Republic of Iraq
Ministry of Higher Education & Scientific research
Al-Mustaqbal University
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Biochemistry Department

Analytical Chemistry Instrumental Analysis

For

Second Year Student/course 1

Lecture 10

By

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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.

Components of HPLC

1. Solvent Reservoir

2. Pumps

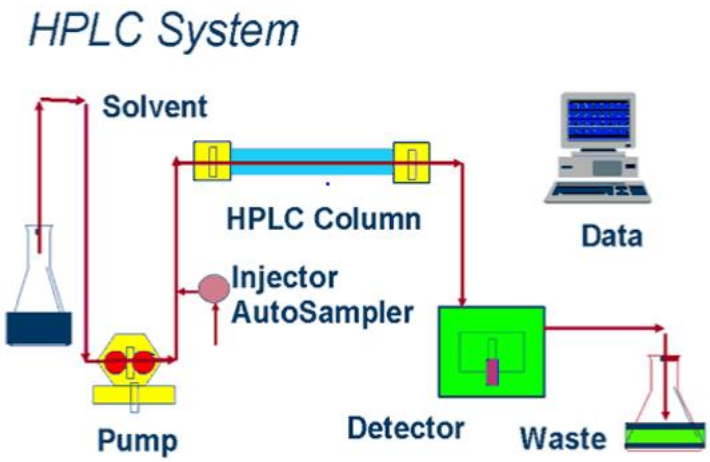
3. Sample Injection System

4. Columns

5. Detectors

6. Data Processing

7. Waste



Types of HPLC Retention Time

1. **Normal–phase chromatography**
2. **Reversed-phase chromatography**

- Normal–phase chromatography was one of the first kinds of HPLC that chemists developed. Also known as normal-phase HPLC (NP-HPLC) this method separates analytes based on their affinity for a polar stationary surface such as silica

- NP-HPLC uses a non-polar, non-aqueous mobile phase (e.g. Chloroform), and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase.
- Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase , moderately polar mobile phase. One common stationary phase is a silica which has been surface-modified with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis). An investigator can increase retention times by adding more water to the mobile phase.

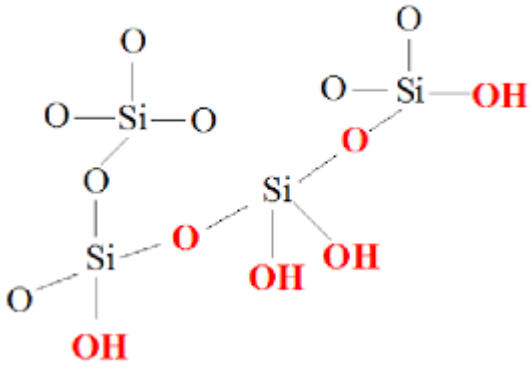
Stationary Phases

• Polar ("Normal" Phase):

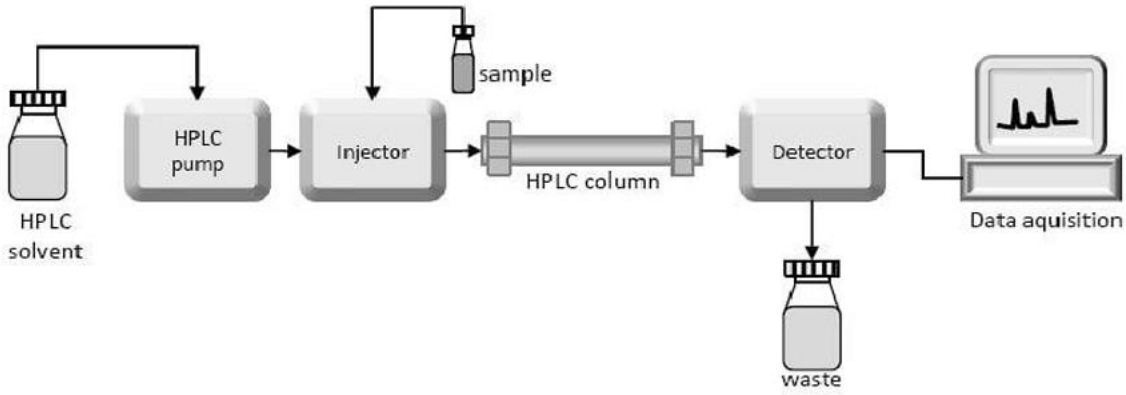
– Silica, alumina

• Non-Polar ("Reversed Phase")

– ODS Silica gel – C18, C8



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

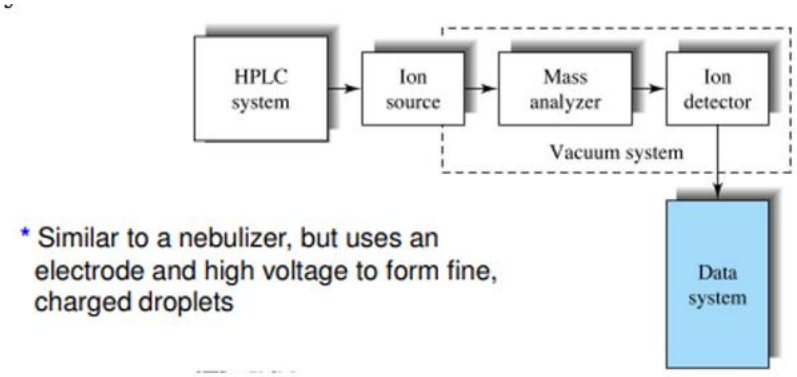
D) Column

- ➢ 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

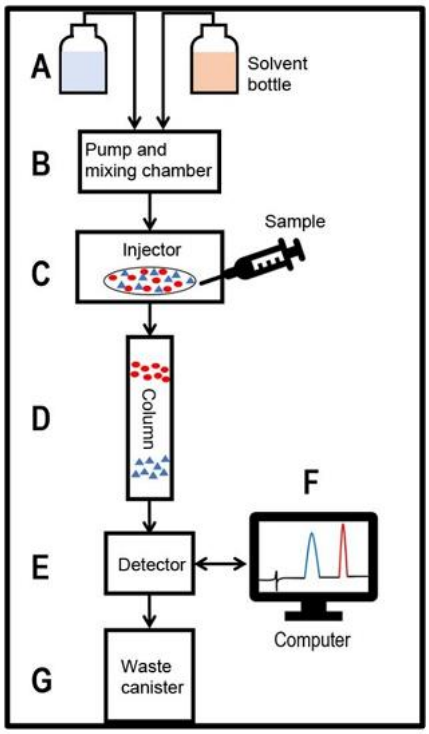
E) Detector:

- o Different design from those of GC detectors because the components are dissolved in a liquid m.p. (vs. gas in GC)
- o 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.

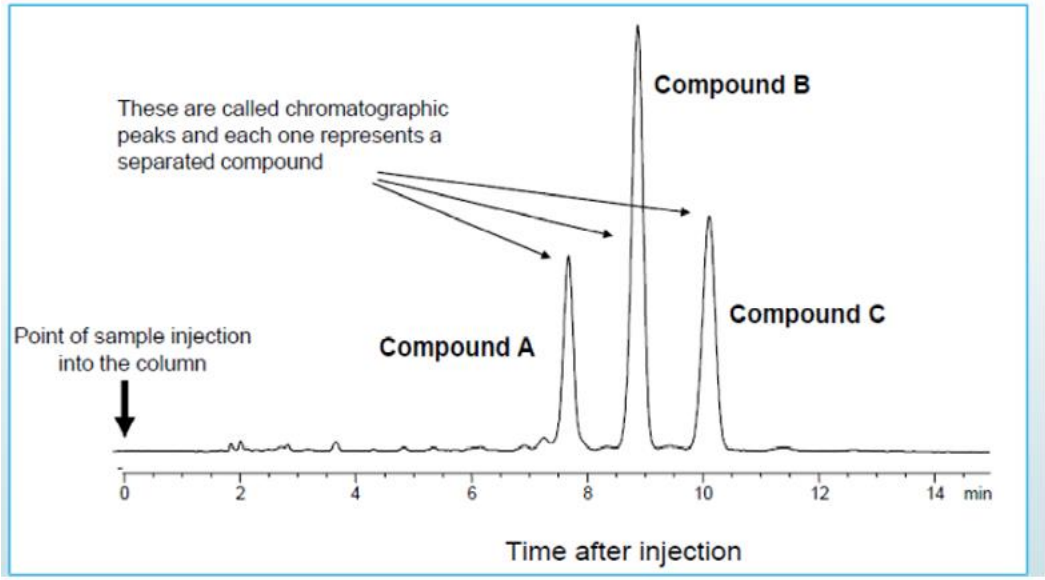


F) Computer: Frequently called the data system, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative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



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.

The factors which influence the HPLC performance

1. Internal diameter of column - the smaller in diameter, the higher in sensitivity
2. Pump pressure - the higher in pressure, the higher in separation
3. Sample size
4. The polarity sample, solvent and column

5. Temperature

- the higher in temperature, the higher in separation

Advantages

1. Needs a small sample with a high accuracy and precis
2. Non-destructed sample during operation compared to GC.

Disadvantages

1. Need a skill to run the instruments
2. Solvents consuming

Comparison between LC and GC Techniques:

Gas Chromatography (GC)	Liquid Chromatography (LC)
1. Stationary phase: Solid/liquid	1. Stationary phase: Solid/liquid
2. Mobile phase: GAS	2. Mobile phase: LIQUID
3. Mobile phase does not take part in separation	3. Mobile phase takes active part in separation
4. Volatile organic/inorganic compounds only	4. Volatile and non-volatile compounds can be separated
5. Works at comparatively low pressure	5. Works at high pressure
6. Works on packed as well as capillary columns	6. Only packed columns are used
7. Fast and better efficiency obtained	7. Slow and poor efficiency
8. Selective columns for applications	8. Very few selective columns available
9. Range of selective detectors available for application	9. Few selective detectors available
10. Environmental friendly technique	10. Solvents need proper disposal (less environmentally friendly)