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SEPARATION BY DIFFERENT TYPES OF CHROMATOGRAPHY - HPLC

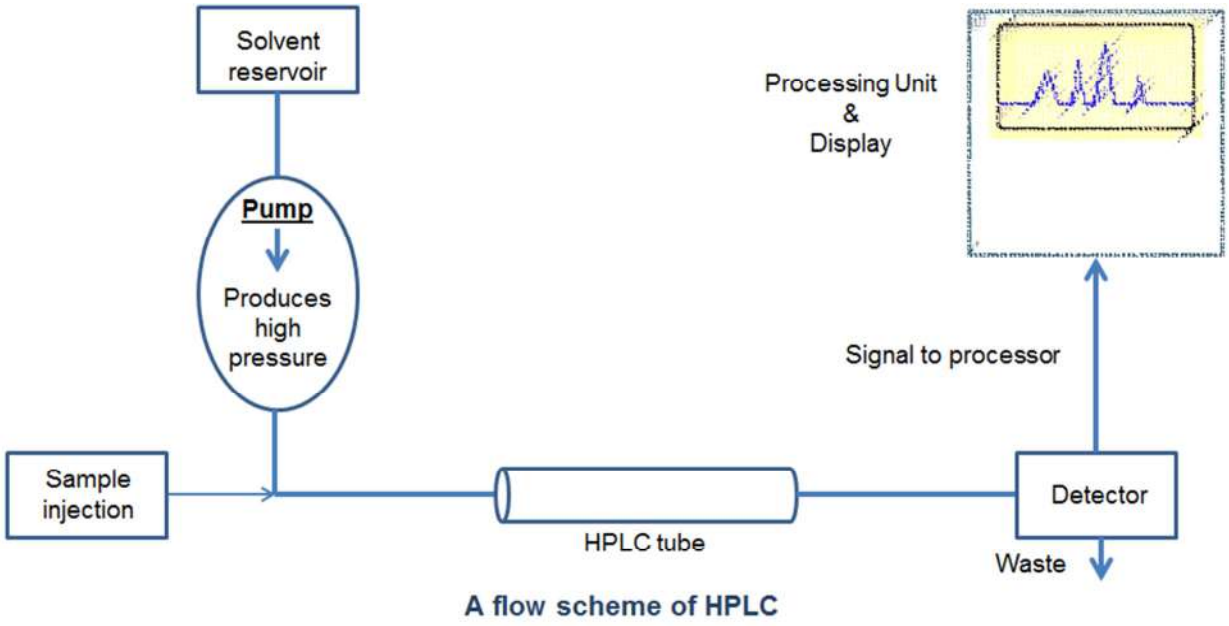
High performance liquid chromatography or HPLC is a type of column chromatography that pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material or stationary phase. It is an analytical technique used to separate, each component in a mixture which are later identified and quantified by spectroscopy. It is after the advent of column chromatography technique, the liquid chromatography (LC) with its high-pressure adapted metal columns was developed in 1960 to be used instead of low-pressure suitable glass column which led the conceptualization of high performance liquid chromatography. HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressure of up to 400 atmospheres.

In this chromatographic technique, the sample is carried by a moving carrier gas stream of helium or nitrogen. It has the ability to separate, and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion. Because of this versatility, HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals. Here, sample retention time will vary depending on the interaction between the stationary phase, the molecules being analyzed, and the solvent, or solvents used. As the sample passes through the column it interacts between the two phases at different rate, primarily due to different polarities in the analytes. Analytes that have the least amount of interaction with the stationary phase or the most amount of interaction with the mobile phase exit the column faster.

Principle of HPLC

The separation principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). The purification of components in a sample takes place in a separation column between a stationary and a mobile phase. The stationary phase is a granular material with very small porous particles in a separation column. The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column. The analyte is injected into the mobile phase flow from the pump to the separation column using a syringe via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel. Subsequently, the individual components of the analyte migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase. Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time “on-column”. Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved. A detection unit (e.g. UV detector) recognizes the analytes after leaving the column. The signals are converted and recorded by a data management system (computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units, a fraction collection unit or to the waste. In general, a HPLC system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and

a data processing unit as shown in figure below. The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. To keep the drift and noise of the detector signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve.



A flow scheme of HPLC

General instrumentation of HPLC

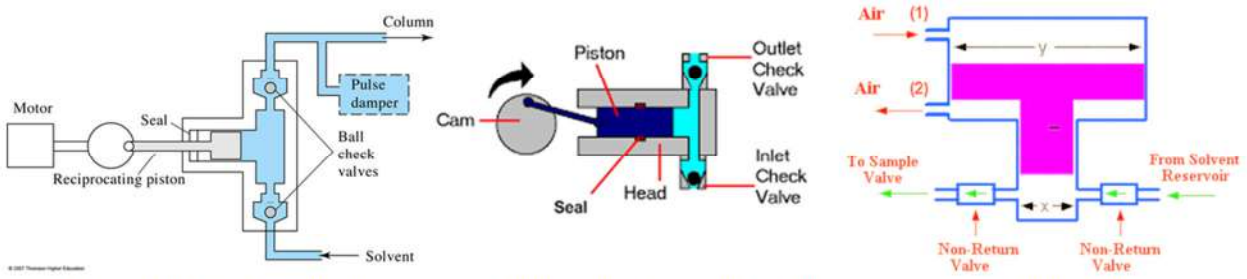
HPLC instrumentation is typically made up of seven main components; pump, mixing unit, solvent degassing, injector, column, detectors, and recorders.

Pump: The role of the pump is to force a liquid (mobile phase) through the liquid chromatography at a specific flow rate, expressed in millimeters per min (ml/min). Normal flow rates in HPLC are in the range of 1 – 2ml/min. During the chromatographic analysis, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient). It is considered to be best component for the analysis of complex samples. The pumps used in the HPLC system are of mainly three types; constant flow reciprocating pump, syringe type pump, and pneumatic pump.

Constant flow reciprocating pump: The term reciprocating describes any continuously repeated backwards and forward motion. As its name implied, reciprocating piston moves back and forth continuously and doing so, solvent is sucked during back stroke and gets delivered to the column in forward stroke. It is widely used type of pump in HPLC system.

Syringe or displacement type pump: It consists of large syringe like chamber. It is suitable for small bore column.

Pneumatic pump: In this type of pump used in the HPLC, gas is used to pressurize the mobile phase present in a collapsible solvent container.

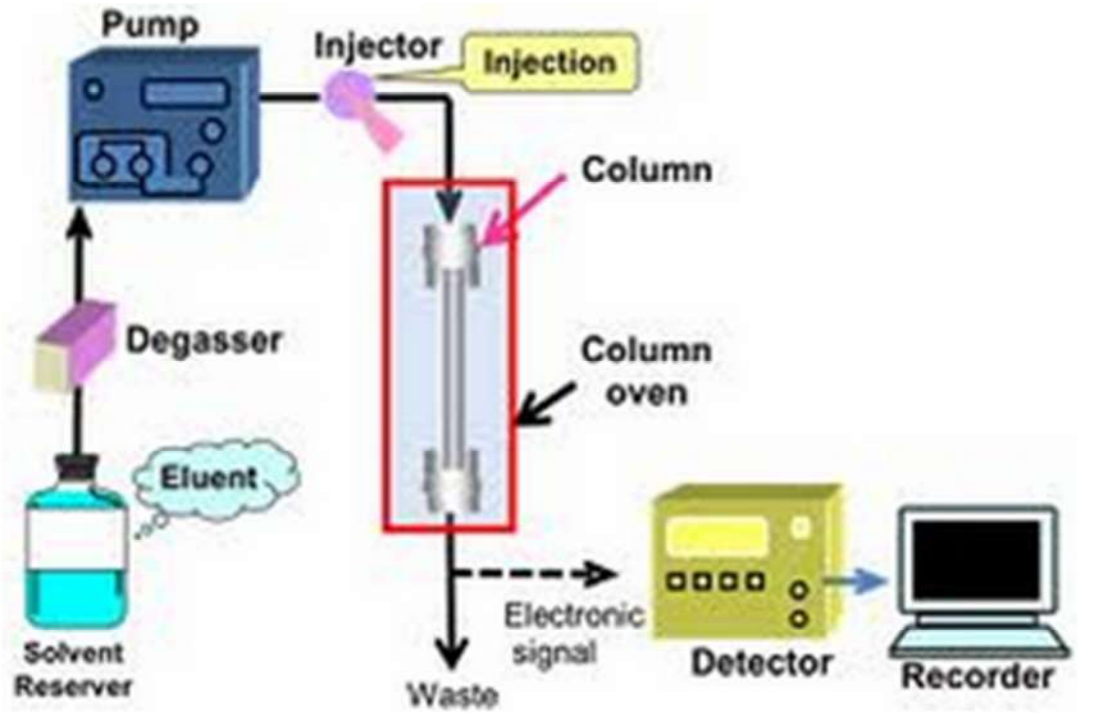


Constant Flow Reciprocating Pump | Syringe or Displacement Type Pump | Pneumatic Pump

Mixing Unit: It is used to mix the solvents in different proportions and pass through the column. There are two types of mixing units; low pressure mixing chamber unit and high pressure mixing chamber unit.

- Low pressure mixing chamber is uses helium for degassing the solvents.
- High pressure mixing chamber does not require helium for degassing solvents.

Mixing of solvents is done either with a **static mixer** which is packed with beads or a **dynamic mixer** which uses magnetic stirrer and operates under high pressure.



General set up of HPLC system

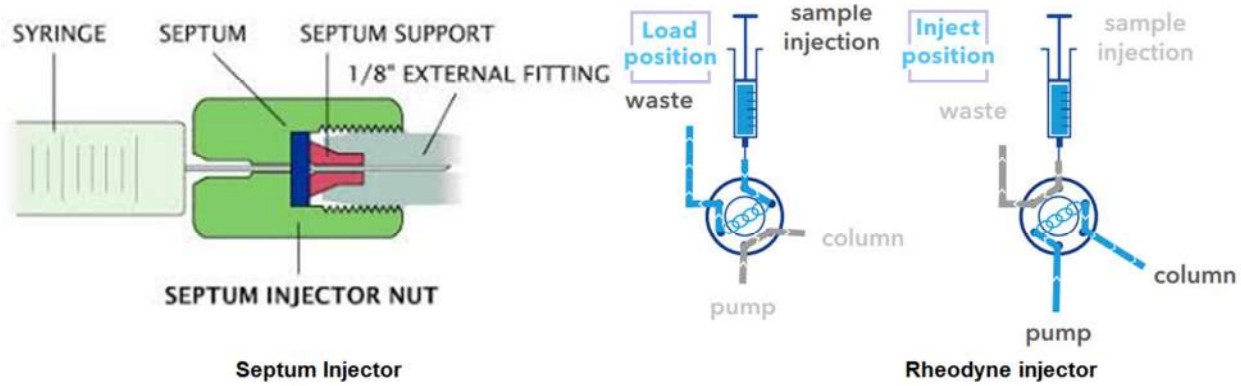
Source: https://www.pharmaguideline.com/2011/01/hplc-caibration.html.

Solvent degassing: several gases are soluble in organic solvents. When solvents are pumped under high pressure, gas bubbles are formed which interfere with the separation process, steady baseline and the shape of the peak, therefore degassing of solvents is necessary. The degassing of solvents is usually done by following techniques;

- ***Vacuum filtration:*** Through the utilization of vacuum in a solvents chamber, degassing is done. It can remove only air bubbles, but it is not always reliable and complete.
- ***Helium purging:*** By passing helium through the solvents, degassing is achieved. But, this method is expensive; however it is very effective method.
- ***Ultrasonification:*** By using Ultrasonicator, which converts ultra-high frequency to mechanical vibrations, degassing is sometimes done.

Injector: An injector is placed next to the pump. It serves to introduce the liquid sample into the mobile phase. Typical sample volumes are 5 – 20 microliters. The simplest method is to use a syringe, and the sample is introduced to the flow of eluent. The most widely used injection method is based on sampling loops. The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set of scheduled-timing. The autosampler is generally used when the user has many samples to analyze or when manual injection is not practical. There are generally three types of injector used in the HPLC system, which are;

- ***Septum injectors:*** For injecting the samples through a rubber septum, septum injectors are designed. This is not common, since the septum has to withstand high pressure of liquid flow.
- ***Stop flow:*** It is the type of injectors in which, the flow of mobile phase is stopped for a while and the sample is injected through a valve device.
- ***Rheodyne injector:*** This type of injector is the most common and popular injector. It has a fixed volume loop like 20µl or 50µl or more. Injector has 2 modes, i.e. load position when the sample is loaded in the lopp and inject mode when the sample is injected.



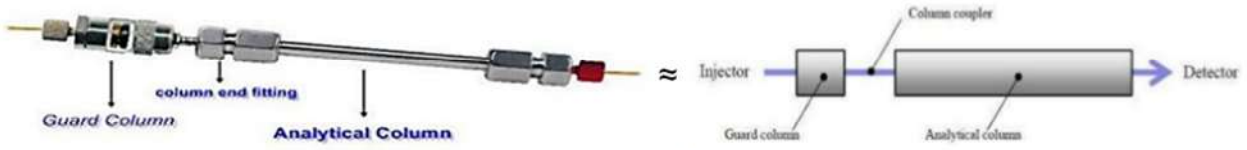
Septum Injector

Rheodyne injector

Column: It is said to be heart of the chromatogarphy. Columns of varied length is used in chromatographic techniques. Specification of column used in the HPLC system is as follwos;

- ***Length:*** It varies from 5cm to 30cm.
- ***Diameter:*** It varies from 2mm to 50mm.
- ***Partcile size:*** It varies from 1µm to 20µm.
- ***Particle nature:*** Spherical, uniform sized, porous materials are used.
- ***Materials of construction for the tubing:*** Generally, stainless steel is used because of its property to withstand high pressure. However, glass is also utilized for making column but it is usually restricted to biomolecules. A new materal PEEK (poly ether ether ketone) polymer is used due to its biocompatibility and chemically inertness to most solvents.

- ***Packing material:*** The packing material is prepared from silica particle, alumina particle and ion exchange resin. Porous plug of stainless steel or teflon are used in the end of the columns to remain the packing material.



Column of HPLC system

Detector: Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation. The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences. This difference is monitored as a form of an electronic signal. There are different types of detectors available, which are following;

UV detectors

- Refractive index detectors
- Flourimetric detectors
- Conductivity detectors
- Amperometric detectors

Recorder: The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes. In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer-based data processor (integrator) is more common. There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only performs data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

Types of HPLC technique

There are many ways to classify liquid column chromatography. It is mainly classified on the basis of mode of chromatography, principle of separation, elution techniques, scale of operation, and type of analysis. The types of HPLC are following;

Classification of HPLC technique on the basis of mode of chromatography: It is of mainly two types; normal phase mode and reverse phase mode.

- ***Normal phase HPLC:*** In this type of HPLC, the stationary phase is polar in nature and the mobile phase is nonpolar. It is generally not opted in pharmaceutical application since most of the drug molecules are polar in nature and takes longer time to be eluted and detected.
- ***Reverse phase HPLC:*** In this type of HPLC technique, the stationary phase is nonpolar and the mobile phase is polar in nature. Unlike to normal phase HPLC technique, this

technique is also applied for quantitative analysis of most of the drugs and pharmaceuticals since in this technique, they are not retained for a longer time and eluted faster.

Classification of HPLC technique on the basis of principle of separation: It is of three types; adsorption chromatography, ion exchange chromatography and size exclusion chromatography.

- ***Adsorption HPLC chromatography:*** In this technique of HPLC, separation of components takes place because of the difference in affinity of compounds towards stationary phase.
- ***Ion exchange HPLC chromatography:*** An ion is used to separate a mixture of similar charged ions in this type of HPLC.
- ***Size exclusion or gel permeation chromatography:*** In this technique, a mixture of components with different molecular sizes is separated by using gels which acts as sieve.

Classification of HPLC technique on the basis of techniques of elution: Based on elution techniques, it is classified into two types; isocratic separation HPLC and gradient separation HPLC.

- ***Isocratic separation HPLC:*** In this type of HPLC, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.
- ***Gradient separation HPLC:*** In this technique, a mobile phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.

Classification of HPLC technique on the basis of scale of operation: Based on the scale of operation, it is classified into two types; analytical HPLC and preparative HPLC.

- ***Analytical HPLC:*** In this technique, only analysis of the samples is done and recovery of the samples is not performed.
- ***Preparative HPLC:*** In this technique, the individual fractions of pure compound can be collected using fraction collector. The collector samples are reused.

Classification of HPLC technique on the basis of type of analysis: Based on the type of analysis, it is classified into two types; qualitative analysis HPLC and quantitative analysis HPLC.

- ***Qualitative analysis HPLC:*** This type of HPLC is used to identify the compound, to detect the impurities, to find the number of components, etc.
- ***Quantitative analysis HPLC:*** This type of HPLC is done to determine the quantity of the individual or several components in a mixture. This can be done by comparing peak area of the standard and sample.

Classification of HPLC technique on the basis of type of analysis: Based on the type of analysis, it is classified into two types; qualitative analysis HPLC and quantitative analysis HPLC.

Procedure: It is formulated for caffeine in a in a diet soda[1].

1. **Making the Mobile Phase**
    a. Prepare the mobile phase by adding 400ml of acetonitrile to approximately 1.5 liter of purified deionized (DI) water.
    b. Carefully add 2.4ml of glacial acetic acid to this solution.
    c. Dilute the solution to a total volume of 2.0 liter in a volumetric flask with purified DI water. The resulting solution should have a pH between 2.8 to 3.2.
    d. Adjust the pH to 4.2 by adding 40% sodium hydroxide, drop-wise with the use of a calibrated digital pH meter. Add very slowly once the pH reaches 4.0. This should take around 50 drops to accomplish.
    e. Filter the mobile phase through a 0.47-µm Nylon 66 membrane filter under vacuum to degas the solution and to remove solids that could plug the chromatographic column. It is important to degas the mobile phase to avoid having a bubble, which could either cause a void in the stationary phase at the inlet of the column or work its way into the detector cell, causing instability with the UV absorbance.
2. **Creating the component solutions as for example for caffeine:** Three components that need to be made are caffeine (0.8mg/ml), potassium benzoate (1.4mg/ml), and aspartame (L-aspartyl-L-phenylalanine methyl ester) (6.0mg/ml). These concentrations, once diluted in the same fashion, put the standards at the levels found in the soda samples.

    a. Add 0.40g of caffeine to a 500ml volumetric flask, then dilute to the 500ml mark with DI water.
    b. Add 0.70g of benzoate to a 500ml volumetric flask, then dilute to the 500ml mark with DI water.
    c. Add 0.60g of aspartame to a 100ml volumetric flask, then dilute to the 100ml mark with DI water. Place this solution in a refrigerator to avoid decomposition during storage.

3. **Making the seven standard solutions**: Three components all have different distribution coefficients which affects the interaction with both of the phases. The larger the distribution coefficient, the more time the component spends in the stationary phase, resulting in longer retention times in reaching the detector.

    a. Following the chart in figure below, pipet the proper amount of each component into a 50ml volumetric flask.
    b. Dilute each of the stock solutions to the 50-mL mark on the volumetric flasks with mobile phase.
    c. Pour each standard solution into labeled small vials in a sample rack.

[1] https://www.jove.com/t/10156/high-performance-liquid-chromatography-hplc

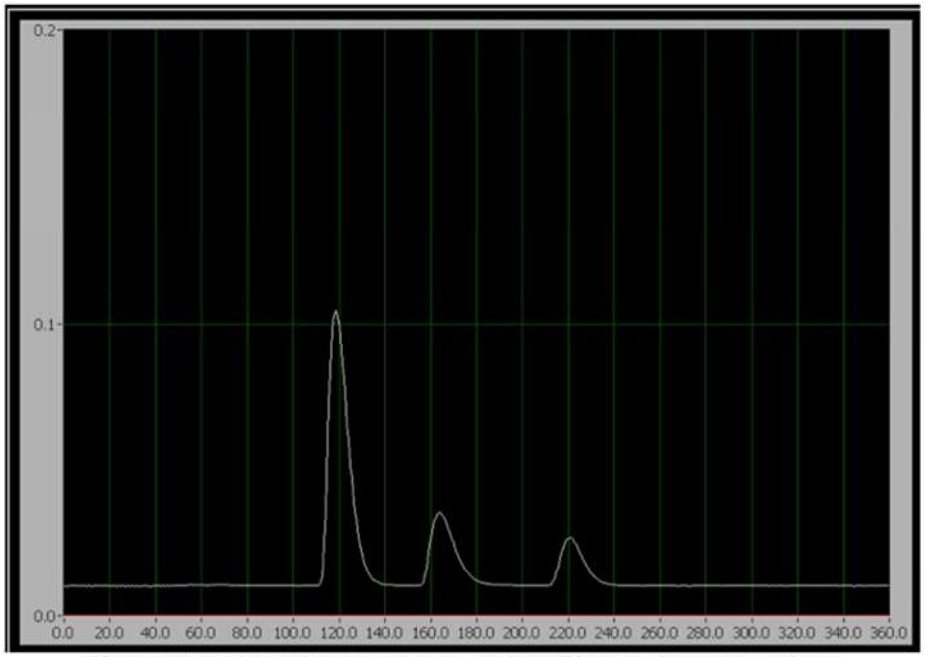
d. Store the racks of samples in a refrigerator, along with the remaining solutions in the 50ml volumetric flasks.

Number	Caffeine (ml)	Benzoate (ml)	Aspartame (ml)
1	4	0	0
2	0	4	0
3	0	0	4
4	1	1	1
5	2	2	2
6	3	3	3
7	5	5	5

Volumes of stock standards used to prepare the seven provided working standards (total volume of each standard is 50ml).

4. **Checking the initial settings of the HPLC system**
    a. Confirm that the waste line is in a waste container and is not recycling back into the mobile phase.
    b. Verify that the flow rate of the mobile phase is set to 0.5 mL/min. This is high enough to allow all peaks to elute within 5 min and slow enough to allow for nice resolution.
    c. Verify that the minimum and maximum pressure and the flow rate are set to the correct values on the front panel of the solvent delivery system (the pump).
        i. Minimum pressure setting: 250 psi (this is to shut off the pump, if a leak occurs).
        ii. Maximum pressure setting: 4,000 psi (this is to protect the pump from breaking, if a clog forms).
    d. Press "zero" on the detector's front panel in order to set the blank (the blank is the pure mobile phase).
    e. Rinse a 100-µL syringe with deionized water, then with several volumes of one of the working standards to be analyzed, and fill the syringe with that solution. Start with the 3 single-component samples, which allows for identifying the peak of each component of interest.
5. **Manually injecting the sample and data collection**
    a. With the injector handle in the load position, slowly inject 100µl of solution through the septum port.
    b. Verify that the data collection program is set to collect data for 300s, which allows enough time for all 3 peaks to elute through the detector.

c. When ready to start the trial, rotate the injector handle to the inject position (which injects the sample into the mobile phase) and click "Start Trial" on the computer data collection program immediately. For standards 1-3, only one of the three sequential peaks appears on the screen during the run (see figure given below).
d. Once 300 s have passed, the data collection sends a prompt to save the data file. Save the data under a suitable file name (*e.g.*, STD#1).
e. Note the time in seconds for the peak of each trial, which is used in identifying that component.
f. Remove the syringe from the septum and repeat the process for each of the remaining working standards, using the same time per chromatogram as determined from the first run.



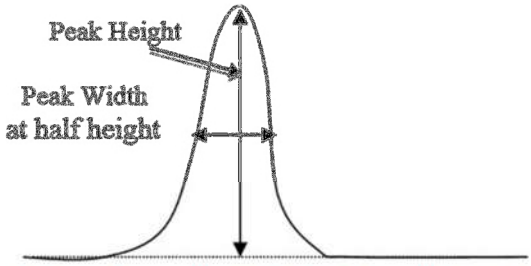
Chromatogram of three components of HPLC analysis of caffeine

6. **The samples of diet sodas:** Diet Coke, Diet Pepsi, and Coke Zero are the "unknowns". They have been left out in open containers overnight to get rid of the carbonation, as bubbles are not good for the HPLC system. This sufficiently gets rid of any gases in the samples.

a. Draw around 2ml of the diet soda into a plastic syringe.
b. Attach the filter tip to the syringe via Luer-Lok by twisting it in place.
c. Push the liquid in the syringe through the filter and into a small glass vial. This gets rid of unwanted particulates that could potentially clog the separation column.
d. Dilute each sample with an equal amount of DI water, so they are at 50% purity.
e. Inject 100µl of the sample into the sample loop, and run trials with the same parameters as for the standards.

7. Calculations

a. From the concentrations of the component solutions, calculate the concentration of all of the components in the standards, based upon the dilutions that were made for the seven samples.
b. Determine a peak area on the chromatograms for each standard and the unknown samples by the triangular method, which equals peak height times the width at ½ heights. After determining which peak corresponds to each component based upon the time it takes for each component to show their respective peak, enter these peak areas into a computer spreadsheet.



A basic example of a curve's peak height and width, which are to be multiplied (peak height times width at ½ height)

c. Create calibration curves of peak area vs. concentration (mg/l) in the standards for all three components.
d. Determine the least-squares fit for each calibration curve.
e. Calculate the concentration of each component in the diet sodas from the peak areas shown from the HPLC trials for the samples. Remember that the diet soda was diluted by a factor of 2 prior to injecting into the HPLC system.
f. Calculate the amount, in mg/l, of each component in the diet sodas.
g. Based upon the results, calculate the milligrams of each component found in a 12oz can of soda. Assume 12oz = 354.9ml.

Applications of HPLC

HPLC is a widely used technique in the separation and detection for many applications in almost all areas of chemistry, biochemistry and pharmacy. Some of the applications are as follows;

- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals
- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components
- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

Reference

1. https://www.chemguide.co.uk/analysis/chromatography/hplc.html

2. https://www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=en_US
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