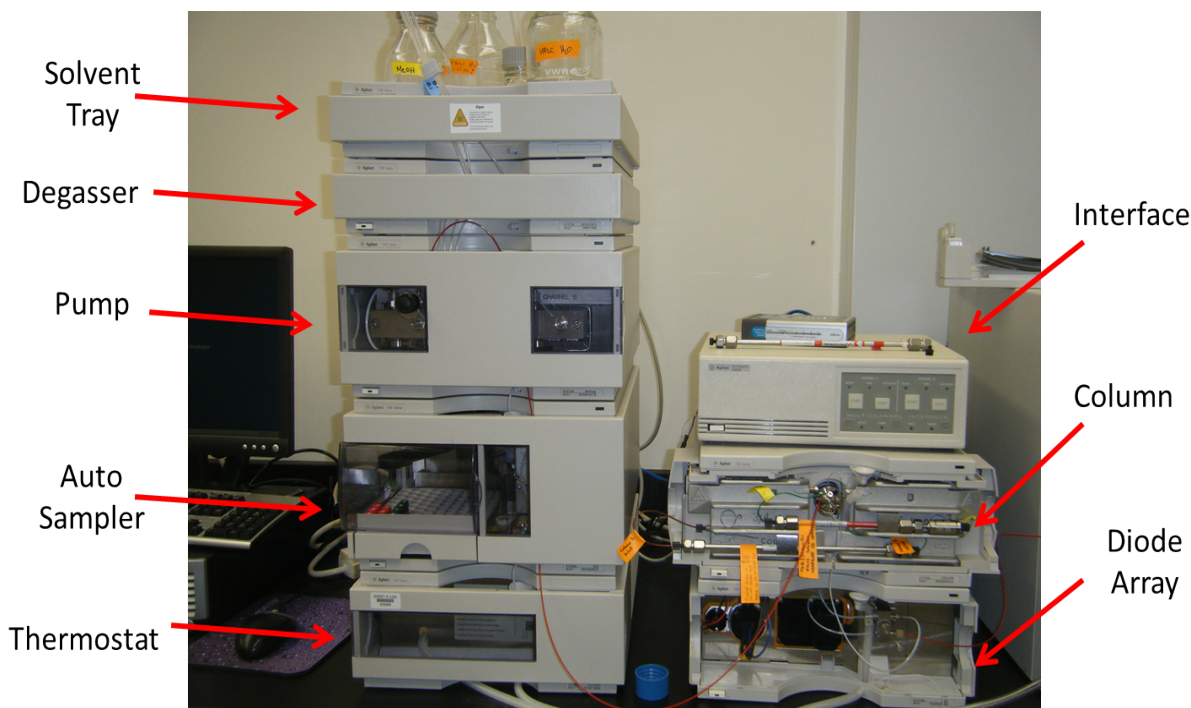


Figure 2: Labeled Agilent 1100 Series HPLC at UAF

HPLC data is given in the form of a chromatogram, which looks much like data from other instruments. (Figure 3)

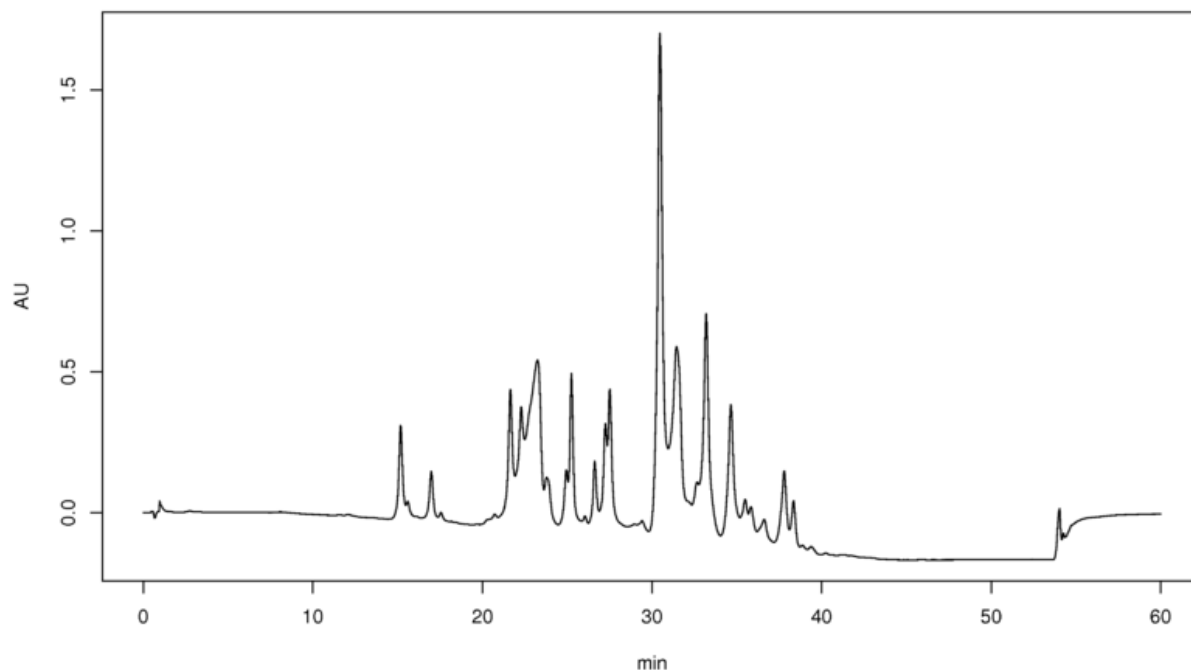


Figure 3: Example HPLC Chromatogram (Mixture of Perfume and Water)¹

The x-axis is labeled with a time unit, typically minutes, and the y-axis can be a variety of things depending on the specifics of each experiment. For example, if you were using a UV-VIS element as a detector, the y-axis would be labeled absorbance. From the chromatogram, compounds can be both identified and their concentrations quantitated. The tools found within the HPLC software on the computer can be extremely useful in analyzing a chromatogram. You can determine peak height, peak area and also see the specific spectrum associated with a given peak.

¹ <http://en.wikipedia.org/wiki/File:Hplc-perfume-chromatogram.png>

Individual Component Diagrams

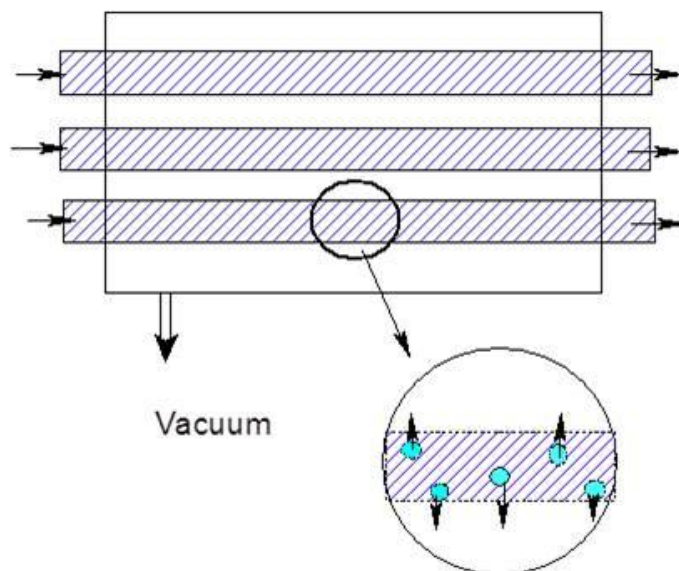


Figure 4: Degasser for the HPLC. This is has small tubes with small holes in them that allows the escape of the gas as it travels through the vacuum. It does not allow any liquid to go through though.

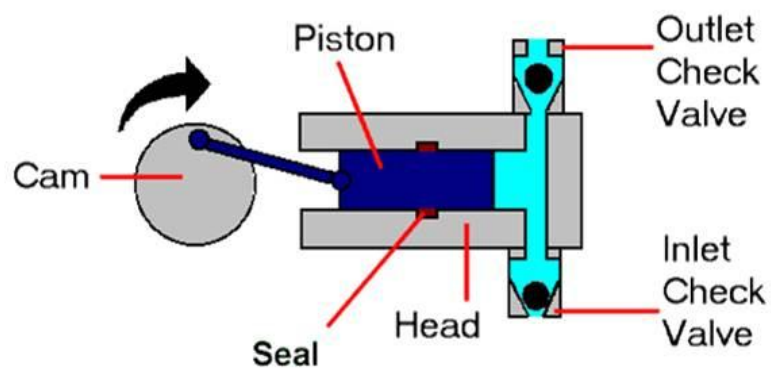


Figure 5: The pump of the HPLC, moves the solvent from the jars on the top of the machine to the auto sampler



Figure 6: Autosampler, this allows for multiple samples to be run with the same method without having to have an individual standing there monitoring and changing the sample every 30 minutes.

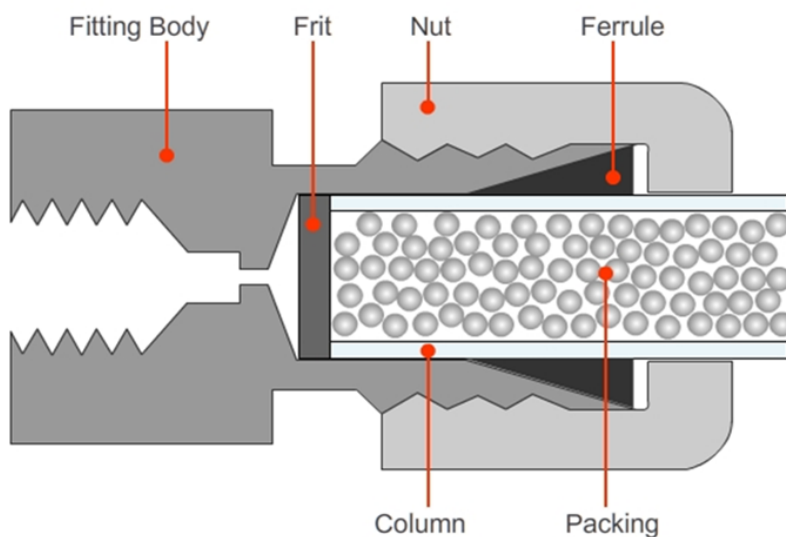


Figure 7: The column. This is where the sample and the solvent are passed through allowing the sample to cling to the packing at different times due to the difference in the chemical composition. These differing times are called retention times. And can be used to help analyze the sample.

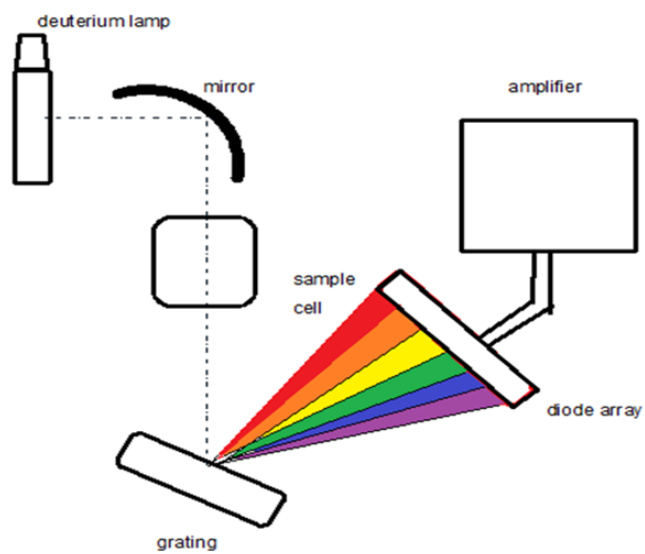


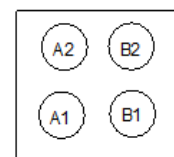
Figure 8: The diode array.

HPLC Instrument Operation

Safety Precautions: Make sure all waste is properly disposed of in designated containers. Make sure you've reviewed the MSDS's for the solvents that you are using.

1. **Check solvent bottles and waste container.** Be sure the necessary solvents are present and bottles are full. Also, note the location of the solvent bottles (A1, A2, B1, B2) you will use (see diagram right). All solvents must be HPLC-grade and filtered. Also, make sure that your waste container has adequate space.

Wall



2. **Connect your column.** After deciding which column you should use, mount it on the column holder (Figure 2) and connect the injection and waste lines to the column. The arrow(s) on the column will indicate the direction of flow. All connections to the pump should be finger tight.



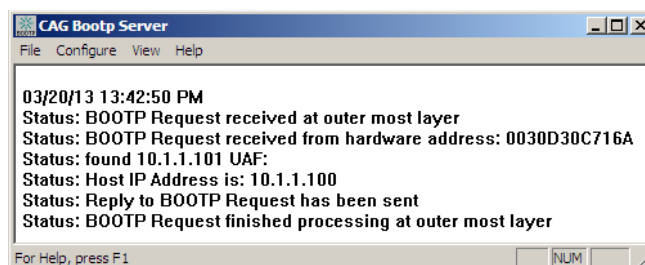
3. **Add samples to autosampler.** Insert your sample(s) into the autosampler and note of which slot your samples are in.

Samples that are to be analyzed with HPLC should have a concentration of about 1 mg/mL (ref). All samples must be filtered using a syringe and ~25 μ m filter (ref). Unfiltered samples run the risk of damaging the column. Degassed samples are best, as bubbles can cause complications with the instrument. The solvents used in preparing your samples should be the same mixture as the mobile phase you plan to run through the column.

4. **Turn on the instrument in correct order.** It is essential to turn on the components of the HPLC in the correct order:

- a. Enter your name and solvent information in the log book.
- b. Log onto to computer.
Username: Administrator and
password: 3000hanover.

- c. Turn on the interface and wait until it beeps and reads "not ready" (~1 minute).
- d. Make sure that the interface and computer are communicating by looking at the CAG Bootup server.

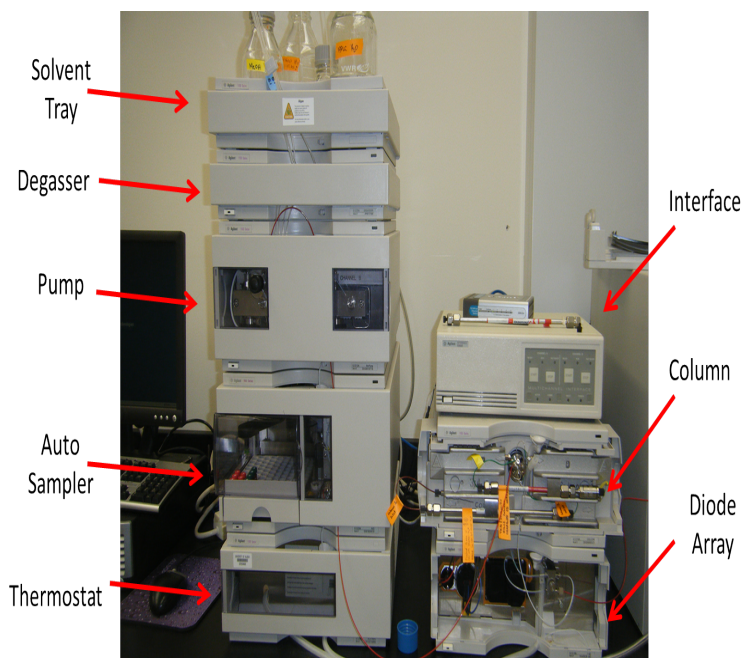


- e. Turn on the 5 components of the HPLC in order:

- i. column,
- ii. diode array,
- iii. autosampler,
- iv. pump, and
- v. degasser.

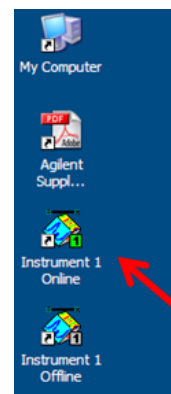
Allow these systems to power up for 2-3 minutes.

- f. Before you open the software on the computer, allow time for the autosampler to stop moving.



This indicates the instrument is ready. Click on the Instrument 1 ONLINE icon on the desktop.

- g. If you plan to use the Evaporative Light Scattering Detector (ELSD):
- i. turn on the instrument by switch in the back.
 - ii. turn on the gas tank near the instrument (flow should be 3.5 bar or 51 psi)
 - iii. Turn on light
 - iv. Temperature should be 60 C for a aqueous mobile phase and 40 C for an organic mobile phase. Flow rate should be 1ml/ min.
 - v. Connect the detector inlet after the UV detector.



5. **Flush lines with proper solvent mix.** If you were not the last user, be sure to flush the lines with the solvent mixture you will be using. To do this,

- a. Disconnect column inlet and drain inlet line into a waste container.
Cap the column so the column does not dry out.
- b. Select your solvent mixture. Under the Instrument tab at the top select "Set up Pump". This will bring you to the setup menu.
 - i. Enter your desired flow rate, stop time, and solvent mixture. The flow should be anywhere from 1-2 mL/min, depending on the manufacturer recommendations for your specific column.

- ii. To change the solvent mixture, adjust the percent of solvent B and the computer will automatically adjust the percent for solvent A.
- iii. Make sure that the numbers in the Timetable (outlined in red) match the numbers entered for time, %B, flow and max pressure above,

Set up Pump : Instrument 1

Control
 Flow: 1.000 ml/min
 StopTime: 20.00 min
 PostTime: Off min

Solvent A
 15.0 %
 1: water
 2: NH4CO2H

Solvent B
 85.0 %
 1: CH3OH 0.9%
 2: CH3CN 15% H

Pressure Limits
 Max: 400 bar
 Min: 0 bar

Timetable

	Time	%B	Flow	Max. Press.
1	0.00	85.0	1.000	200

Buttons: Insert, Append, Cut, Copy, Paste, Display: Timetable, OK, Cancel, Help

- otherwise the system will freak out. Hit “OK” and now you can flush the lines.
- iv. Under the Instrument tab at the top select “System On” to turn on the pump, thermostat and DAD. Allow the lines to flush until the pressure is stable.
- v. Once the lines are done flushing, you can turn off the pump by clicking on the pump icon and selecting “Controls”.
- vi. Select “Off” in the upper left hand corner and click OK. Once the pump is off, you can reconnect the injection line to the column.
- vii. Once the column is connected turn the pump back on by going back into the pump controls and selecting “On” then hitting “OK”. Monitor the pressure to make sure it doesn’t exceed the specifications of your column.
- viii. If you are using the ELSD, connect it behind the UV detector after flushing column for a few minutes.

6. Create a method.

- a. Under the Method tab, select “Load Method,” or “New Method.”
- b. Under the Method tab, select “Edit Entire Method.” This will walk you through the setup of your method.
- c. Edit “Edit Method” pop up window. Check all boxes and click “OK.”
- d. Edit “Method Information.” Here you can make notes about your sample, the solvents used and any other information you might need. Click “OK.”

Edit Method: Instrument 1

Check Method Sections to Edit

- ☒ Method Information
- ☒ Instrument/Acquisition
- ☒ Data Analysis
- ☒ Run Time Checklist

Buttons: OK, Cancel, Help

Method Information: Instrument 1

Method Comments:

c18 column flow rate 1.5 ml/min ambient 80/20 water methanol

Buttons: OK, Cancel, Help

- e. Edit “Set up Pump.” These should be the same parameters you used to flush the lines. Be sure that the flow rate, solvent mixture, and max pressure are the same in the top portion of the screen are the same as in the “timetable” portion below. Click “OK.”

Set up Pump : Instrument 1

Control
 Flow: 1.000 ml/min
 StopTime: 20.00 min
 PostTime: Off min

Solvent A
 15.0 %
 1: water
 2: NH4CO2H

Solvent B
 85.0 %
 1: CH3OH 0.9%
 2: CH3CN 15% H

Pressure Limits
 Max: 400 bar
 Min: 0 bar

Timetable

	Time	%B	Flow	Max. Press.
1	0.00	85.0	1.000	200

Buttons: OK, Cancel, Help

Display: Timetable

- f. Edit “Set up Injector.” This is the amount of your sample that will be injected. Standard injection volumes are 3-5 μL (ref?). The optimization for most experiments can be set to “none”.

Set up Injector : Instrument 1

Injection
☒ Standard Injection
☐ Injection with Needle Wash
☐ Use Injector Program

Injection Volume: 10.0 μL
 Wash Vol: 0
 Total Lines: 0 Edit...

Optimization: none 0.00 min. after Injection

Buttons: OK, Cancel, Help, More >>

- g. Edit “ADC1 ADC Signal.” This window controls the Light Scattering Detector (ELSD). The default settings seem to function. Select “OK.”

- h. Edit “ADC1 Timed Event Table.”

ADC1 ADC-Signal: Instrument 1

Signal
 SignalSource
 Description: ADC1 CHANNEL A
 Source: Signal
 Testplot

Unit: mAU
 Units/Volt: 2000.000

Peak Width: 0.0260 Min.
 (Data Rate: 10 Hz.)

Stoptime: noLimit Min.

Data Storage: ☒ All
 Start Data: 0.00 Min.
 Stop Data: 0.01 Min.

Buttons: OK, Cancel, Help

ADC1 Timed Event Table: Instrument 1

Start Signal Source
☐ 35900 Will Start External Devices
☒ External Device Will Start 35900

Timed Event Table

Time	When	Event
Minutes		

Buttons: Add, Delete, Replace, OK, Cancel, Help

- i. Edit “DAD Signals.” In this tab, select which wavelengths you want to monitor. You can monitor up to 5 wavelengths at a time, A-E.

Make sure that you have the boxes checked next to the wavelengths you want to monitor.

You can select UV and/or VIS lamps by checking the boxes next to them on the right. You can also select which spectra you want stored, but it's a good idea to save it all.

- j. Edit “Column Thermostat Method.”
Next you need to set up your thermostat and select which column you are running.
For most experiments 24°C is an acceptable temperature.
Select either “Column 1” or “Column 2” from the drop down menu on the right.
The injection lines for the respective columns are labeled with small pieces of colored tape so you know when column you have connected, 1 or 2.

- k. Edit “Signal Details.” From the pulldown menu, select which signals to monitor in the main window. Add a signal to your method by selecting which signal you want in the pulldown menu and clicking “Add to Method”. Click “OK.”

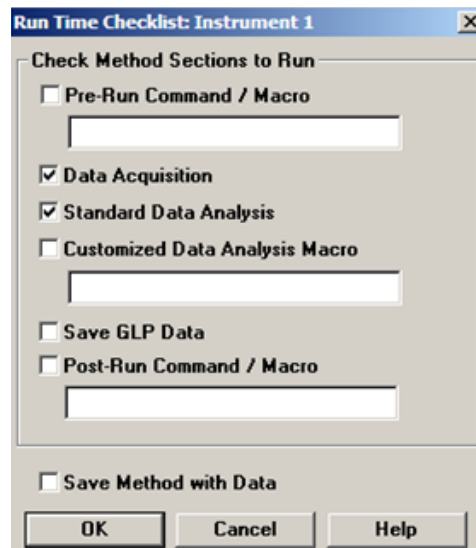
Signal Description	Start	End	Delay	Align	Peak
DAD1 A, Sig=254.4 Ref=360,100	0.000	0.000	0.000	No Alignment	0.0
PMP1, Pressure	0.000	0.000	0.000	No Alignment	0.0
DAD1 C, Sig=210.8 Ref=360,100	0.000	0.000	0.000	No Alignment	0.0

- l. Edit “Integration Information.” The default settings for this are adequate so just click “OK”.
- m. Edit “Specify Report.” The next step is the report menu. Select where and how you want your data to be saved. DO NOT select to print your data since the instrument is not hooked up to a printer.
Other than the “Destination” parameters, all the default setting are adequate.
- n. Edit “Instrument Curves.” Select which signals you overlayed in the data report. For example, you can select the “Flow”

Time	Integration Events	Value
Initial	Slope Sensitivity	1
Initial	Peak Width	0.04
Initial	Area Reject	1
Initial	Height Reject	1.7
Initial	Shoulders	OFF

option and a spectrum of the flow rate will be overlaid on the spectral data collected from the UV.

- o. Edit "Run Time Checklist." Enter any commands to be carried out before or after you run a sample. Typically, these commands are unnecessary and you can just leave it blank and select "OK".
- p. Save your method. Under the Method tab select "Save Method" and you can name your method and save it to a location of your choosing. Make sure that you don't save over an existing method.



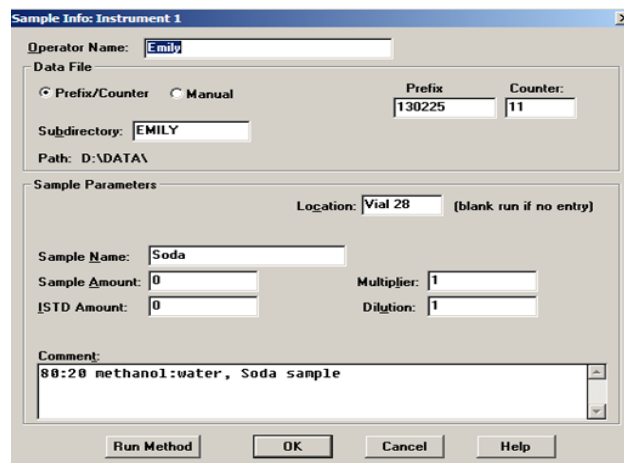
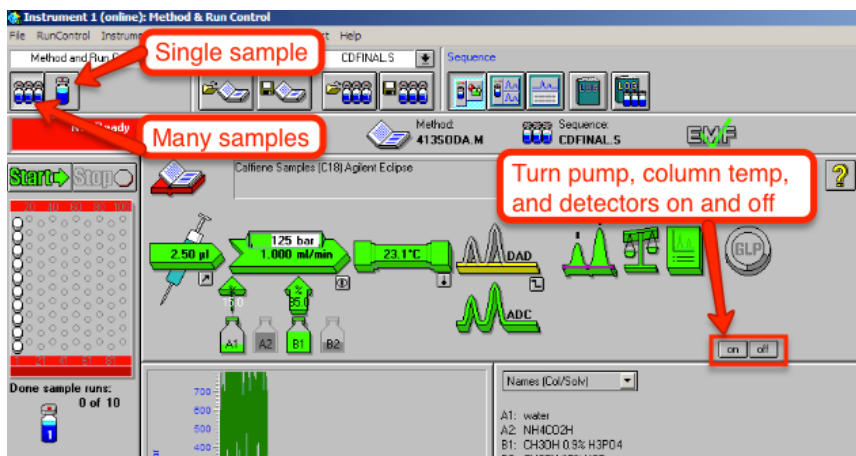
7. Set up Sample Run.

- a. Load Method. Under method tab, click "Load Method." Review your method parameters by selecting "Edit Entire Method" under the Method tab.
- b. Next, go to the Instrument tab and select "System On". This will turn on the pump, lamp and thermostat. This can also be done by clicking "on" button on the bottom right of the window showing the column conditions.

8. Setting up a single sample.

Start run by clicking on the Single Sample Icon. From here, click on the vial icon, below the green "Start" button, and select

9. "Sample Info". From this menu, you can edit the information about your sample. You can edit the operator name, the subdirectory where the file will be saved, the prefix and sample number for your sample, as well as any additional information in the bottom box. It's a good idea to make the prefix for the sample the date that it was collected. You also need to input the location of the sample, the numbered slot your vial is in within the autosampler.



Hit the green “Start” button. The data collected will appear on the screen and be automatically saved to the location you designated previously.

10. **Setting up several samples.** You can also run a sequence of samples, so long as they’re all using the same mobile phase.

- Under the Sequence tab, select “Sequence table.” Edit this table with the appropriate information. Be especially careful to select the appropriate method and use the correct autosampler position.
- Select the sequence icon in the upper left of the screen.
- Click the green “Start” button

Sequence Table: Instrument 1

Currently Running
Line: Method: Location: Inj:

Sample Info for Vial 25:
p-toluene-Beta-OH Ethyl Ester (Racemic)

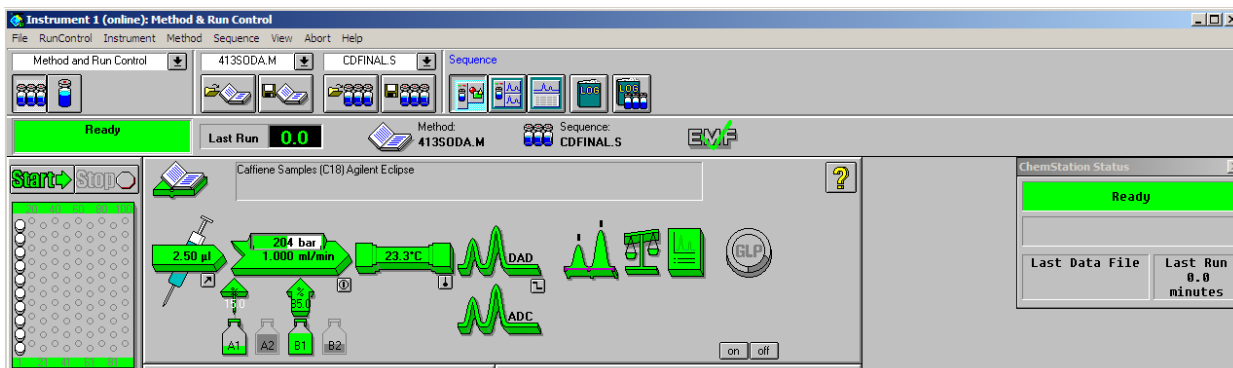
Line	Location	Sample Name	Method Name	Inj/Location	Sample Type	Cal Level	Update RF	Update RT
1	Vial 25	Racemic	ADAISHT		2 Sample			
2	Vial 14	Enzyme14	ADAISHT		2 Sample			
3	Vial 15	Enzyme15	ADAISHT		2 Sample			
4	Vial 16	Enzyme16	ADAISHT		2 Sample			
5	Vial 17	Enzyme17	ADAISHT		2 Sample			
6	Vial 18	Enzyme18	ADAISHT		2 Sample			
7	Vial 19	Enzyme19	ADAISHT		2 Sample			
8	Vial 20	Enzyme20	ADAISHT		2 Sample			
9	Vial 21	Enzyme21	ADAISHT		2 Sample			
10	Vial 22	Enzyme22	ADAISHT		2 Sample			
11	Vial 23	Enzyme23	ADAISHT		2 Sample			
12	Vial 24	Enzyme24	ADAISHT		2 Sample			

Insert Cut Copy Paste Append Line Undo All Undo Wizard
Insert/FillDown Wizard Run Sequence Read Bar Code OK Cancel

Sample location (leave empty for a non-injection blank)

11. Running samples.

- Once the sample information has been entered and the system is on and ready to run a sample, many parts of the window will turn green. Including the autosampler,



the instrument injector, column, and detector. Also the green box under the single/multiple sampler.

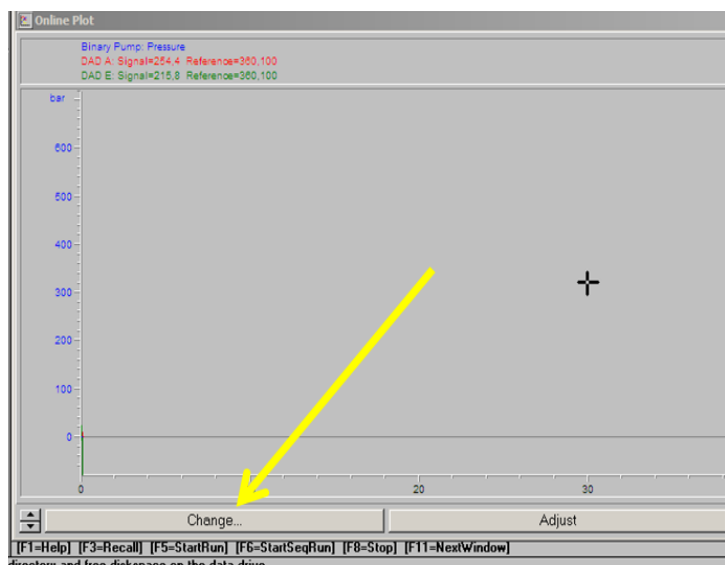
- click the green “Start” button to start the run.
- Once a run has started, the green boxes will turn blue.

12. **Viewing your data.** The data collected will appear on the screen and be automatically saved to the location you designated previously.

You can also choose which spectra you want to view in the main window by clicking the “Change” button and adding or removing spectra from the viewing window. Pressure is one that you should always be monitoring.

13. **Shut down Instrument.** Before closing the software, turn of the pump by clicking on the pump icon, selecting “Controls” and selecting the “Off” option in the upper left-hand are of the screen. You can then exit the software.

When asked if you’d like to turn off the pump, thermostat and lamp select “Yes”. Once the software is closed, you can turn off the instrument components and lastly the interface. You should turn off the instrument in the reverse order that you turned it on.



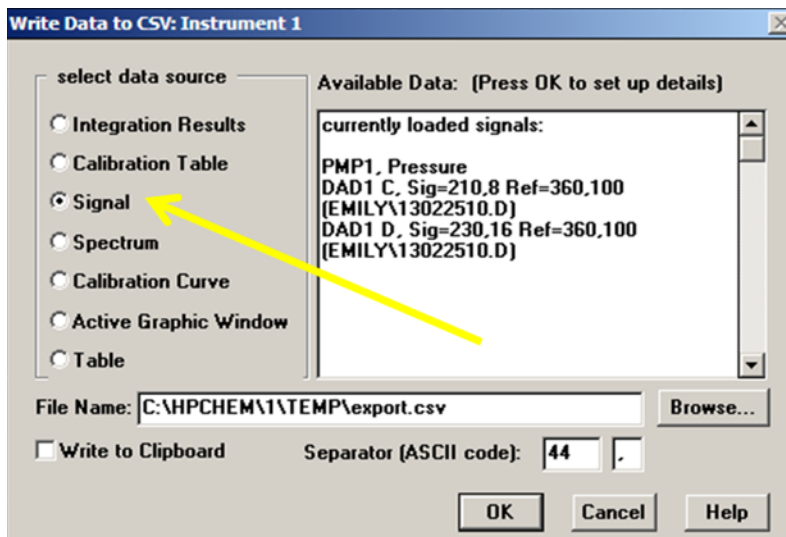
Be sure to clean up the instrument area, empty waste bottles, remove your solvent bottles, et when you are finished.

14. **Viewing and exporting your data.** You can export the data that you collect from the computer as a CSV file to be opened in Excel later.

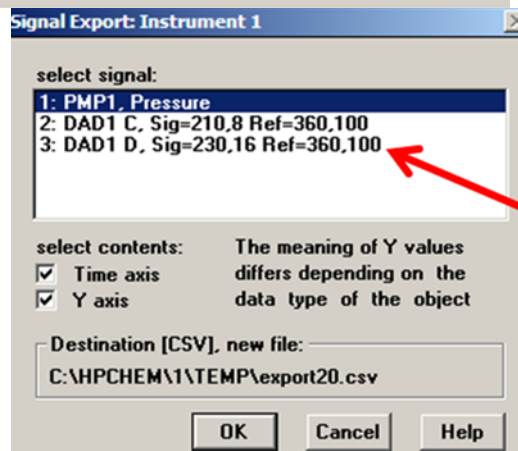
- a. Open the “Instrument 1 Offline” software. From here you can open your saved data by going to “File” and “Load Signal”. Find your saved data and select which sample data you’d like to look at. All data is saved in the d:/ drive and within the “Data” folder. You should’ve created a folder when you ran your samples.

If you have trouble connecting your data files to samples, use the “file information” button just below the files listed. This will give you the information from the file run sequence. With the file naming convention (ex: 001-0203) is the first three digits correspond to autosampler position (001) the next two (02) correspond to position in run sequence, and the last two digits correspond to replicates (03).

- b. Once you have your data open, you can go to “File” then “Export” and select “CSV”. A box will pop up and here you can select which signal you want to export. Select the “Signal” option on the left-hand side and click OK.



- c. Make sure you note where the computer is saving the data to. Also, add a number after “export” in the file name, otherwise the software will just keep replacing the same file over and over. After that you can select which signal you want to export.
- d. Once you have exported the data, you can copy the file to a flash drive to be opened in Excel later.



Troubleshooting

In case of disaster contacts: Dr. Hayes, Dr. Iceman, Dr. Green or his lab members, Dr. Rasley.

One of the most common errors is not turning on the instrument components in the correct order. Take the time to make sure you do this to avoid any problems. If the software gives you a specific error code, consult the various manuals, located in a big stack next to the HPLC computer, and once you find the specific error code, the manuals will walk you through how to correct the error. Otherwise Mike Jaramillo, Adai, and Professor Rasley can all help with the HPLC.

If the pressure randomly drops, this could indicate a leak. Make sure that no solvent drips onto the instrument otherwise sensors will register a leak and shut the instrument down.

Instrument Maintenance

Always make sure that you empty any waste containers after using the HPLC. It is a good idea to run a test for a column that hasn't been used recently or is old, to make sure that it is working properly and hasn't been contaminated.

References

- Introduction to Modern Liquid Chromatography, 2nd ed., New York: Wiley, 1979
- S. Lindsay, High Performance Liquid Chromatography, New York, Wiley, 1992
- Operating Manual, High Performance Liquid Chromatograph, Agilent 1100; VWD, DAD, FLD, and RID.
http://share.psu.ac.th/system/assets/media/files/000/007/356/original_Operating_Manual_HPLC_1100.pdf?1306827231
- Jaramillo, Mike. SOP for Agilent 1100 Series HPLC Instrument