High-Performance Liquid Chromatography (HPLC)

- High performance liquid chromatography (HPLC) is a chromatographic technique that uses a solvent under pressure to separate, identify, or quantify substances in a mixture.
- An analytical separation technique that involves the high-pressure flow of a liquid through a column that contains the stationary phase.
  Mobile phase: Liquid
  Stationary phase: Can be a solid (LSC) or a liquid (LLC)
- Liquid chromatography is a separation technique that involves: •The placement (injection) of a small volume of liquid sample into a tube packed with porous particles (**stationary phase**) where individual components of the sample are transported along the packed tube (column) by a liquid moved by gravity.

1- The components of the sample are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.

2- The separated components are collected at the exit of this column and identified by an external measurement technique, such as a spectrophotometer that measures the intensity of the color, or by another device that can measure their amount.

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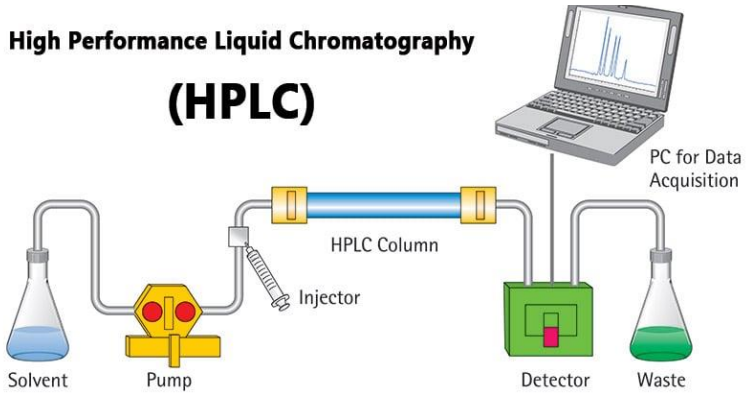


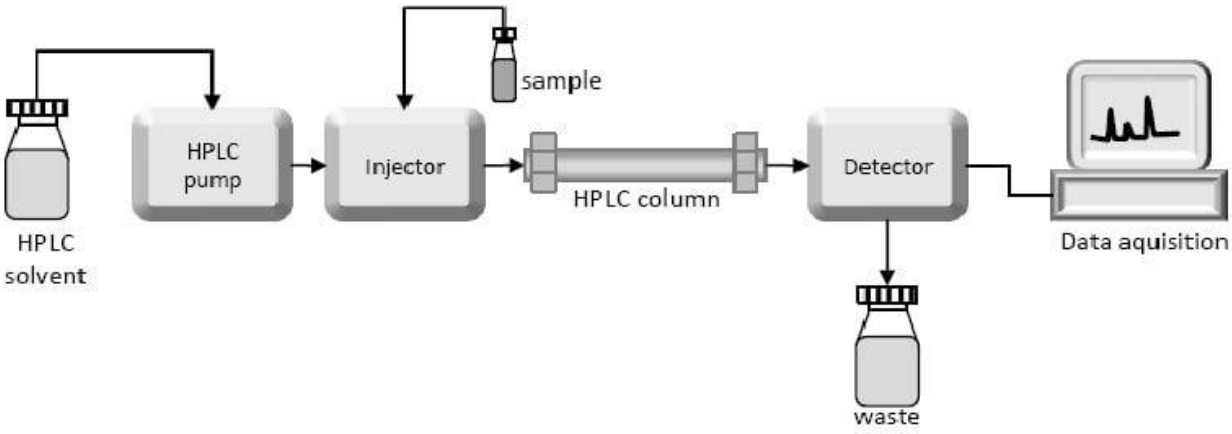
Comparison with GC:

Parameter	GC	HPLC
Basis of separation	Interaction of solutes with the stationary phase.; solute vapor pressure	Interaction of solutes with both the stationary phase. and mobile phase
Analysis time	Fast (a few minutes for simple mixtures)	Slower than GC (several minutes for a simple mixture
Temperature for separation	Usually requires a high temperature (>40 0C)	Usually, a room temperature technique
Applications	Separation of volatile and thermally stable compounds - cannot be used for high MW and highly polar compounds	Separation of a wider range of compounds, high MW, polar, and ionic compounds

Principle of High-Performance Liquid Chromatography (HPLC)

- The purification 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.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.
- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.
- The chromatogram allows the identification and quantification of the different substances.

Instrumentation of High-Performance Liquid Chromatography (HPLC)



-Major components:

A) Solvent or mobile phase

- ❖ Usually, a mixture of an organic solvent (Ex. methanol, IPA) and water.
- ❖ Solvent polarity affects the separation process.
- ❖ Sometimes buffered - keeps solutes in electrically neutral form.

Mobile phase considerations Must be filtered (to prevent tiny solids from depositing at the column head) and degassed

- ✓ Bubbles could interfere with detection.
- ✓ Degassing is done by helium sparging.

B) pump

- ✓ Role is to pump the solvent at a high pressure (usually from 1000 to 6000 psi) through the packed column
- ✓ **Pump Module–types**:

1. **Isocratic pump** -delivers constant mobile phase composition; •solvent must be pre-mixed; •lowest cost pump
2. **Gradient pump** -delivers variable mobile phase composition; •can be used to mix and deliver an isocratic mobile phase or a gradient mobile phase.
   a. Binary gradient pump –delivers two solvents.
   b. Quaternary gradient pump –four solvents.

C)Sample introduction system

- ✓ Usually a loop injector .
- ✓ Introduces the injected sample to the flowing mobile phase
- ✓ Automated injectors are common

D) Column

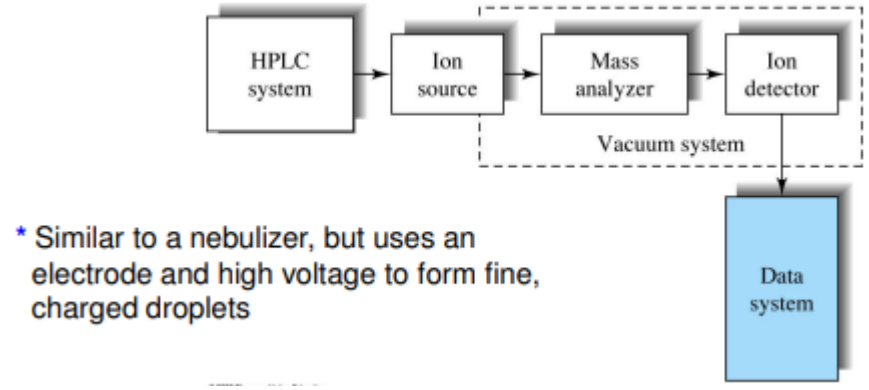
- ✓ A small metal tube (typically 5 to 30 cm long; 1-5 mm i.d.) that contains the stationary phase (Cont.)
- ✓ Role is to separate the components of a mixture

Much shorter than columns used in GC --- Why?

- ❖ Highly efficient separations achieved in HPLC due to interactions of both m.p. and **s**.p. with the components of a mixture.
- ✓ No need for long columns
- ✓ vs. GC, where only the s.p. interacts with components.

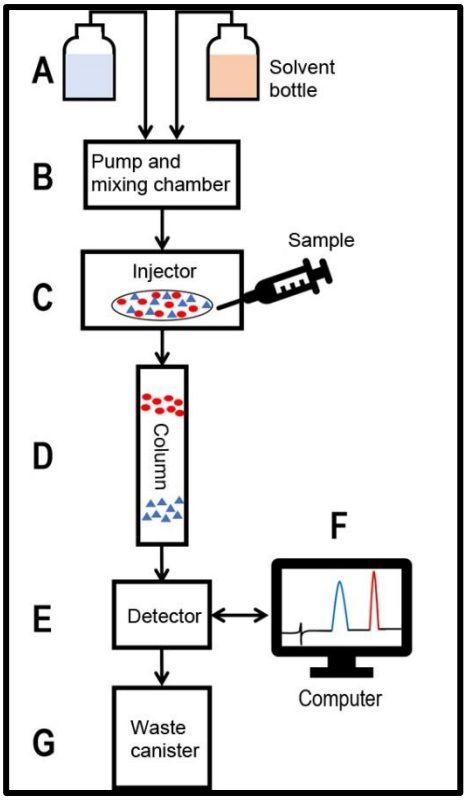
E. Detector:

- ❖ Different design from those of GC detectors because the components are dissolved in a liquid m.p. (vs. gas in GC)
- ❖ The detector can see (detect) the individual molecules that come out (elute) from the column.
- ❖ A detector serves to measure the amount of those molecules so that the chemist can quantitatively analyze the sample components.
- ❖ The detector provides an output to a recorder or computer that results in the liquid chromatogram (i.e., the graph of the detector response).
- ❖ **UV detectors** – most common UV absorption cell for HPLC Applications: Respond to substances that absorb light in the range 180 to 350 nm Z-shaped flow cell - > more time for UV light to pass th/ π systems (aromatics, alkenes, alkynes) Carbonyls.



F.Computer:

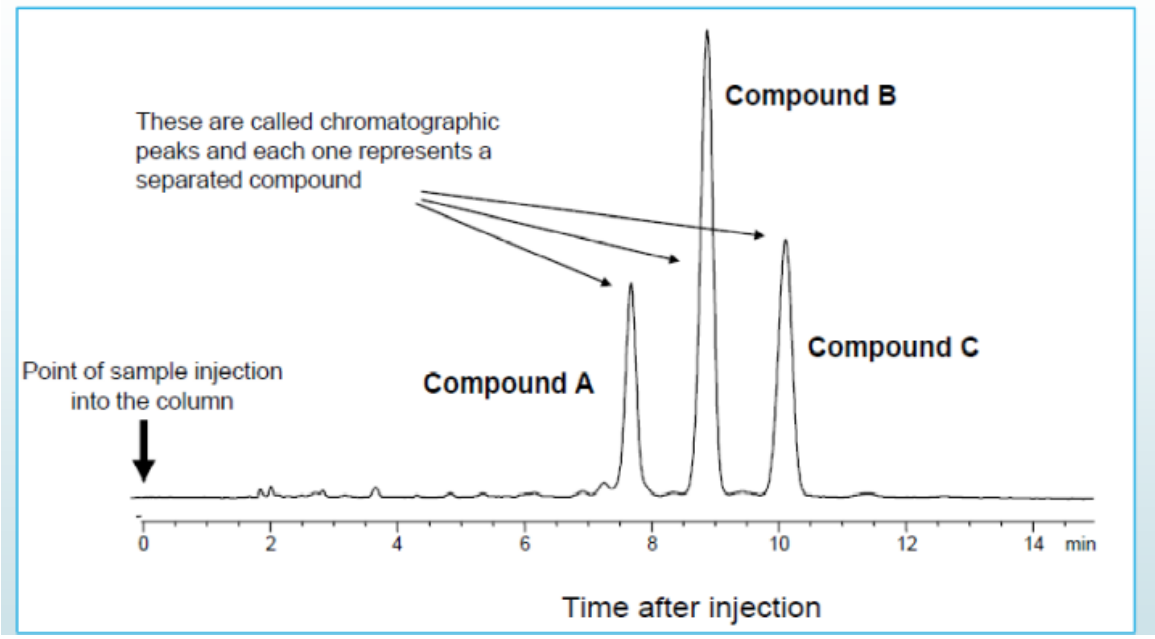
Frequently called the **data system**, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (**retention time**) of the sample components (**qualitative analysis**) and the amount of sample (**quantitative analysis**).



How HPLC technique Work

1- The injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (µm) in diameter called the stationary phase)
2- Where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump
3- These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.
4- These separated components are detected at the exit of this tube (column) by a flow-through device (**detector**) that measures their amount.
5- An output from this detector is called a **"liquid chromatogram".**
6- In principle, LC and HPLC work the same way except the speed, efficiency, sensitivity and ease of operation of HPLC is vastly superior

What Does a Liquid Chromatogram Look Like



This is the chromatogram resulting from the injection of a small volume of liquid extracted from a vitamin E capsule that was dissolved in an organic solvent. Modern HPLC separations usually require 10-to 30-minutes each.

What is HPLC used for?

- ✓ Separation and analysis of non-volatile or thermally-unstable compounds .
- ✓ HPLC is optimum for the separation of chemical and biological compounds that are non-volatile.
- ✓ **NOTE:** If a compound is volatile (i.e. a gas, fragrance, hydrocarbon in gasoline, etc.), Gas chromatography is a better separation technique.

Typical non-volatile compounds are:

1. Pharmaceuticals like aspirin, ibuprofen, or acetaminophen (Tylenol)
2. Salts like sodium chloride and potassium phosphate
3. Proteins like egg white or blood protein
4. Organic chemicals like polymers (e.g. polystyrene, polyethylene)
5. Heavy hydrocarbons like asphalt or motor oil
6. Many natural products such as ginseng, herbal medicines, plant extracts
7. Thermally unstable compounds such as trinitrotoluene (TNT), enzymes , hormones

Qualitative analysis by HPLC

The identification(ID) of individual compounds in the sample; the most common parameter for compound ID is its retention time(the time it takes)for that specific compound to elute from the column after injection); depending on the detector used, compound ID is also based on the chemical structure, molecular weight or some other molecular parameter

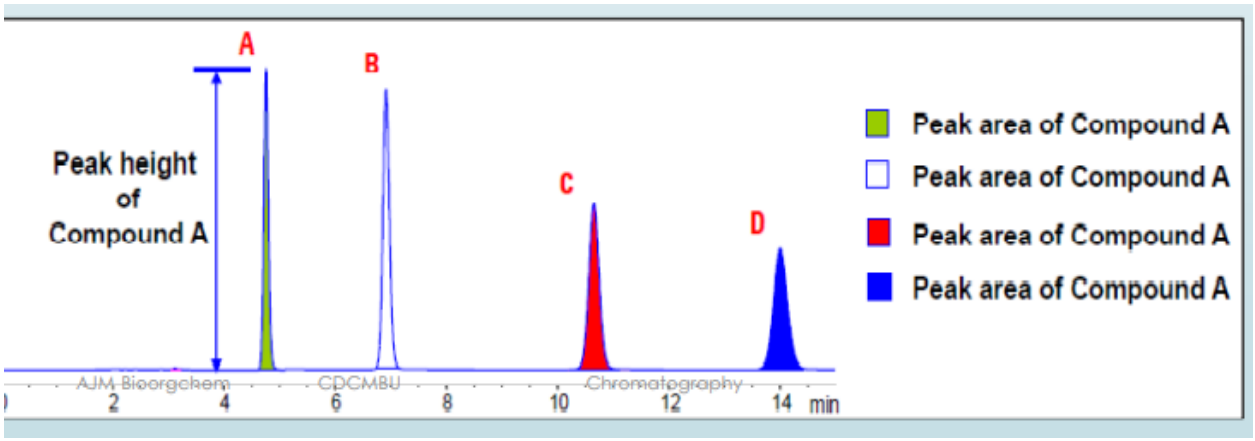
Quantitative analysis by HPLC

There are two main ways to interpret a chromatogram (i.e. perform quantification):

a. determination of the peak height of a chromatographic peak as measured from the baseline.

b. determination of the peak area (see figure below).

In order to make a quantitative assessment of the compound, a sample with a known amount of the compound of interest is injected and its peak height or peak area is measured. In many cases, there is a linear relationship between the height or area and the amount of sample



. Preparation of Pure Compound(s)

preparative chromatography.

1- By collecting the chromatographic peaks at the exit of the detector,
2- and concentrating the compound (analyte) by removing/evaporating the solvent,
3- a pure substance can be prepared for later use (e.g. organic synthesis, clinical studies, toxicology studies, etc.).