



# “HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN MODERN ANALYTICAL SCIENCES: A REVIEW OF TECHNIQUES AND APPLICATIONS”

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## ABSTRACT:

High Performance liquid chromatography (HPLC) is a specialized form of column chromatography extensively used in biochemistry and analytical chemistry for the separation, identification, and quantification of chemical compounds. It is especially prevalent in drug analysis for detecting, separating, and quantifying pharmaceuticals. The development and validation of HPLC techniques are crucial for novel drug discovery, development, and manufacturing, as well as for a variety of research applications involving both human and animal studies. This involves verifying the performance of the HPLC system, including checks for column efficiency, resolution, and reproducibility, to ensure it meets the required standards. By addressing these factors, an HPLC method can be developed to be reliable, reproducible, and suitable for its intended analytical tasks, thus supporting drug discovery, quality control, and various research endeavours's.

**Key Words:** High performance liquid chromatography, instrumentation, elution, applications, mobile phase.

## INTRODUCTION:

High-Performance Liquid Chromatography (HPLC),<sup>[1]</sup> also known as High-Pressure Liquid Chromatography, is a specialized type of column chromatography frequently employed in biochemistry and analytical chemistry. It is designed to separate, identify, and quantify active chemical compounds. In an HPLC system, a column filled with packing material, known as the stationary phase, plays a crucial role. A pump circulates the mobile phase(s) through this column, while a detector measures the retention times of the molecules. The retention time of each molecule is influenced by its interactions with the stationary phase, the solvent(s) used, and the specific properties of the molecules being analyzed. The sample to be analyzed is introduced in a small volume into the mobile phase stream. As it moves through the column, the sample components interact with the stationary phase, causing them to be delayed or retarded. This retardation results from specific chemical or physical interactions between the analytes and the stationary phase.<sup>[2]</sup> The extent of this delay depends on the nature of the analytes and the composition of both the stationary and mobile phases. The time it takes for a specific analyte to exit the column, known as its retention time, is used to identify and quantify the analyte. Common solvents used in HPLC include various combinations of water and organic liquids, with methanol and acetonitrile being the most frequently utilized. To improve separation, the mobile phase composition can be varied during the analysis—a

technique known as gradient elution. Gradient elution separates analyte mixtures based on their affinity for the changing mobile phase composition. The choice of solvents, additives, and the specifics of the gradient are tailored to match the nature of the stationary phase and the properties of the analytes.<sup>[3]</sup>

### HPLC Principle<sup>[4]</sup>

- 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.

### TYPES OF HPLC <sup>[5]</sup>

High-Performance Liquid Chromatography (HPLC) is a versatile analytical technique that relies on various phase systems to separate compounds in a mixture. The types of HPLC are generally categorized based on the nature of the stationary and mobile phases used. Here are the main types of HPLC commonly used in analysis:

1. Normal-Phase HPLC:
  - Stationary Phase: Polar (e.g., silica gel, alumina).
  - Mobile Phase: Non-polar (e.g., hexane, chloroform).
  - Applications: Used for separating polar compounds. It is often used in cases where the stationary phase is more polar than the mobile phase.
2. Reverse-Phase HPLC:
  - Stationary Phase: Non-polar (e.g., C18, C8 bonded phases).
  - Mobile Phase: Polar (e.g., water, methanol, acetonitrile).
  - Applications: The most common type of HPLC. It is used for separating non-polar to moderately polar compounds. It is widely used in pharmaceuticals, environmental analysis, and biochemistry.
3. Ion-Exchange HPLC:
  - Stationary Phase: Charged (e.g., anion-exchange or cation-exchange resins).
  - Mobile Phase: Typically, an aqueous buffer with varying pH and ionic strength.
  - Applications: Useful for separating ionic species, such as proteins, amino acids, and other charged biomolecules.
4. Size-Exclusion HPLC (SEC):
  - Stationary Phase: Porous gel (e.g., dextran, agarose).
  - Mobile Phase: Usually an aqueous buffer.
  - Applications: Used for separating molecules based on size (molecular weight). It is commonly used for analyzing polymers, proteins, and other macromolecules.
5. Affinity HPLC:
  - Stationary Phase: Modified with specific ligands (e.g., antibodies, enzymes).
  - Mobile Phase: Varies depending on the affinity interaction.
  - Applications: Used for highly specific interactions, such as separating proteins based on their binding affinity to a ligand.
6. Chiral HPLC:
  - Stationary Phase: Chiral (e.g., using chiral selectors or columns).
  - Mobile Phase: Varies, often similar to reverse-phase or normal-phase solvents.
  - Applications: Used for separating enantiomers (optical isomers), which is crucial in pharmaceuticals and other fields where chiral purity is important.

Each type of HPLC has its unique advantages and is chosen based on the specific requirements of the analysis, including the nature of the compounds to be separated and the desired resolution.

## PARAMETERS

In analytical chemistry, especially in techniques like chromatography, the parameters I mentioned are crucial for obtaining accurate and reliable results.

### 1. Internal Diameter (ID) of the Column:

- **Impact:** The internal diameter of the column affects the resolution and the amount of sample that can be injected. A smaller ID usually results in higher resolution and more efficient separation, but it also means that the sample capacity is lower. Conversely, a larger ID can handle more sample but may have lower resolution.
- **Adjustment:** Different compounds or samples might require different column diameters to optimize separation and sensitivity.

### 2. Particle Size of the Stationary Phase:

- **Impact:** Particle size affects the efficiency of separation. Smaller particles (e.g., 3-5  $\mu\text{m}$ ) provide better separation and resolution because they increase the surface area and lead to a more uniform flow. However, they also increase backpressure. Larger particles (e.g., 10  $\mu\text{m}$ ) result in lower pressure and less resolution.
- **Adjustment:** Depending on the complexity of the mixture and the required resolution, you might choose different particle sizes. For high-resolution needs, smaller particles are preferred.

### 3. Pore Size of the Stationary Phase:

- **Impact:** Pore size determines the capacity of the column to interact with larger molecules. Smaller pores are suitable for smaller molecules, while larger pores are necessary for larger molecules like proteins or polymers.
- **Adjustment:** Selecting the right pore size is essential for ensuring that the analyte molecules can enter and interact with the stationary phase effectively.

### 4. Pump Pressure:

- **Impact:** Pump pressure affects the flow rate of the mobile phase and the separation efficiency. Higher pressures can lead to better separation but may require more robust equipment. Lower pressures may not adequately force the mobile phase through the column, leading to poor separation.
- **Adjustment:** The optimal pressure depends on the column dimensions and the stationary phase characteristics. It's important to adjust the pressure to avoid issues like column clogging or inefficient separation.

## INSTRUMENTATION:

### 1. Injection of the sample:<sup>[6]</sup>

- Injecting While the Mobile Phase is Flowing:** Many liquid chromatography systems inject the sample while the mobile phase is continuously flowing. This approach, often used with auto-samplers or manual injection valves, allows the sample to mix immediately with the mobile phase, leading to consistent results. This method is preferred for routine analyses because it integrates seamlessly into the chromatographic process.
- Injecting When the Mobile Phase is Stopped:** In some systems, especially older or manual setups, the mobile phase might be stopped to facilitate sample injection. This method can help avoid sample dispersion or ensure precise timing but may introduce inconsistencies if the system does not re-equilibrate properly after the injection.
- Advanced Rotary Valve and Loop Injector:** Using an advanced rotary valve and loop injector enhances the reproducibility of sample injections by allowing precise control over the injected volume and ensuring consistent sample introduction. This system can be set up to inject samples either while the mobile phase is flowing or when it is paused, depending on the system's configuration and the method requirements.

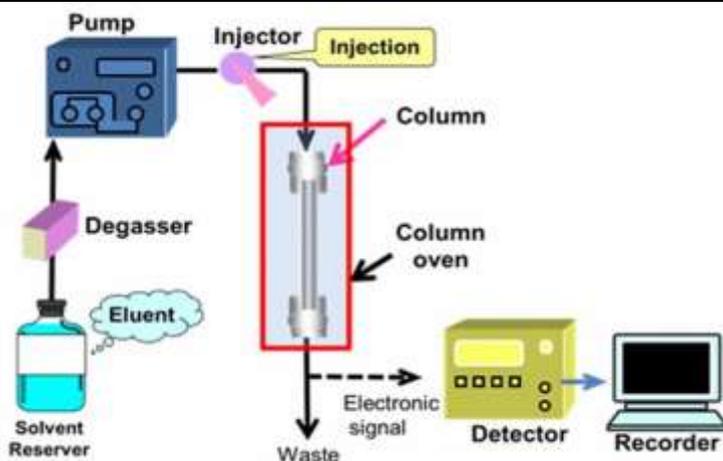


Figure 1: High-Performance liquid Chromatography (HPLC)

2. **The detector:** The UV detector measures the absorbance of the UV light by the compounds in the sample. Each compound absorbs UV light at specific wavelengths characteristic of its structure, known as its  $\lambda_{\max}$ . The detector generates a signal proportional to the amount of light absorbed, which is directly related to the concentration of the compound in the sample. The detector produces a chromatogram, which is a plot of absorbance versus time (or volume). Peaks on the chromatogram correspond to different compounds eluting from the column, with the area or height of each peak related to the concentration of the respective compound. By comparing the UV absorbance spectra of the peaks with known spectra, you can identify the compounds.

#### Interpreting the output from the detector:

- They Represent each peak in the chromatogram corresponds to a different compound in the sample that has passed through the HPLC column and been detected by the UV detector.
- **Retention Time:** The position of the peak along the x-axis (retention time) indicates the time it took for the compound to travel through the column and be detected. Compounds with different properties interact differently with the stationary phase and thus elute at different times.

#### Peak Area:

- **Proportional to Quantity:** The area under each peak is proportional to the amount of the corresponding compound in the sample. A larger peak area indicates a higher concentration of the compound.
- **Automatic Calculation:** The HPLC software automatically calculates the area under each peak. This area can be used to quantify the concentration of the compound if calibration standards are used to create a calibration curve.

#### Detailed Steps in Interpreting the Output:

##### Identify Peaks:

- **Retention Time Matching:** Compare the retention times of peaks in your sample with those of known standards. This helps in identifying which peaks correspond to which compounds.
- **Peak Shape and Width:** The shape and width of the peaks can provide information about the compound's interaction with the stationary phase and the efficiency of the separation.

##### Quantify Compounds:

- **Calibration Curve:** To determine the concentration of each compound, you need a calibration curve created from standards with known concentrations. By comparing the peak area of your sample to the calibration curve, you can calculate the concentration of each compound.
- **Integration:** Ensure the peak areas are accurately integrated by the software. Peaks should be well-defined and not overlapping for precise quantification.
- **Peak Analysis Tools:** Most HPLC systems come with software that offers tools for peak integration, baseline correction, and quantification. Familiarize yourself with these tools to ensure accurate data analysis.
- **Data Review:** Regularly review the chromatograms and data reports generated by the software for accuracy.

## APPLICATIONS:

High-Performance Liquid Chromatography (HPLC) is a widely used analytical technique with numerous industrial applications across various fields.

### 1. Pharmaceutical Industry:<sup>[7]</sup>

- **Drug Development and Quality Control:** HPLC is essential for determining the purity of pharmaceutical compounds, identifying and quantifying active ingredients, and ensuring that drug products meet regulatory standards. It is used in all stages of drug development, from formulation to manufacturing.
- **Stability Testing:** HPLC helps in evaluating the stability of pharmaceutical products by analyzing changes in the drug's composition over time under different conditions.

### 2. Biotechnology:

- **Protein and Peptide Analysis:** HPLC is used to analyze proteins, peptides, and other biomolecules, including their purity, structure, and concentration. It is crucial for the development and quality control of biologics and biosimilars.
- **Purification:** It is used in the purification of recombinant proteins and other biomolecules during the production process.

### 3. Environmental Monitoring:

- **Pollutant Detection:** HPLC is employed to detect and quantify pollutants and contaminants in air, water, and soil. This includes the analysis of pesticides, heavy metals, and other harmful substances.
- **Environmental Compliance:** Ensuring that industrial processes meet environmental regulations and standards often involves HPLC analysis.

### 4. Food and Beverage Industry:

- **Quality Assurance:** HPLC is used to analyze and ensure the quality of food products, including the detection of additives, preservatives, and contaminants. It also helps in verifying the authenticity of ingredients.
- **Nutritional Analysis:** It is used to determine the nutritional content of food products, such as vitamins, amino acids, and fatty acids.

### 5. Forensic Science:<sup>[8]</sup>

- **Drug Testing:** HPLC is used in forensic laboratories to analyze biological samples for the presence of drugs and their metabolites. This is crucial for toxicology, criminal investigations, and legal proceedings.
- **Trace Evidence Analysis:** It helps in identifying trace substances found at crime scenes.

### 6. Cosmetics and Personal Care:

- **Product Development and Quality Control:** HPLC is used to analyze the components of cosmetic and personal care products, including active ingredients, preservatives, and potential contaminants.

### 7. Chemical Industry:<sup>[9]</sup>

- **Product Purity:** In the production of chemicals, HPLC is used to monitor the purity and concentration of chemical compounds. This ensures that chemical products meet the required specifications.
- **Process Monitoring:** It is used to track the progress of chemical reactions and to optimize production processes.

### 8. Clinical Diagnostics:<sup>[10]</sup>

- **Biomarker Analysis:** HPLC is employed in clinical laboratories to analyze biomarkers in patient samples, aiding in diagnosis and monitoring of diseases.
- **Therapeutic Drug Monitoring:** It helps in measuring drug levels in patients to ensure effective and safe medication dosage.

## CONCLUSION:

Based on the review, it can be concluded that High-Performance Liquid Chromatography (HPLC) is a highly versatile and reproducible chromatographic technique. It is widely applicable across various fields for both the quantitative and qualitative estimation of active molecules in drug products. This version emphasizes HPLC's versatility and reproducibility while clearly stating its applications.

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