High Performance Liquid Chromatography (HPLC)

Using Agilent 1100 with Chemstation software

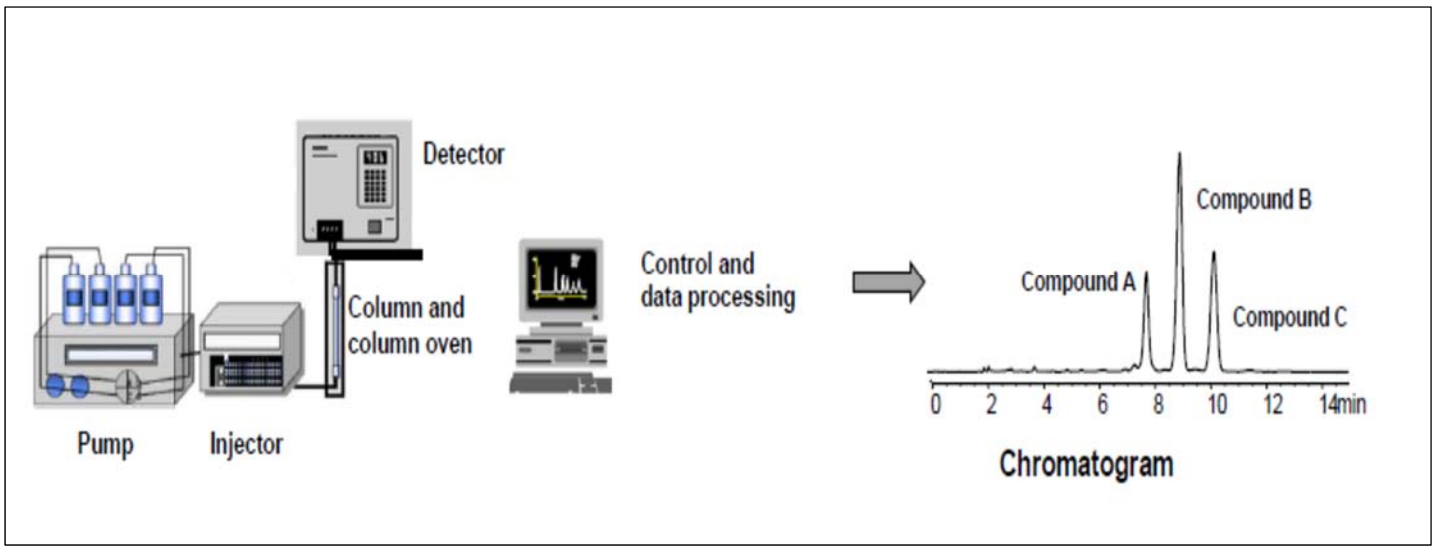

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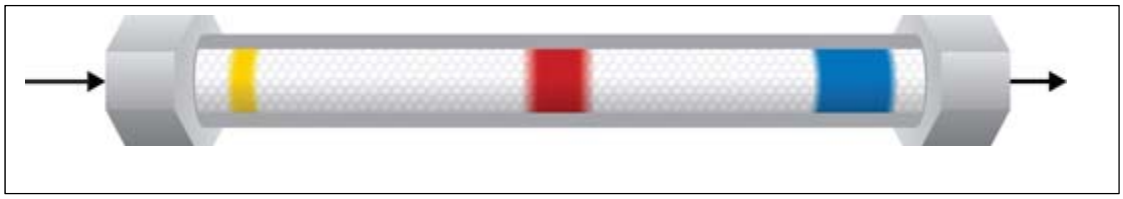
Table of Contents

1. **Important Items:**
   a. Check Waste bottle.
   b. Check Solvent bottles on top of instrument.
   c. Filter sample through 0.22 micron filter.
   d. You can always STOP a run.
   e. The instrument will stop itself if it detects a leak.
   f. You can place a "blank" run at the end of your run to slow down the flow rate. Use the 0.1mL/min method. This will keep the instrument at 0.1mL/min after the run.
   g. Use HPLC grade solvent
   h. Prime if you are changing solvents or instrument hasn't been run in a while.
   i. Shut off system if you can to save the life of the detectors.

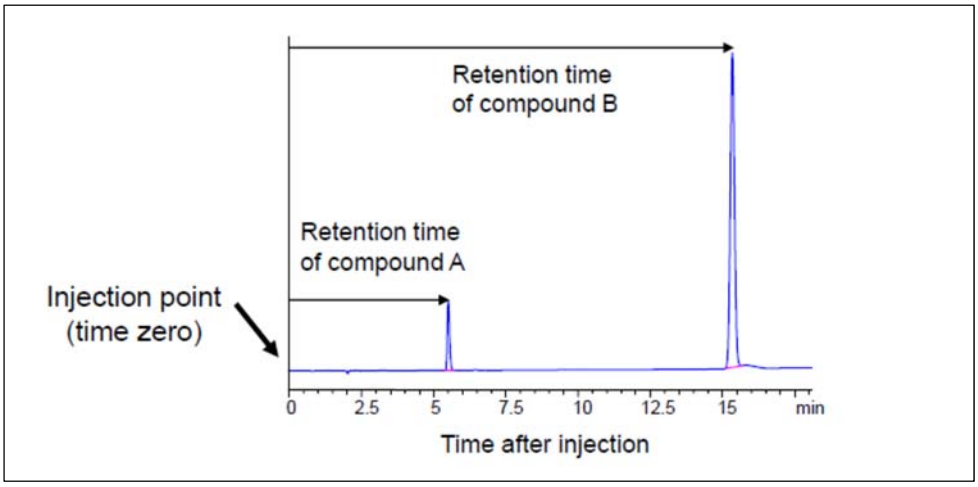
2. What Is High Performance Liquid Chromatography?

High Performance Liquid Chromatography is a powerful analytical technique. HPLC can separate, identify, and quantitate compounds that are present in any sample that can be dissolved in solvent. HPLC can purify chemical and biological compounds that are non-volatile.

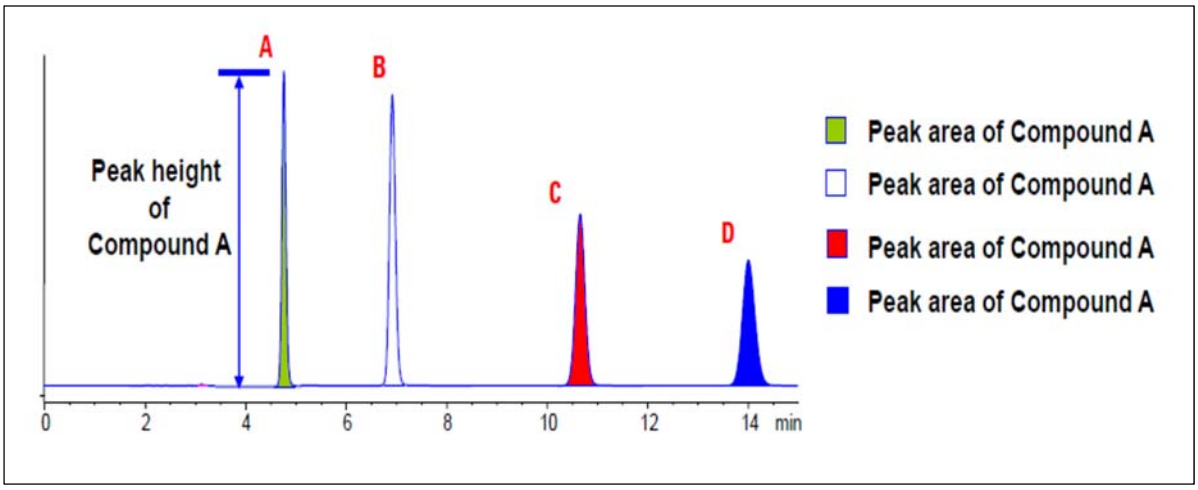


In HPLC components are separated based on their affinity for the stationary phase or the mobile phase. The separated components are detected based on UV absorbance. The detectors qualify substances based on their retention time and quantify substances based on peak intensity and peak area.

Qualitative analysis is based on peak retention time. It gives the ability to identify compounds based on different times through the Column.

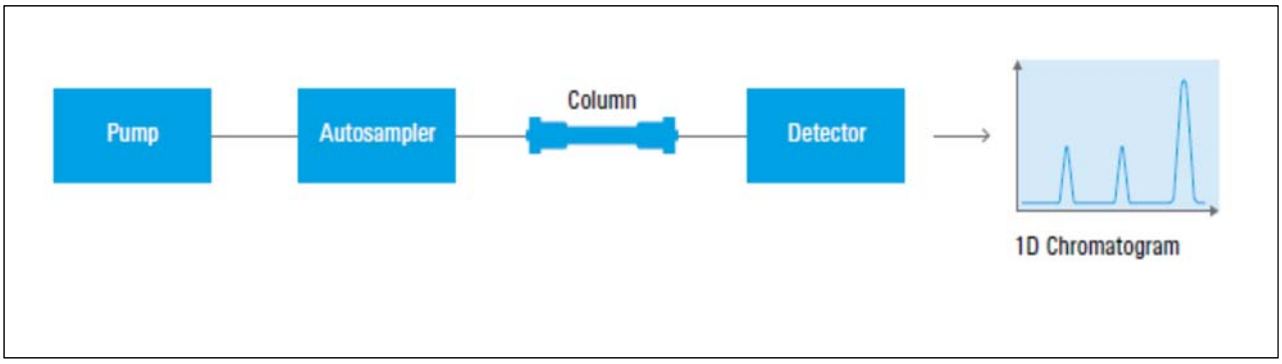


Quantitative analysis is a measurement that gives amount or concentration of sample. It is based on peak area and peak height.

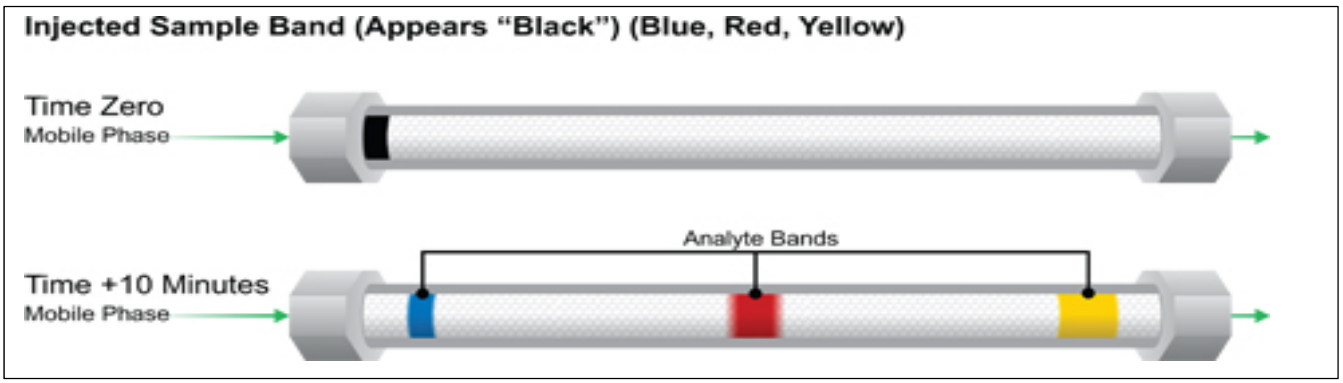


The mobile phase is the solvents that run through the column. They are stored above the unit since gravity aids in priming the system. A high-pressure quaternary pump delivers the mobile phase at a specified flow rate. The autosampler introduces the sample into the mobile phase stream that carries the sample to the HPLC column. The column contains packing material that enables separation of components. Stationary phase is the name of this packing material. The detector sees the material as they elute from the column. The mobile waste then goes to a waste container.

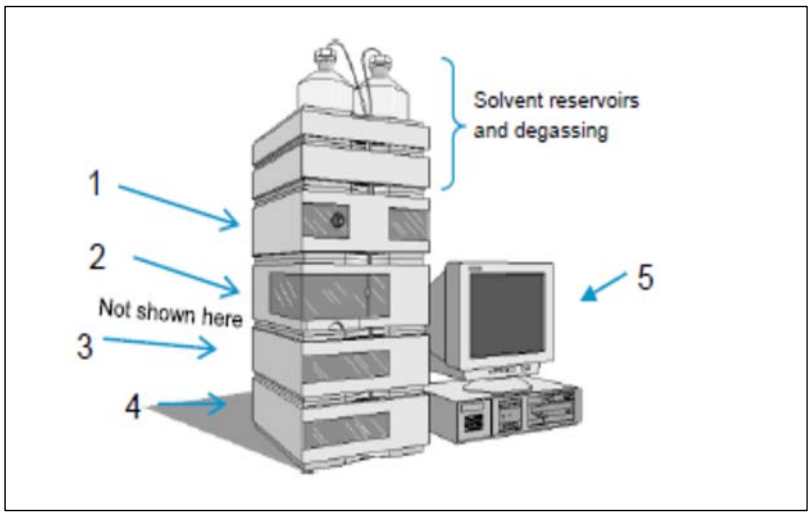
3. 4 Key Components of HPLC System



Mobile phase enters the column from the left, passes through the particle bed, and exits at the right. Flow direction is represented by green arrows. First, consider the top image; it represents the column at time zero [the moment of injection], when the sample enters the column and begins to form a band. The sample shown here, a mixture of yellow, red, and blue dyes, appears at the inlet of the column as a single black band. [In reality, this sample could be anything that can be dissolved in a solvent; typically the compounds would be colorless and the column wall opaque, so we would need a detector to see the separated compounds as they elute.]



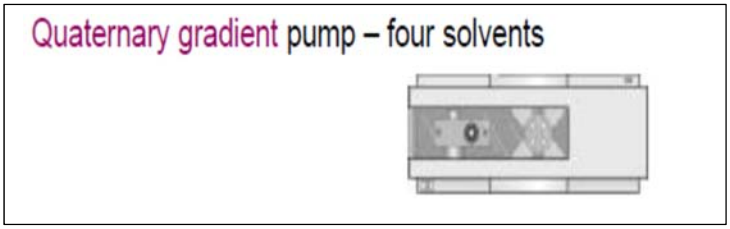
After a few minutes [lower image], during which mobile phase flows continuously and steadily past the packing material particles, we can see that the individual dyes have moved in separate bands at different speeds. This is because there is a competition between the mobile phase and the stationary phase for attracting each of the dyes or analytes. Notice that the yellow dye band moves the fastest and is about to exit the column. The yellow dye likes [is attracted to] the mobile phase more than the other dyes. Therefore, it moves at a *faster* speed, closer to that of the mobile phase. The blue dye band likes the packing material more than the mobile phase. Its stronger attraction to the particles causes it to move significantly *slower.* In other words, it is the most retained compound in this sample mixture. The red dye band has an intermediate attraction for the mobile phase and therefore moves at an *intermediate* speed through the column. Since each dye band moves at different speed, we are able to separate it chromatographically.



A. Pump

The role of the pump is to force a liquid (called the mobile phase) through the column at a specified flow rate of mL/min. A pump can deliver constant mobile phase composition (isocratic) or increasing mobile phase composition (gradient). On the Agilent 1100, we have a quaternary pump. Meaning up to four solvents can be pumped and mixed at the same time. Before delivering the mobile phase to the column, the pump mixes the solvents in either constant proportion (isocratic) or in varying proportion (gradient).

In gradient mode, the mobile phase composition of component B is changed in proportion to component A. Solvent A is always aqueous while solvent B is always organic. In gradient mode, it goes from lower to higher percentage of solvent B. Above the pumps is a degassing unit to remove dissolved air in the solvents. By degassing solvents, there is a decrease in fluctuations of the detector baseline such as noise and drift.



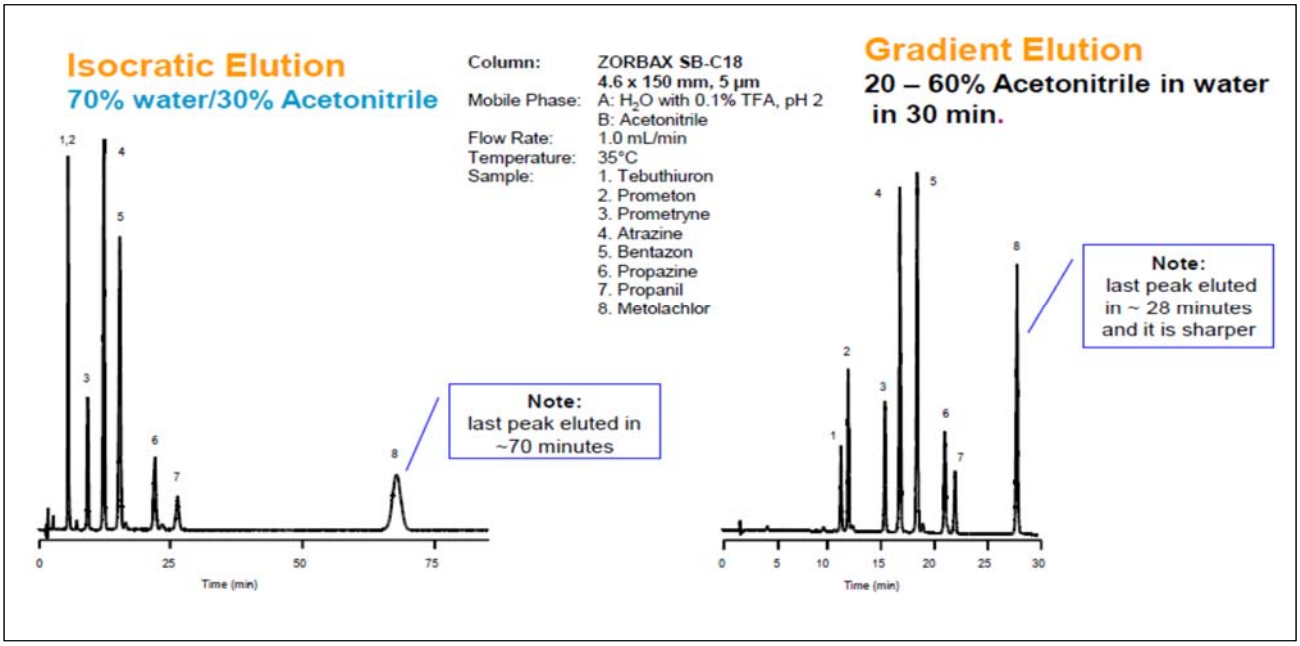
1) Pressure Range

Pumps for HPLC deliver mobile phase through a column containing stationary phase. Standard HPLC columns contain 5µm silica-based particles. Pumps can deliver flow at pressures up to 400 bar (6,000 psi). Always check the pressure and flow rate of each column. If running 100% organic, the pressure will be lower than if running only 5% organic.

2) Solvent Mixing

The pumps must mix solvents as well as delivery of mobile phase. The pumps must give precise constant ratio of solvents for isocratic elution as well as create gradients. Creating gradients proportions two or more solvents change over time. The ability to mix solvents precisely and to deliver reproducible flow rates determines the retention-time reproducibility of the pump. Retention time is a fundamental parameter in HPLC, and it is used to identify sample components.

This instrument contains a quaternary pump which mixes solvents at low pressure. Up to four solvent lines lead to a multi-channel valve, which combines and mixes preset proportions. This pump can deliver binary, tertiary, and quaternary gradients.



B. Autosampler and injector

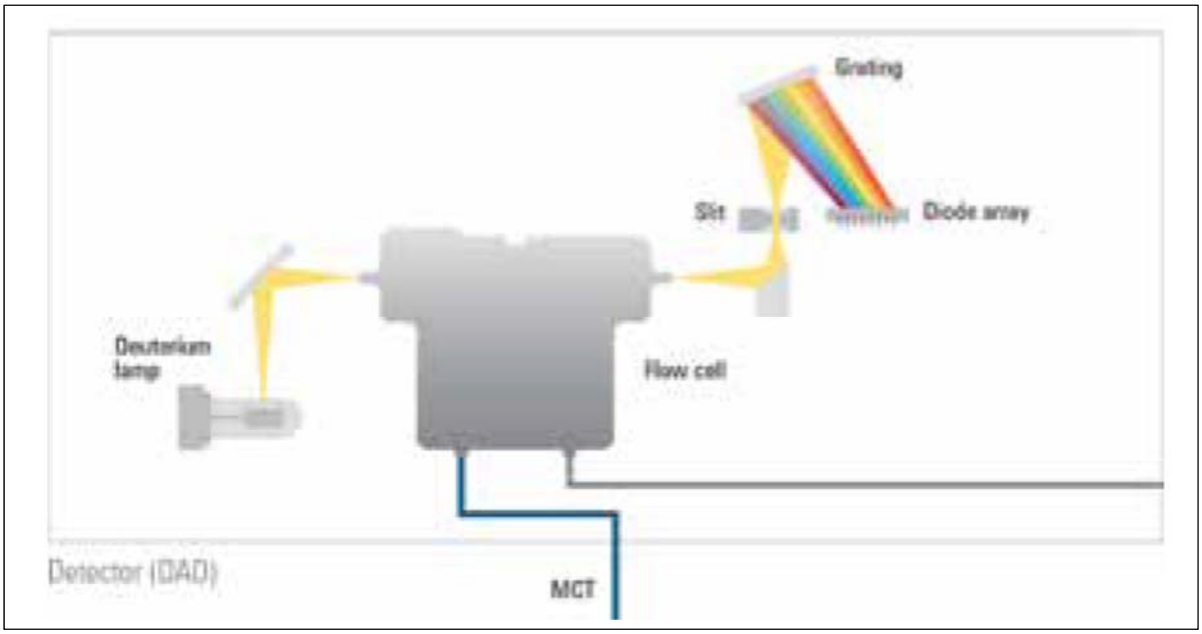
The injector introduces the sample into the mobile phase. The autosampler measures the appropriate sample volume, injects the sample, and flushes the injector to be ready for the next sample. This allows unattended automatic operation. The autosampler holds 1.5ml sample vials. It contains a 100µl sample loop. It can inject volumes from 0.1 to 100 µl. To prevent carry over, it is recommended to use the needle wash vial in vial 91 to clean outside of needle. If you still see carry over, you may need to run a blank injection in between injections**.**

C. Column Thermostat

The majority of users will run the HPLC at room temperature. For reproducibility and a stable environment, using a programmed temperature is important. The ambient temperature in the laboratory can change daily, so programming in a temperature will eliminate this change. Temperature influences retention time and impacts resolution of a sample. The column thermostat goes from 10 degrees below ambient until 80°C. Be sure to put on cover of column heater unit for best efficiency.

D. Detector

The detector has the ability to detect the components of the sample versus the mobile phase as they flow through the flow cell. This detector is a diode array detector (DAD), and it is nondestructive. You can simultaneously look at up to 5 different wavelengths. A compound must absorb UV/Vis light in the 190nm to 600 nm region to be detected. Light of all wavelengths from 190 to 600 nm pass through the flow cell, the emerging light is separated by a grating into its constituent wavelengths and an array of photodiodes acquires the resulting spectral information. It contains a separate UV and Visible lamp. The UV lamp is a deuterium lamp and the visible lamp is a tungsten lamp.



E. Data Control

The computer controls the instrument and the data analysis. We use Chemstation software. There is an online version to control your experiment and an offline version for data analysis.

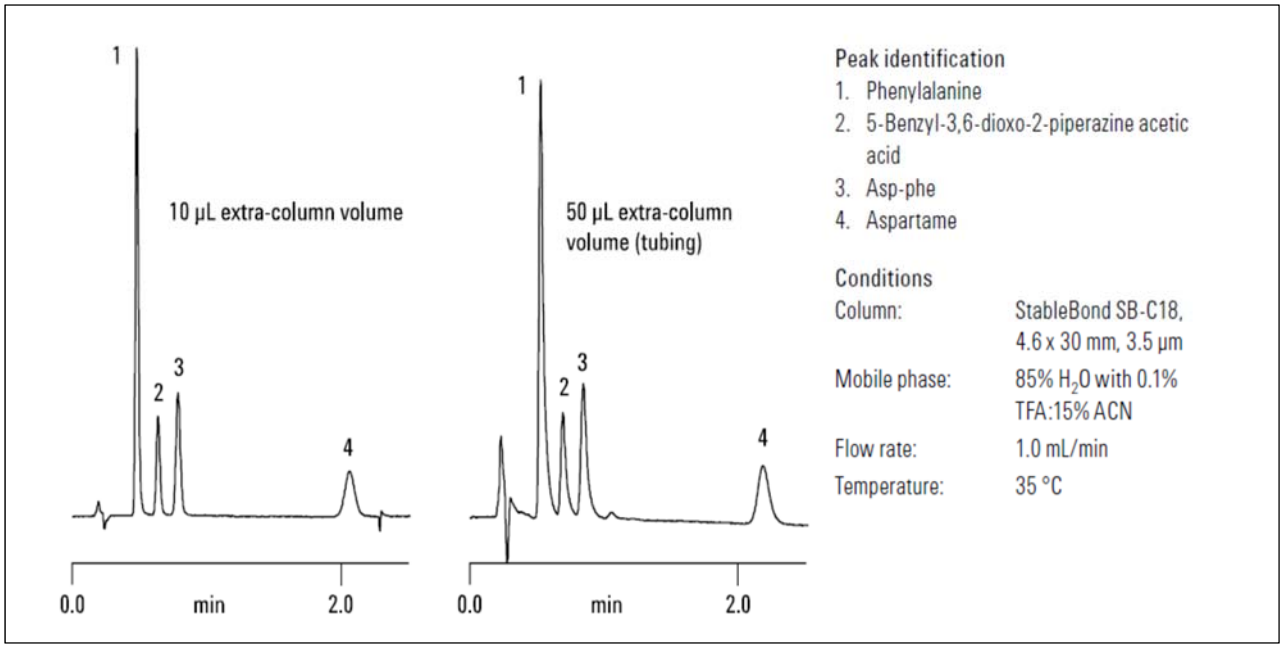
4. HPLC Columns

Silica Gel is commonly used as a stationary phase. The surface of the silica gel is covered with silanol groups. In normal phase HPLC, the surface of the silica gel is covered in strongly polar silanol groups that use a non-polar mobile phase. Normal phase works well for analytes that are insoluble in water. In reverse phase HPLC, the silica particles are chemically modified to be non-polar or hydrophobic and the mobile phase is a polar liquid. Reverse phase is the most common type of chromatography.

Silica is an ideal material for chromatography, and it has been the main base packing material for bonded phase HPLC columns. Silica particles are rigid and resist compaction due to flow, and their extremely large surface areas provide adsorptive capacity for HPLC. The silanols or Si-OH groups on the particle surface are ideal bonding sites for functional carbon chains.

The most common bonded phase for reverse phase chromatography is the C18 (octyldecylsilane, ODS). Some other popular choices are C8, C4 and phenyl. Larger molecules, like proteins, tend to be separated better on short-chain reverse phase columns, like C4, while small molecules and peptides tend to have better separation on C18 and C8 columns.

It is important to reduce extra column volume for efficiency. Extra-column volume refers to the volume that is part of your system. This refers to the connecting tubing that carried your sample between HPLC components. Unnecessary extra-column volumes can lead to loss of efficiency and tailing of peaks.



A. Gradient and isocratic methods

The more complex your sample, the more likely you will use a gradient method. Isocratic is easy to use, but it can lead to peak broadening of late-eluting peaks because peak width increases with retention time. Gradient elution overcomes this problem by decreasing the retention of late-eluting peaks. Gradient elution leads to sharper peaks, and it allows faster separation methods and less solvent use.

Gradient separation uses two to four mobile phase components. Solvent A is usually water and the weaker solvent allowing the analyte to slowly elute from the column. Solvent B is stronger and causes the analyte to elute more rapidly. Solvent B is an organic solvent which is miscible with water. Solvent B choices are acetonitrile, methanol, isopropanol, or tetrahydrofuran.

Isocratic

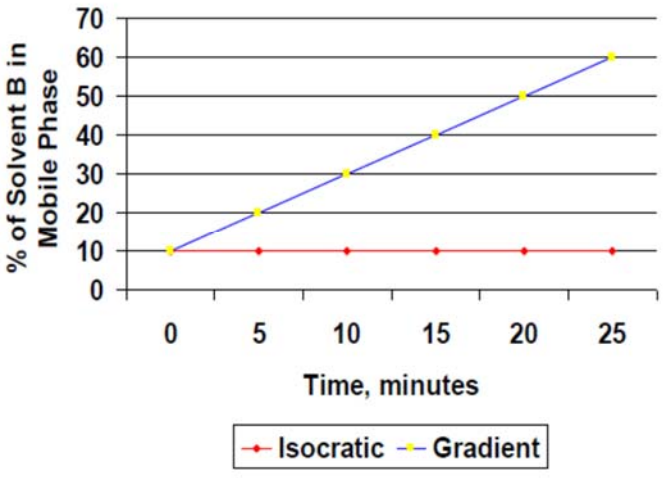
mobile phase solvent composition remains constant with time

- Best for simple separations
- Often used in quality control applications that support and are in close proximity to a manufacturing process

Gradient

mobile phase solvent ("B") composition increases with time

- Best for the analysis of complex samples
- Often used in method development for unknown mixtures
- Linear gradients are most popular (for example, the "gradient" shown at right)



B. Here are the key steps to creating a gradient:

1) Start by running a linear gradient from 5% to 100% organic over time.
2) Hold at 100% organic for additional time to be sure all sample components have eluted.
3) Examine chromatogram to determine the appropriate initial gradient composition and gradient profile.
4) Be sure to re-equilibrate column in intial mobile phase so that retention times are reproducible. This is the time it takes to return column to starting mobile phase conditions after running a gradient. Ten column volumes is the recommended, but during method development, you can get away with shorter time.

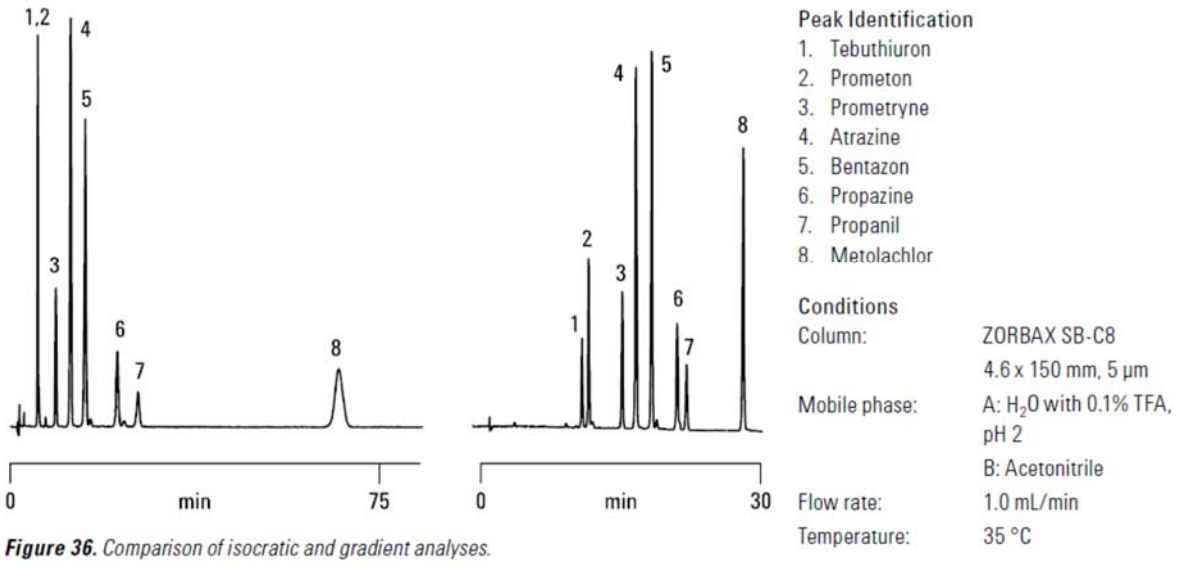


Figure 36. *Comparison of isocratic and gradient analyses.*

C. Column Maintenaince

Over time, there may be changes in retention due to column aging. Column aging can be due to the various mobile phases, analytes, etc. If you are unsure of column performance, run a known test sample(caffeine). Column contamination is the leading cause of performance problems of HPLC columns. Filtering sample through 0.22 micron flter can help prevent sample contamination.

To alleviate high pressure or peak tailing, you can clean your column. Before cleaning, disconnect from detector and run solvents straight into waste container from column. A good way to flush column is to turn it upside down, and flush at 0.1ml.min with methanol overnight.

Steps to backflush or clean your column:

1. Disconnect the column from the detector, attach tubing to the end of the column and place it in a beaker to capture the liquid.
2. Start with your mobile phase without buffer salts (water/organic)
3. Next, use 100% organic (methanol and acetonitrile)
4. Check pressure to see if it has returned to normal; if not, then
5. Discard column or consider stronger conditions: 75% acetonitrile/25% isopropanol
6. 100% isopropanol
7. 100% methylene chloride*
8. 100% hexane*

*When using either hexane or methylene chloride, the column must be flushed with isopropanol prior to use and before returning to your reversed-phase mobile phase.

Buffer salts can also precipitate out and cause backpressure build-up inside column. If this ocurs, slowly pump warm water through the column to remove the salts. Wash solutions containg IPA can generate higher pressuredue to viscosity of solvent. Reduce the flow rate during this stage of cleaning.

5. Method development

The overall goal in method development is to optimize resoltuion in the shortest possible. You want to reduce the use of solvents and the time it takes to purify your sample. Usually gradients are the best choice for a short, clean separation.

In reversed phase chromatography, the analyte is partioned between the polar mobile phase and the non-polar stationary phase. One sees non-polar, non-specific interaction of anayltes with hydrophobic staionary phase. More polar analtyes are less retained than non-polar analytes.

The mobile phase for RP chromatography is

Water with optional buffer, acid or base to adjust pH-(TFA-TEA)

Water miscible organic solvent- methanol or acetonitrile

RP chromatography can be used to separate non-polar, polar, ionizable, and ionic molecules. To improve retention and peak shape, a modifier, like TFA, is added to control pH and retention.

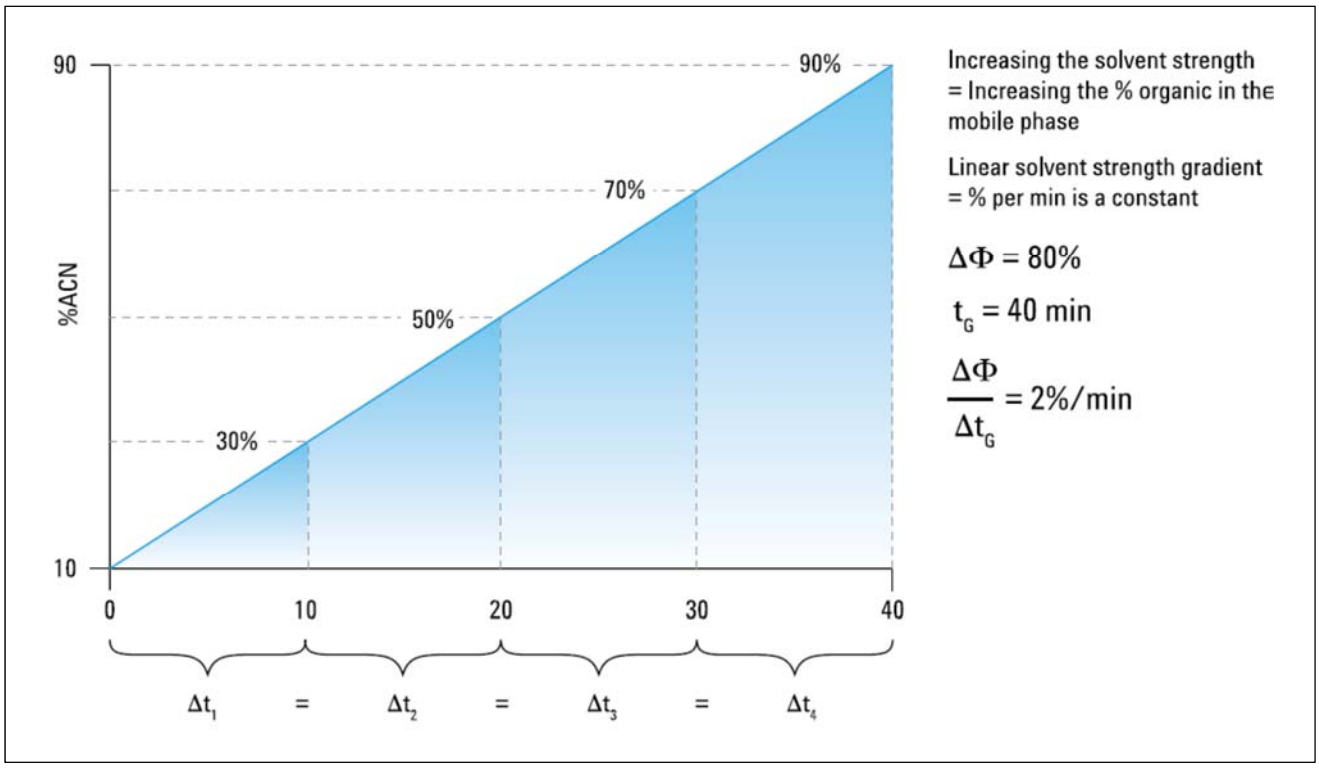
It is best to start with a C18 column. If there is too strong of a retention on the C18 column, you could try the C8 column.

A. **Mobile phases**

Typical mobile phases for RP are methanol and acetonitrile for organic solvents. Always use HPLC grade solvents. HPLC grade are used to be sure nothing UV is in the solvent. Sample solubility will determine the solvent choice.

B. **Gradient Optimization**

For samples containing a wide variety of components, it is best to use gradient elution. Some sample components may be polar and elute quickly in RP, while hydrophobic will stick more strongly to a C18 column. If you change the mobile phase compostion over time, you can separate multiple components easier. Start with a weak highly aqueous mobile phase and increase the % organic solvent over time, in a linear manner.



6. Priming the HPLC

- Anytime that you are switching solvents
- When the instrument has been sitting without flow for long periods of time
- maybe when lowering the solvent bottles to refill you have lost prime
- periodic perturbations in the baseline

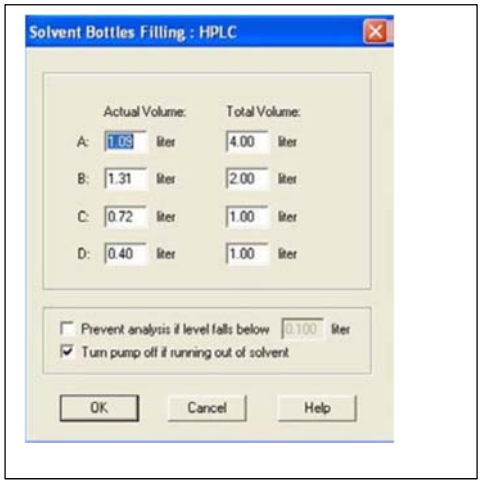
a. Place waste container at the outlet tube from the purge valve.
b. Turn the knob counter clockwise several turns
c. Pump at 100% at 5mL/min for several minutes from each channel that needs to be primed.
    i. Right click on pump to Set up Pump
    ii. Change flow to 5mL/min of 100% of solvent
    iii. Run for several minutes to ensure no air bubbles in tubing.
    iv. Turn flow to 0mL/min
d. Turn know clockwise until closed.

7. Chemstation icons

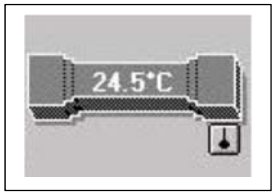
A. **Injector** icon allows you to control injection volume (1 to 100µl) and the wash vial.

B. **HPLC pumping** icon allows you to see pressure, flow rate, % Composition, and amount of solvents.

Right click on bottles icon to open solvent filling. Here you can add the amount of mobile phase in each bottle. Be sure to check off the two bottom boxes so that the instrument will shut off if the solvent volume is too low.

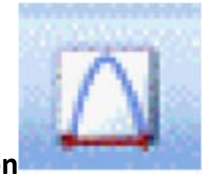


C. **Column icon** allows you to set up column type and temperature.



D. **Detector** icon allows you set up your wavelengths.

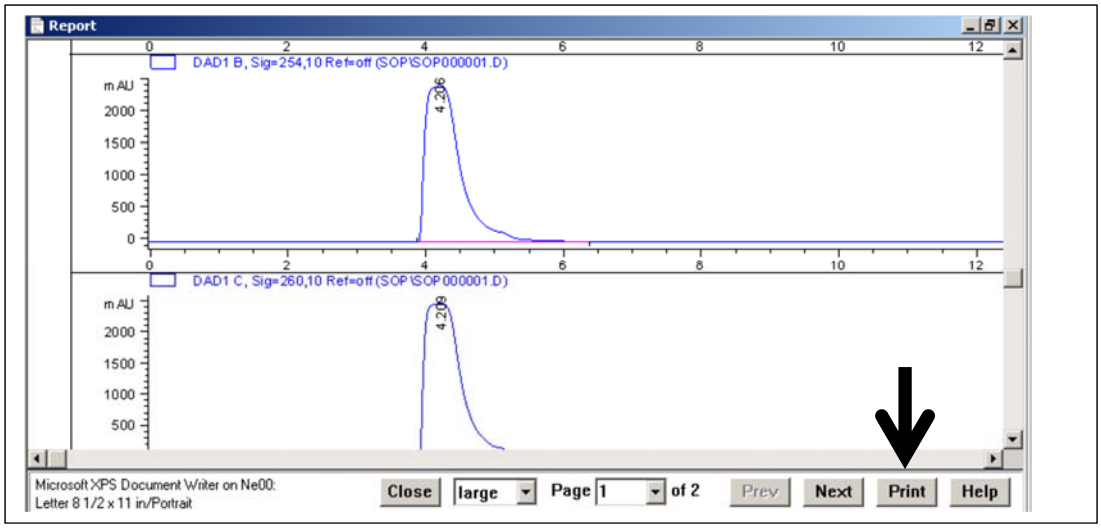
8. Integration using Chemstation software

- Chemstation will automatically integrate every spectrum.
- You can manually integrate.
  - **a.** Go to **Chemstation Offline**
  - **b. Open dataset**
  - **c.** Click on the **Delete Integration icon.** 
  - **d.** Click on every peak you want **integration removed.**
  - **e.** Click on the **Integration icon** 
  - f. **Click and hold** from each side of the peak you need integrated.

9. Print Reports

a. Once you are happy with integration, under **File, Choose Print, Specify Report and Print.**

b. A report will appear on screen. To save report, hit the **Print** button at the bottom of report. Save as pdf on computer or USB drive.



10. Shutdown Instrument

Please shut down instrument when experiment complete.

a. Close Chemstation software. Go under File, and choose Exit.
b. Say **Yes** to close BCL Online.
c. Say **No** to shut off instruments.
d. Shutdown computer.
e. Turn off each module of the Agilent 1100.