Int. J. Sci. R. Tech., 2025 2(5)
A Multidisciplinary peer-reviewed Journal
www.ijsrtjournal.com [ISSN: 2394-7063]

Original Article

HPLC and its Hyphenated Techniques: Review

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ABSTRACT

High Performance Liquid Chromatography (HPLC) is a precise analytical technique used to separate, identify, and quantify components within complex mixtures. Developed in the late 1960s, it operates by pumping a liquid mobile phase under high pressure (50–350 bars) through a column packed with solid adsorbents like silica or polymers, which interact differently with each sample component to achieve separation. HPLC is favored in pharmaceuticals, biotechnology, environmental testing, and food industries for its speed, sensitivity, and high resolution. Hyphenated techniques in liquid chromatography combine chromatography with spectroscopic methods, a concept introduced by Hirsch Feld in 1980. These integrated systems, such as LC-MS, LC-NMR, and LC-FTIR, use appropriate interfaces to enable simultaneous separation and detailed detection, offering enhanced qualitative and quantitative analysis. These approaches are crucial in modern drug discovery and chemical analysis, providing comprehensive data in a single run.

Keywords: High Performance Liquid Chromatography (HPLC) , Analytical technique, Separate, Identify, Quantify, Complex mixtures

INTRODUCTION

Chromatography is a powerful analytical method often used to separate, identify and determine chemical constituents in complex mixtures. It was first demonstrated in 1906 by Russian botanist Milkhail Tswett. [1] High Performance Liquid Chromatography (HPLC) is an analytical technique that uses a liquid mobile phase to separate mixed components, pumped through a column with a solid or liquid stationary phase. [2]

High Performance Liquid Chromatography (HPLC):

High Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. Today it is applied to a variety of fields, including pharmaceuticals, biotechnology, the environment, polymers and food industries. HPLC has become a choice method for analyzing a wide range of connections over the past decade. [3] High-performance chromatography is also considered high-pressure liquid chromatography. This is a well-known mixture of analytical methods used to separate, identify, and quantify each component. HPLC is an excellent approach to column liquid chromatography. The solvent usually supports gravity and flows through the column. However, in the HPLC method, the solvent is placed under pressure under excessive pressing up to 400 atmospheres. [4-10] The pump is used in HPLC to transfer the compressed solvent and sample mixture to a column packed with solid adsorbent. Each sample component interacts differently, leading to different flow rates for each component, and ultimaty leads to the separation of column components. Adsorption is part of the bulk. exchange process that creates chromatography. The pump is used in HPLC to place the liquid and sample mixture under pressure through the ADS-fill section separating the sample segments. Adsorbents, which are dynamic segments of sections, are particulate matters, often made up of solid particles anging from 2 m to 50 m in size, such as silicon dioxide and polymers. They separate different levels of connectivity between sample mix/mixture segments and retaining particles. The mobile phase under pressure is usually a mixture of solvents (such as water, acetonitrile, methanol). Its structure and temperature have a major impact on the connection between the sample segment and the adsorbent as the partitioning process works. [11-18] HPLC can be distinguished from the latter, as it essentially works with Higher Press (50 to 350 Bars) as traditional (“low

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weight") liquid chromatography. Traditional liquids Chromatography is often based on the force of gravity that moves portable levels into segments. Scientific HPLC separates very small sample volumes. Therefore, column section measurements range from 2.1 mm to 4.6 mm and from 30 mm to 250 mm. Additionally, smaller SORB particles (up to 50 m at normal molecular size) are used to generate HPLC segments. Due to its excellent decision or resolution (ability to identify components), HPLC is a well-known chromatography technology. [19-28]

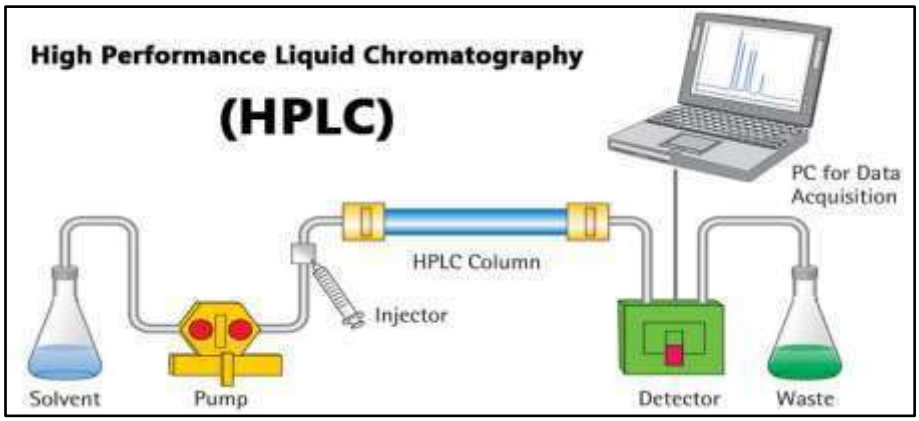

Figure 1: High Performance Liquid Chromatography.

❖ HISTORY:

Prior to HPLC, traditional liquid chromatography was slow and inefficient, and was based on the gravity of the solvent stream. Gas chromatography was not suitable for thermally unstable connections. In the 1960s, scientists such as Giddings and Huber used smaller particles and higher HPLC is a separation technique: Injection of small volume liquid tests of tubes containing 3-5 micrometers (events) in diameter (henm) as steady phase phase). There are individual components of the package pipe (gaps) of the liquid (mobile phase) package pipe (mobile phase) that passes through the column through the liquid (working phase) and through the liquid through the liquid. I moved to a column. These components are separated from each other by column packages containing various chemical and/or physical interactions between the molecule and the pack. These individual components are recognized at the output of this pipe (column) by the flow (detector) through the device that measures the amount. As a rule, the output from this detector is called HPLC, and although LC and HPLC operate in the same way, HPLC's speed, efficiency, sensitivity and simple features are much better. Although HPLC maintains the main subject of the analytics credits to improve LC efficiency. Innovations such as Zipax and gas amplifier pumps have improved performance. In the 1970s, advances in molecular technology and instrumentation shaped the latest HPLC despite the challenges associated with handling pressure and particulates. [29]

- **Historical Perspective:**

- 1903 Tswett – plant pigments separated on chalk columns

•1931 Lederer & Kuhn – LC of carotenoids
•1938 TLC and ion exchange
•1950 reverse phase LC
•1954 Martin & Synge (Nobel Prize)
•1959 Gel permeation
•1965 instrumental LC (Waters)

The term high-performance liquid chromatography (HPLC) was first used to distinguish it from the latest high-performance technology invented in the 1930s from traditional low-pressure column chromatography. [30]

Method development on HPLC:

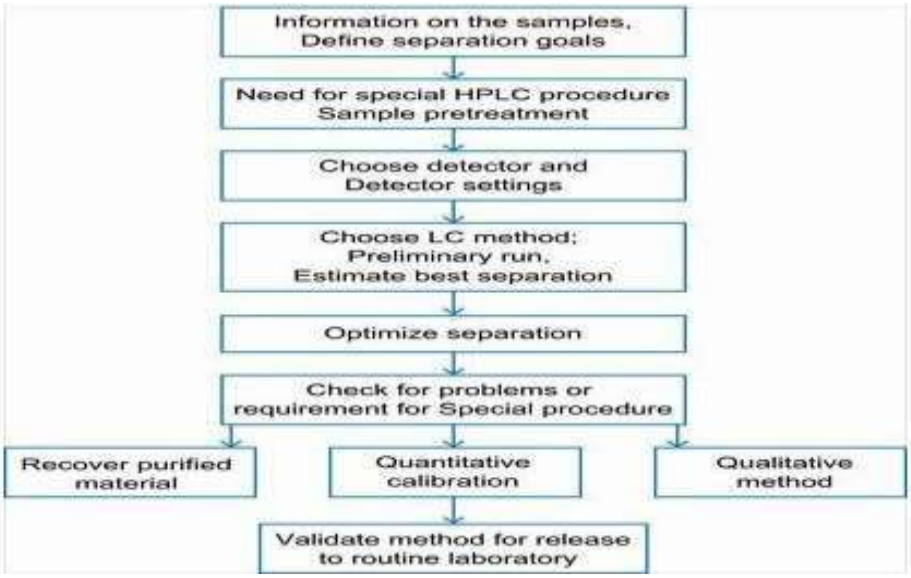

Figure 2: Steps involved in HPLC Method development [31]

▪ HPLC PRINCIPLE:

High-performance liquid chromatography (HPLC) is a separation technique in which differences in distribution are used by connecting two phases, called stationary phase and mobile phase. The stationary phase shows the thin layer formed on the surface of the fine particles, while the mobile phase shows the liquid flowing through the particles. In certain dynamic states, each component in the sample has a different distribution balance depending on the solubility of the phase and/or molecular size. As a result, the components travel at various speeds beyond the stationary phase, thus separating them from each other. This is the principle behind HPLC. [32]

• TYPES OF HPLC:

• Based on modes of separation HPLC is classified into the following two types

1. Normal-phase chromatography, or NP
2. Reversed-phase chromatography, or RP

• Based on the separation mechanism and type of stationary phase used

1. Ion exchange chromatography
2. Size exclusion chromatography
3. Affinity chromatography
4. Chiral phase chromatography
5. Adsorption chromatography
6. Partition chromatography

1. Normal-phase chromatography, or NP:

Also known normal phase HPLC (NPHPLC), this method separates the analytes based on polarity. NPHPLC uses polar stationary and non-polar mobile phases. Polar analytes interact and are kept by the polar stationary phase. Adsorption strength increases with increasing analytical polarity, and the interaction between the polar analyte and the polar analyte increases the elution time. [33]

2. Reversed-phase chromatography, or RP:

Reverse phase HPLC (RP-HPLC or RPC) has non-polar stationary phases and aqueous slightly polar mobile phases. RPCs work on the principle of hydrophobic interactions due to repulsion forces between polar eluents, relatively non-polar analytes, and non-polar stationary phases. The binding of the analyte to the stationary phase is proportional to the contact interface around the nonpolar segment of the analyte molecule when associated with the ligand in an aqueous solvent. [34]

• Based on the separation mechanism and type of stationary phase used

1. Ion-exchange chromatography:

In ion exchange chromatography, retention is based on the attraction between dissolved ions and chargedregions bound during stationary phase. ion with the same charge is excluded. This type of

chromatography is often used for cleaning water and for ligand exchange chromatography, ion exchange chromatography of proteins, and high-quality exchange chromatography of carbohydrates and oligosaccharides, etc. [35,36]

2. Size exclusion chromatography (SEC):

Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. SEC also useful to determine the tertiary and quaternary structures of Purified proteins. SEC is primarily used for the analysis of large molecules, such as proteins and polymers. SEC works by capturing these small molecules into the pores of particles. Large molecules are too large to enter the pores, so they simply pass through them. Therefore, larger molecules are faster and more retention times than smaller molecules.

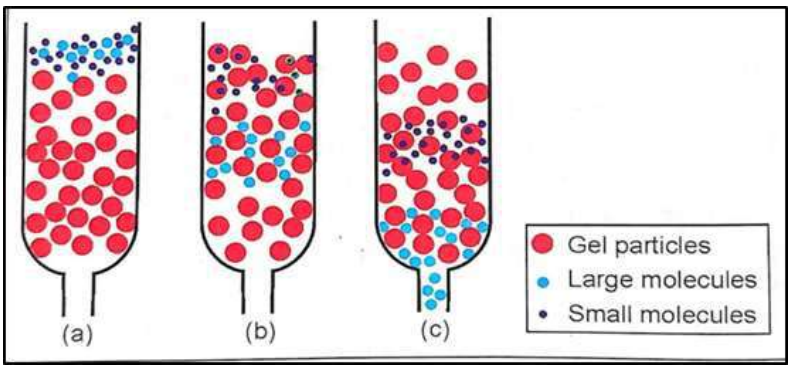

Figure 3: Size exclusion chromatography (SEC)

This technique is often used to measure the molecular weight of polysaccharides. In SEC, there is no interaction between sample connections and column packaging materials. Instead, the molecules diffuse and diffuse into the pores of the porous medium. Molecules are separated according to size compared to pore size. Molecules larger than the opening in the pore will not diffuse into the particles, but molecular smaller penetrates and separates the particles more than the opening in the pore. The large molecules elute first. Smaller molecules elute later. [37]

3. Affinity Chromatography:

Chromatograph includes covalently bonding, a reagent known as the Affinity ligand, for solid support. Typical affinity ligand are antibodies, enzyme inhibitors, cofactors/coenzymes or other molecules that reversibly and selectively bind to the analytical analysis of samples. The principle is that the stationary phase consists of a support medium (such as cellulose beads), as the substrate (or coenzyme) is linked to a covalent bond, exposing the reactive groups essential to the enzyme binding. Because the protein mixture is pass through the chromatographic column, those proteins that have a binding site for the immobilized substrate will bind to the stationary phase, while all other proteins will be eluted in the void volume of the column.

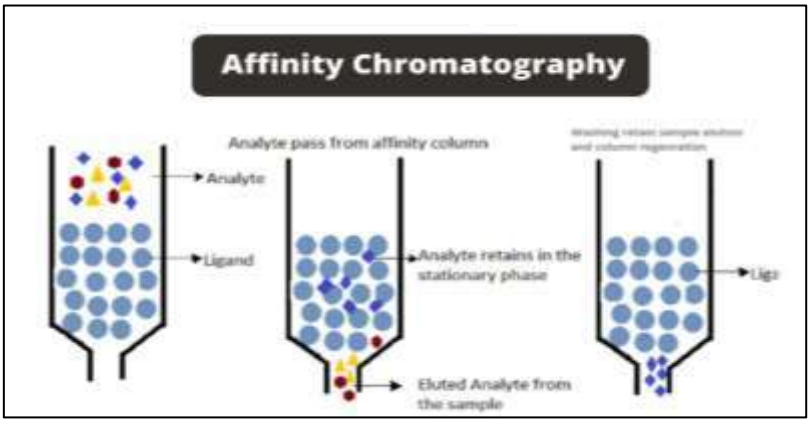

Figure 4: Affinity Chromatography.

When a sample passes through the column, only molecules that selectively bind to the affinity ligand are retained. Non-bound molecules pass the columns through the mobile phase. After the unwanted molecules have been removed, the retained analyte may be eliminated by changing mobile phase conditions. As soon as they bounded themselves later, it must be separated from the bounded stationary phase using another solvent which has a good capacity for separation. Mostly it is help in the separation of biomolecules like protein. [38]

4. Chiral Phase Chromatography:

Chiral phase chromatography is a variant of column chromatography in which the stationary phases contain a single enantiomer of chiral connections rather than achiral. Two enantiomers of the same analyte compound at different times due to different affinities of the individual enantiomer station phases. chiral stationary phases can be grown by attaching appropriate chiral connections to the surface of the achiral support, such as silica gel that produce chiral stationary phases (csps). many frequent chiral stationary phases are based on oligosaccharides such as cellulose and cyclodextrins (especially in particular with β- cyclodextrin, a seven-sugar molecule). the chiral stationary phase is much more expensive than comparable achiral stationary phases such as c18. [39,40]

5. Adsorption chromatography:

The chemical mixture is needed to be passed over an adsorbent bed. The different compounds present in this mixture get adsorbed on the bed at different rates. The process is mostly carried out for analytical separation. [41]

6. Partition chromatography:

This type of separation technique, a mixture is separated by making use of the partition of a solute between two solvents. In this process, one of the solventsis immobilized with the help of a substance present in the filter paper or column. [42]

Instrumentation of HPLC:

a. Solvent Reservoir

Table 1. The overall study of techniques. [43]

Sr. No	Mode	Solvent type Used	Type of Compound used
1	Reversed-Phase	Water/ Buffer, CAN, Methanol	Neutral or non-ionized Compound which can be dissolved in water.
2	Ion-Pair	Water/ Buffer, CAN, Methanol	Ionic or Ionizable Compound
3	Normal Phase	Organic Solvent	A mixture of isomer & compound not Soluble in Organic / Water mixture
4	Ion Exchange	Water/ Buffer	Inorganic ions, Protein, nucleic acid, organic acid.
5	Size exclusion	Water, Chloroform	High Molecular weight compound

b. Pump

- ✓ High pressure-1000 to 5000 psi

c. Injector

- ✓ Low pressure-stop the flow
- ✓ High-pressure value

d. Column

- ✓ Normal Phase-organic (water-free) mobile phase
- ✓ Silica gel-non-aqueous
- ✓ Adsorption
- ✓ Reverse phase (C8, C18)-aqueous mobile phase
- ✓ Partitioning
- ✓ Ion-exchange-aqueous mobile phase

- ✓ Molecular sieve-aqueous mobile phase
- ✓ Size

e. Detector

- ✓ Specific
- ✓ Absorbance
- ✓ Fluorescence
- ✓ Electrochemical
- ✓ Non-specific
- ✓ Refractive index
- ✓ Radioactivity
- ✓ Conductivity [44,45]

Table 2: Table of Different detectors & Type of compounds detected by them. [46]

Sr. No	Detector	Type of compound can be detected
1	UV-Visible & Photodiode array	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
2	Fluorescence detector	Fluorescent compounds, usually with fused rings or highly conjugated planer system
3	Conductivity detector	Charged compounds, such as inorganic ions and organic acid.
4	Electrochemical detector	For easily oxidized compounds like quinines or amines
5	Refractive Index detector & Evaporative light scattering detector.	Compounds that do not show characteristics usable by the other detectors, eg. polymers, saccharides.

f. Data system (Computer, Software)
g. Waste / fraction collector.

- **Hyphenated Techniques Used in Liquid Chromatography:**

Over the past decade, the requirements for analytical support for drug discovery have increased. As a result, new technologies continue to develop to master these challenges. Furthermore, the use of more established methods is improved through incremental improvements in technology and protocols. Bind stabbing (combination) analytical techniques [47,48] are approaches that allow modern pharmaceutical analysts to meet the needs of today's industry. Hirsch Feld used the term "hyphen" in 1980 to refer to the fusion of two or more instrumental analytical techniques in a single run. Compared to the results of using a single analytical approach, binding is intended to provide comprehensive detection for both identification and quantification. Mating or binding of two different analytical approaches using the appropriate interface is called hyphenated methodology. Spectroscopic techniques are primarily paired with chromatography. Pure or almost pure part of a chemical components are separated in combination, and selection information for standard or library spectra are generated by spectroscopy. Pairing products as a result of separation methods and online spectroscopic recognition systems. The hyphenation procedure consists of using or combining two different analytical techniques, along with the help of the appropriate interface. In hyphenation, many other methods are included in separations and strategies for separation, separation, identification. [49]

- **Types of Hyphenated Techniques Used In Liquid Chromatography:**

1) Liquid chromatography-Mass spectrometry (LC/MS)
2) Liquid chromatography-Nuclear magnetic resonance spectroscopy (LC/NMR)
3) Liquid chromatography-gas chromatography (lc-gc)
4) Liquid chromatography-Fourier-transform infrared spectrometry (LC-FTIR)
5) Liquid chromatography-Inductively coupled plasma mass spectrometry (LC-ICPMS)

1) Liquid chromatography-Mass spectrometry (LC/MS):

Principle: [50-52]

Liquid chromatography mass spectrometry is a technique that performs separations through liquid chromatography and mass analysis with the help of mass spectrometry. With the help of HPLC impurities and degradation products, we can separate and mass spectrometry can maintain the same identification as the molecular weight. The LC-MS is highly selective and sensitive. LC-MS is described as specific, and therefore the presence of other chemicals leads to the detection and identification of chemicals. The flow rate of HPLC is 1 ml/min, which is difficult in the mass spectrometric vacuum system of the diluent used, and damages the thermolabile compounds due to excessive heating via hyphenation of these two techniques capacity of both techniques were increased. There are various methods used in Quantitative Analysis which may be broadly classified as

□ Chemical/classical Method (Titrimetric, Volumetric and Gravimetric method)
□ Instrumental Method (Spectrophotometry, Polarography, HPLC, GC)

LC-MS also plays a role in the field of molecular pharmacognosy, particularly in regards to differences in components in aspects of phenotypic clones. The most important factor to consider is how the greatest difference in active ingredient in plant cells between a plant test group and a controlled one.

- **Instrumentation and Working:** [53-55] LC-MS equipment can be connected with electro spray, particle beams, and thermal spray. Electrospray is the most extensive interface.

- Spray needles are used as a bridge to connect liquid chromatography with mass. However, individual emitters are flexible and comfortable. LC-MS is divided into three parts chromatography, interfaces, and spectrometry. In liquid chromatography, separation is performed.
- This is proven in the help of detectors such as photodiode array, ultraviolet, and fluorescent.
- These individual components were transferred to the interface. At the interface, the liquid is a liquid and the is transmitted to the MS. With the help of various ionization techniques, the compound is ionized and is analyzed by a mass analyzer.
- A variety of mass analyzers are used. Mass analysis of Quadrupole ion traps, Time to Flight (TOF), Time to Flight Reflection (TOFR) and Ion Cyclotron Resonance (ICR).
- Mass analyzers in This technology is separated by LC and provides a clear mass spectrum through recording of mutual signals.

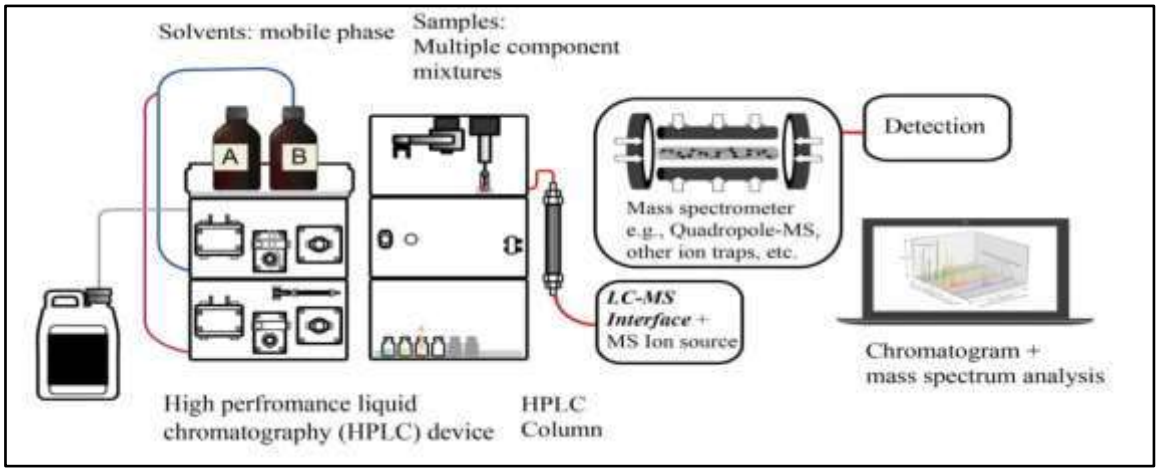

Figure 5: Liquid chromatography-Mass spectrometry

2) Liquid chromatography-Nuclear magnetic resonance spectroscopy (LC/NMR)

Principle:

This combined high performance liquid chromatography (HPLC) with nuclear magnetic resonance spectrometer (NMR) resulted in the analysis of unknown components such as impurities

and metabolites in pharmaceuticals, natural products and synthetic polymers [56,57], ever since they were first reported in 1978. [58]

- **Instrumentation and Working:** A typical online LC-NMR system consists of standard LC devices connected to an NMR detection device where a flow probe is inserted. The LC device consists of a pump system that pushes the liquid solvent through the system, a column on which the separation is located, and a detector with a flow cell that uses light to measure the components during elution.

➢ This light detector can be an ultraviolet/visible light (UV/VIS) detector, refractive index Index (RI) detector or an infrared detector (IR). The most common these days is the use of Diode Array Detectors (DADs).

➢ This is a type of ultraviolet/vis detector that can measure multiple optical wavelengths simultaneously.

➢ Essentially, all types of detectors can be used as long as you do not modify or destroy the sample. The NMR system consisted of huge radiofrequency (RF) magnets, where non-rotating flow cells were oriented vertically.

➢ This orientation allows laminar flow and allows easy acquisition of bubbles in the mobile phase.

➢ The RF coil is wrapped around the cell, so the appropriate filling coefficient is preserved, and the difference between the detection volume and the coil volume is only glass containing the flow cell. [59]

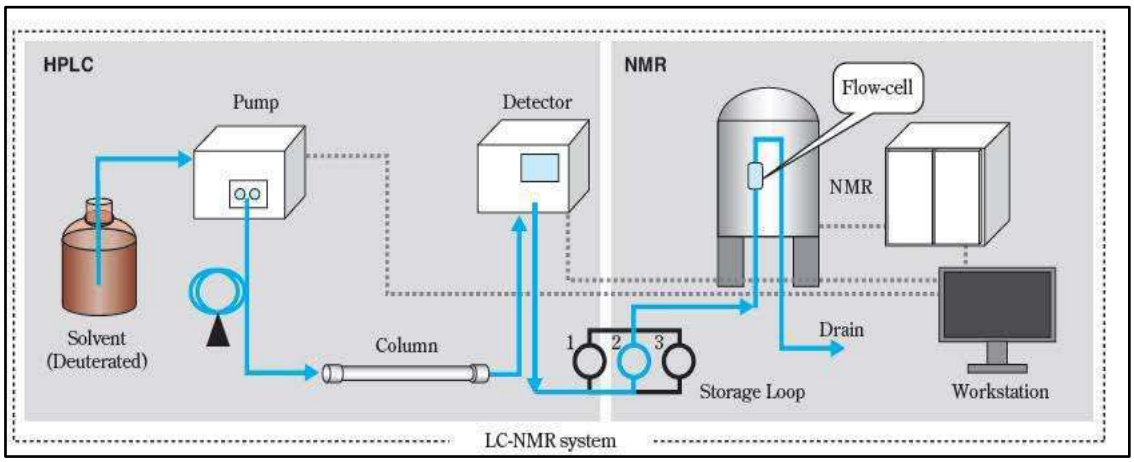

Figure 6: Liquid Chromatography-Nuclear magnetic resonance spectroscopy (LC/NMR). [60]

3) Liquid chromatography-gas chromatography (lc - gc):

Principle: Chromatography in all forms includes stationary and mobile phases. In all other chromatographic formats, when encountered at this level, the mobile phase is a liquid. In gas liquid chromatography, the mobile phase is a helium-like gas, and the stationary phase is a high boiling point liquid adsorbed into a solid. How quickly a particular connection drives through the machine depends on how much time it travels with the gas, rather than being attached to the liquid in some way. [61]

- **Instrumentation and Working:** [62,63]

➢ **Injection of the Sample:**

A minute amount of the sample intended for analysis is introduced into the instrument using a fine syringe. The needle pierces a thick rubber membrane called a septum, which reseals itself once the needle is withdrawn. This injection port is housed within a temperature-controlled oven. The oven is maintained at a high temperature to ensure the sample vaporizes completely. The resulting vapor is then transported into the column by a flow of helium or another carrier gas.

➢ **Working of the Column:**

In gas-liquid chromatography, two primary types of columns are used. One type is a long, narrow tube filled with the stationary phase, while the other is an even narrower tube with the stationary phase bonded

directly to its inner wall. These columns are usually constructed from stainless steel and range from 1 to 4 meters in length, with internal diameters of up to 4 millimetres. To fit within a temperature-regulated oven, the column is typically coiled.

- **Packing Material:**

The column is filled with finely powdered diatomaceous earth, a highly porous mineral. This material is coated with a liquid that has a high boiling point, usually a wax-like polymer, which acts as the stationary phase in the separation process.

- **Column Temperature:**

The temperature within the column is adjustable, typically ranging between 50°C and 250°C. It is maintained at a lower temperature than the injector oven to allow certain components of the sample to condense initially at the column's entrance. In some analyses, the process begins at a lower temperature, which is then gradually increased using computerized control to improve separation as the analysis continues.

- **Retention Time:**

Retention time refers to the duration a specific compound takes to pass through the column and reach the detector. It is calculated from the moment the sample is injected until the peak for that compound appears on the display, indicating its highest concentration.

- **Detector:**

Various types of detectors are used in gas chromatography, each with its own advantages. One of the most commonly used and easiest to understand is the flame ionization detector, which is described below.

- **Flame Ionization Detector (FID):** [64]

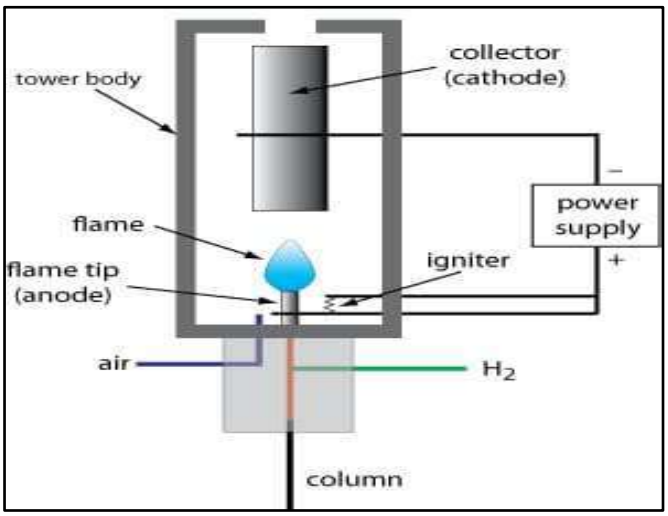

Figure 7: Flame Ionization Detector (FID)

An FID detects organic compounds by measuring ions formed during combustion. When an organic compound enters the flame, it produces ions and electrons. These charged particles generate a small electric current between two electrodes—one acting as the anode and the other as the cathode. The detector is kept at a higher temperature than the column to prevent condensation. The strength of the current, which can be amplified, is directly related to the amount of organic compound present in the sample.

4) Liquid chromatography-Fourier-transform infrared spectrometry (LC-FTIR)

Principle:

HPLC-FTIR combines High-Performance Liquid Chromatography (HPLC) for separating components with Fourier Transform Infrared (FTIR) Spectroscopy for identifying them based on their IR absorption patterns. After separation by HPLC, compounds are either detected directly in a flow cell or collected and dried using a solvent-elimination interface. FTIR then records their unique absorption spectra, revealing

functional groups like –OH, –COOH, etc. This technique helps identify organic compounds but is less sensitive than UV or MS detection. [65]

- **Instrumentation and Working:**

- The technique known as LC-IR or HPLC-IR results from combining liquid chromatography (LC or HPLC) with infrared (IR) or Fourier-transform infrared (FTIR) spectrometry.
- HPLC is a highly effective method for separating components within a mixture, while IR and FTIR spectroscopy are valuable tools for identifying organic molecules.
- This identification is possible because organic compounds absorb infrared radiation at specific wavelengths in the mid-IR region, particularly at frequencies associated with functional groups such as hydroxyl (–OH) and carboxyl (–COOH).
- Despite their individual strengths, integrating HPLC with IR detection presents challenges. One of the main issues is that solvents used in the mobile phase often show strong absorption in the mid-IR range, which can interfere with the relatively weaker signals from the analytes.
- Furthermore, IR detection is inherently less sensitive compared to other common detectors like UV-Vis or mass spectrometry (MS).
- To address these limitations, two principal strategies have been developed for HPLC-IR interfaces: the flow-cell approach and the solvent-elimination approach. In the flow-cell method, detection occurs directly in a cell similar to those used in UV-Vis detection.
- While solvent interference remains a concern, certain IR-transparent regions still allow for effective monitoring—especially when using deuterated solvents like heavy water (D_2O) or deuterated methanol, which reduce background absorption and enhance analyte visibility.
- The solvent-elimination approach is more widely adopted. In this method, the mobile phase is first removed before the sample is subjected to IR analysis. Typically, sample components are deposited on IR-transparent media such as potassium bromide (KBr) or potassium chloride (KCl).
- The volatile solvents are then evaporated by heating, allowing for clearer IR spectra of the analytes.
- Two main interface types are used for the solvent-elimination technique: diffuse reflectance infrared Fourier transform (DRIFT) and the buffer-memory technique.

- More recently, integrated systems have been developed that enable coupling of FTIR with multiple chromatographic techniques—such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and supercritical fluid chromatography (SFC)—using IR microscopy as the detection platform. [66]

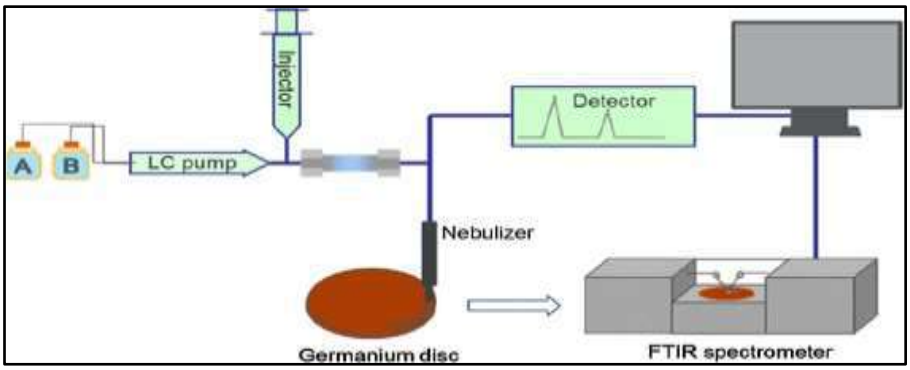

Figure 8: Liquid chromatography-Fourier-transform infrared spectrometry (LC-FTIR)

5) Liquid Chromatography-Inductively coupled plasma mass spectrometry (LC-ICPMS):

Principle:

In environmental speciation studies, the primary HPLC techniques employed are size exclusion chromatography, ion-exchange chromatography, and reversed-phase chromatography. Although capillary electrophoresis is still developing in this field, it shows great promise for speciation analysis due to its high separation efficiency, minimal sample volume requirements (in the nanolitre range), and the lack of

stationary phase packing that could interact with metals or disturb complexation equilibria. [67,68]

- **Instrumentation and Working:**

➢ **Sample Injection:**

A sample containing various chemical species of elements (e.g., As^{3+}, As^{5+}, DMA, MMA) is introduced into the LC system.

➢ **Separation (LC):**

The components travel at different speeds through the column, depending on their interactions with the stationary and mobile phases. This results in separated peaks as each species elutes at a specific time (retention time).

➢ **Introduction to ICP-MS:**

The effluent from LC enters the ICP-MS through a nebulizer and spray chamber. This stream is converted into a fine aerosol.

➢ **Ionization (ICP Plasma):**

The aerosol is introduced into a very high-temperature argon plasma* (\~7000–10,000 K). Molecules are broken into atoms, and atoms are ionized to produce positive ions (e.g., As^{+}, Cr^{+}).

➢ **Mass Analysis and Detection:**

The ions are filtered by a quadrupole (or another type of mass analyzer) based on their m/z values. A detector counts the ions and generates a signal proportional to their concentration.

➢ **Data Interpretation:**

The result is a time-resolved elemental profile showing the concentration of a specific element in its various chemical forms. [69]

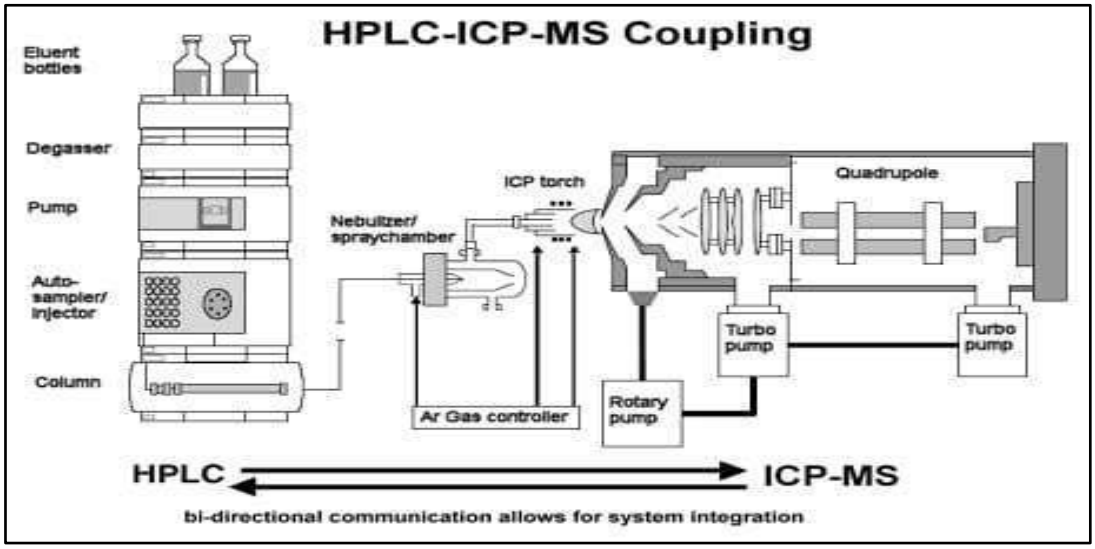

Figure 9: Liquid Chromatography-Inductively coupled plasma mass spectrometry (LC-ICPMS):

❖ **Advantages of Hyphenated Techniques:**

One of the key benefits of hyphenated speciation techniques is their capability to identify unexpected or unknown chemical species, not just the ones initially targeted. This advantage is particularly valuable in areas such as drinking water and wastewater analysis, drug development, biochemical studies, and biotechnological research, which are global research priorities.

1. Enables rapid and precise analysis.
2. Offers a high level of automation, minimizing manual intervention.
3. Supports high sample throughput for efficient processing.
4. Provides improved reproducibility of results.
5. Minimizes contamination risks due to its enclosed system design.
6. Allows simultaneous separation and quantification of analyst
7. Faster analysis times

8. Greater automation capabilities
9. Increased sample handling capacity
10. Improved consistency and reproducibility of results
11. Reduced contamination risk due to enclosed system design
12. Enhanced selectivity through combined techniques, resulting in more comprehensive data. [70]

❖ **Applications of Hyphenated Techniques:** [71- 74]

1. Detecting and identifying drug degradation products.
2. Isolation and analysis of trace-level impurities.
3. Monitoring environmental pollutants like pesticides and herbicides.
4. Separation and profiling of peptide libraries.
5. Utilized in combinatorial chemistry, photochemical studies, and drug discovery.
6. Identification of impurities in pharmaceutical substances.
7. Structural analysis of isomers, such as acid glucuronides and vitamin A derivatives.
8. Direct study of both endogenous and xenobiotic metabolites in biological fluids.
9. Integration of LC-NMR and LC-MS for enhanced compound analysis.
10. Analysis and characterization of polymers.
11. LC-NMR enables isomer differentiation and compound identification without the need for standards.
12. Drug metabolism studies using biological fluids like urine or plasma:
a) 19F NMR provides selective, low-background tracking of fluorinated drugs.
b) Small-volume NMR analysis (e.g., micro-coil probes) can detect ibuprofen metabolites from minimal urine samples.
13. Efficient identification of closely related aporphine alkaloids in plants using significantly less material than traditional NMR methods.
14. Rapid multi parametric data acquisition from microbial bio transformations—for instance, identifying novel warfarin metabolites and antibiotics from Streptomyces species.
15. Separation and identification of chiral compounds, such as photoisomers of azadirachtin (a neem-derived insect anti feedant) using a CH_3CN\:D_2O solvent ratio of 7:13.
16. Detection of vitamin E analogues in palm oil using combined LC-NMR-MS analysis.
17. Rapid screening and analysis of plant extracts and natural products for potential drug candidates.
18. Identification of impurities in bulk drugs during stability studies, with LC-NMR/MS enabling full impurity profiling.
19. Effective for studying unstable or in situ-formed compounds that are difficult to isolate or analyze by other methods.
20. Investigation of drug metabolism by analyzing biofluids like urine or plasma, using tracers such as fluorine-19 (^{19}F) for selective NMR tracking.
21. Structural analysis of ring-fused and heterocyclic compounds with few protons, using LC-2D-NMR to determine carbon shifts and bonding.
22. Composition profiling of chemical mixtures to aid in optimizing manufacturing processes in the chemical industry.
23. Polymer analysis, utilizing high-resolution LC-NMR to study microstructures in synthetic polymers and proteins.
24. Characterization of complex, unclassified natural organic matter (NOM) from environments such as soil, air, and water using LC-NMR and LC-SPE-NMR.
25. Metabolomics applications for disease diagnosis through analysis of body fluids.
26. LC-MS employed for detecting a wide range of compounds, from non-polar polyaromatics to peptides and proteins.
27. Assessment of compound identity and purity through LC-MS analysis.
28. Monitoring environmental contaminants like pesticides and herbicides using LC-MS.
29. Proteomics research, including the analysis of proteins and complex biological samples.
30. Detection, identification, and measurement of various compounds in environmental samples—such as pesticides, oils, and organic pollutants—as well as in medical fields, including drug analysis and monitoring of biological markers.

CONCLUSION:

In conclusion, High Performance Liquid Chromatography (HPLC) is a highly effective and versatile analytical technique essential for separating, identifying, and quantifying components in complex mixtures across various industries. Its ability to

operate under high pressure ensures rapid and precise separations. The development of hyphenated techniques, which combine chromatography with spectroscopic methods, has further enhanced analytical capabilities by providing detailed qualitative and quantitative information in a single run. These advanced approaches are crucial in modern pharmaceutical research, environmental monitoring, and chemical analysis, enabling more comprehensive and accurate detection of diverse substances.

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HOW TO CITE: Komal Zade*, Mrunali Ahirrao, Madhuri Shirsath, Tufail Dana, M. B. Nikam, HPLC and its Hyphenated Techniques: Review, Int. J. Sci. R. Tech., 2025, 2 (5), 584-599. https://doi.org/10.5281/zenodo.15529234