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Understanding HPLC: The Science Behind the Technique

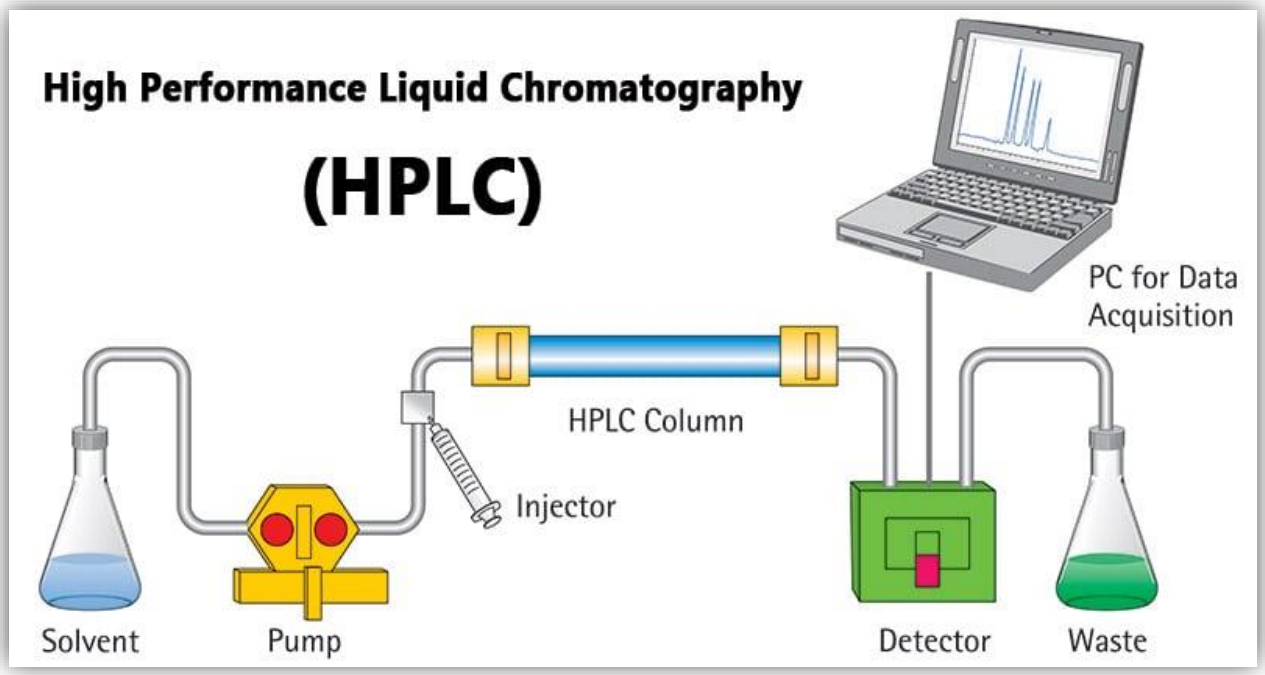
Introduction

High-performance liquid chromatography or commonly known as HPLC, is an analytical technique used to separate, identify or quantify each component in a mixture

The mixture is separated using the basic principle of column **chromatography** and then identified and quantified by spectroscopy.

In the 1960s, the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.

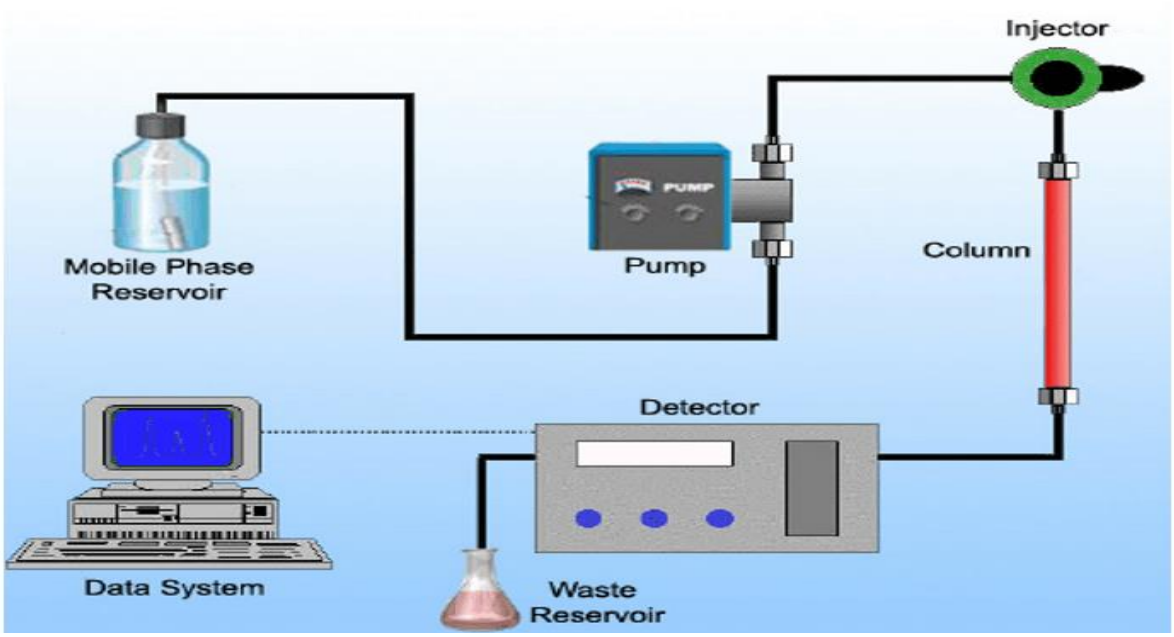
HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.



HPLC Principle

- 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 HPLC



Function of HPLC

The Pump

- The development of HPLC led to the development of the pump system.
- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system.
- High-pressure generation is a “standard” requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.
- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces “pulses”.

Injector

- An injector is placed next to the pump.
- 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 scheduled-timing.

Column

- The separation is performed inside the column.
- The recent columns are often prepared in a stainless steel housing, instead of glass columns.
- The packing material generally used is silica or polymer gels compared to calcium carbonate. The eluent used for LC varies from acidic to basic solvents.
- Most column housing is made of stainless steel since stainless is tolerant towards a large variety of solvents.

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.

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 data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes.

- When gas is present in the eluent, this is detected as noise and causes an unstable baseline.
- Degasser uses special polymer membrane tubing to remove gases.
- The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore.

Column Heater

The LC separation is often largely influenced by the column temperature.

- In order to obtain repeatable results, it is important to keep consistent temperature conditions.
- Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C).
- Thus columns are generally kept inside the column oven (column heater).

Types of HPLC

High-Performance Liquid Chromatography (HPLC) is a powerful and widely used analytical technique in the field of chemistry and biochemistry. It enables the separation, identification, and quantification of components in a mixture. HPLC is preferred for its high resolution, sensitivity, and ability to handle complex samples. Here, we delve into the specifics of different types of HPLC, their mechanisms, and their applications.

1. Normal-Phase HPLC (NP-HPLC)

Mechanism:

- **Stationary Phase:** Polar, often silica.
- **Mobile Phase:** Non-polar, such as hexane or chloroform.
- **Principle:** Analytes are separated based on their polarity. Polar compounds have stronger interactions with the polar stationary phase and elute slower, while non-polar compounds elute faster.

Applications:

- Separation of isomers.
- Analysis of lipids and fats.
- Separation of small organic molecules.

Advantages:

- Good for separating compounds with different polarities.
- Effective for hydrophilic compounds.

Disadvantages:

- Limited to non-polar solvents.
- Less commonly used compared to reverse-phase HPLC due to solvent limitations.

2. Reverse-Phase HPLC (RP-HPLC)

Mechanism:

- **Stationary Phase:** Non-polar, often C18 or C8 chains bonded to silica.
- **Mobile Phase:** Polar, typically water mixed with methanol or acetonitrile.
- **Principle:** Non-polar compounds interact more strongly with the non-polar stationary phase and elute slower, while polar compounds elute faster.

Applications:

- Widely used in pharmaceutical analysis.
- Separation of peptides and proteins.
- Analysis of environmental samples.

Advantages:

- Versatile and suitable for a wide range of compounds.
- Compatible with aqueous and organic solvents.
- High reproducibility and robustness.

Disadvantages:

- May require gradient elution for complex mixtures.
- Limited efficiency for very hydrophilic compounds.

3. Size-Exclusion HPLC (SEC or GPC)

Mechanism:

- **Stationary Phase:** Porous particles that separate molecules based on size.
- **Mobile Phase:** Typically aqueous or organic solvent, depending on the analytes.
- **Principle:** Larger molecules elute first as they are excluded from the pores, while smaller molecules enter the pores and elute later.

Applications:

- Analysis of polymers.
- Protein purification.
- Characterization of molecular weight distributions.

Advantages:

- No chemical interaction between the stationary phase and analytes.
- Gentle on samples, preserving their native state.

Disadvantages:

- Limited to size-based separations.
- Lower resolution compared to other HPLC types.

4. Ion-Exchange HPLC (IE-HPLC)

Mechanism:

- **Stationary Phase:** Charged functional groups attached to a solid support.
- **Mobile Phase:** Aqueous buffer with varying pH or ionic strength.
- **Principle:** Analytes are separated based on their charge. Oppositely charged analytes are attracted to the stationary phase and elute based on their interaction strength and the mobile phase conditions.

Applications:

- Separation of amino acids and peptides.
- Analysis of proteins and nucleotides.
- Purification of charged biomolecules.

Advantages:

- High selectivity for charged compounds.
- Ability to separate closely related substances.

Disadvantages:

- Requires careful control of pH and ionic strength.
- Not suitable for neutral compounds.

5. Affinity HPLC

Mechanism:

- **Stationary Phase:** Ligands or antibodies specific to the target analyte.
- **Mobile Phase:** Aqueous buffer or gradient elution.

- **Principle:** Specific interactions between the analyte and the ligand immobilized on the stationary phase lead to high selectivity. Elution is achieved by changing the mobile phase conditions to disrupt the interaction.

Applications:

- Purification of enzymes and antibodies.
- Analysis of biomolecular interactions.
- Isolation of specific proteins from complex mixtures.

Advantages:

- Extremely high selectivity.
- Capable of purifying target molecules from complex matrices.

Disadvantages:

- Limited to analytes with specific binding partners.
- Ligand immobilization can be complex and expensive.

6. Chiral HPLC

Mechanism:

- **Stationary Phase:** Chiral selectors or derivatized chiral molecules.
- **Mobile Phase:** Aqueous or organic solvents.
- **Principle:** Chiral stationary phases interact differently with enantiomers, leading to their separation based on their stereochemistry.

Applications:

- Separation of enantiomers in pharmaceuticals.
- Analysis of chiral pesticides.
- Resolution of racemic mixtures.

Advantages:

- Essential for the analysis of chiral compounds.
- High resolution and selectivity for enantiomers.

Disadvantages:

- Chiral stationary phases can be expensive.
- Limited to chiral analytes.

7. Hydrophilic Interaction Chromatography (HILIC)

Mechanism:

- **Stationary Phase:** Polar, such as silica or bonded polar groups.
- **Mobile Phase:** Mixture of water and organic solvents like acetonitrile.
- **Principle:** Combines elements of normal-phase and reverse-phase HPLC. Polar analytes have stronger interactions with the stationary phase and elute slower, while less polar analytes elute faster.

Applications:

- Analysis of highly polar compounds.
- Metabolomics and glycomics.
- Separation of nucleotides and amino acids.

Advantages:

- Effective for polar compounds that are poorly retained in reverse-phase HPLC.
- Compatible with mass spectrometry detection.

Disadvantages:

- Requires high organic solvent content, which can be costly.
- Optimization can be challenging due to the dual retention mechanism.

8. Fast Protein Liquid Chromatography (FPLC)

Mechanism:

- **Stationary Phase:** Various resins, including ion-exchange, size-exclusion, and affinity resins.
- **Mobile Phase:** Aqueous buffers.
- **Principle:** Designed for the purification of proteins and other large biomolecules. Operates at lower pressures and uses mild conditions to preserve the biological activity of the samples.

Applications:

- Protein purification and characterization.
- Enzyme isolation.
- Separation of nucleic acids and other biomolecules.

Advantages:

- Gentle on biological samples.
- High throughput and efficiency for protein purification.

Disadvantages:

- Limited to aqueous systems.
- Lower pressure limits the speed compared to traditional HPLC.

APPLICATIONS OF HPLC

The HPLC has developed into a universally applicable method so that it finds its use in almost all areas of chemistry, biochemistry, and pharmacy.

- 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

HPLC Chromatogram

In the high performance liquid chromatography system, a chromatogram can be used for the representation of separation or analysis of samples. A series of peaks is obtained in a computer or recorder which rises from baseline along the time axis.

Different types of peaks are obtained for different compounds. The response should be 0.5 seconds or better for full scale to avoid distortion of rapidly eluting peaks. Chat speed in the HPLC system should be adjustable.

Summary

High-Performance Liquid Chromatography (HPLC) encompasses a range of techniques tailored to different analytical needs. Normal-phase and reverse-phase HPLC offer versatile options for separating compounds based on polarity, while size-exclusion and ion-exchange HPLC provide specialized approaches for analyzing molecules by size and charge, respectively. Affinity HPLC and chiral HPLC offer high selectivity for specific interactions and stereochemical differences, making them invaluable in biochemistry and pharmaceutical analysis. Hydrophilic interaction chromatography (HILIC) bridges the gap for highly polar compounds, and fast protein liquid chromatography (FPLC) caters to the gentle purification of proteins and biomolecules. Understanding the principles, applications, advantages, and disadvantages of each type of HPLC enables chemists and biochemists to select the most appropriate method for their analytical challenges.