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Review Article

Chromatography Hyphenated Techniques for the Analysis of Natural Products (A Review)

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Abstract

Hyphenated techniques for natural product analysis significantly enhance the separation and identification of complex mixtures. Among these, Gas Chromatography-Mass Spectrometry (GC-MS) excels at analyzing volatile and semi-volatile compounds. Food safety, forensics, medicines, environmental analysis, and other fields depend on it heavily, and new technical developments are always improving its abilities and extending its uses. Sensitivity and selectivity of GC-MS have greatly increased in recent years. Automated sample preparation methods improve efficiency, decrease human error, and streamline operations. Machine learning techniques also improve data analysis by allowing automated peak detection, quantification, and compound property prediction. High-throughput analysis is facilitated by ultra-high-performance liquid chromatography (UHPLC) and high-performance liquid chromatography-mass spectrometry (HPLC-MS). When it comes to locating and measuring the bioactive substances in medicinal plants, HPLC-MS is quite useful. Liquid Chromatography-Fourier Transform Infrared Spectroscopy (LC-FTIR) provides complementary benefits by combining liquid chromatography's separation capabilities with FTIR's structural elucidation. LC-FTIR improves molecular composition comprehension and quantitative capabilities, as chemometrics advances. Combining LC-FTIR with additional methods, such as LC-MS, offers a full perspective of sample composition. Along with these technological advancements, green chromatography methods such as eco-friendly solvents, supercritical fluid chromatography (SFC), energy-efficient apparatus, and low-waste sample preparation are gaining popularity for decreasing the ecological impact. This study aims at understanding the principles, advantages, limits, scientific evaluation, applications and differences in modern hyphenated chromatographic technique which have found relevance in natural products analysis.

Introduction

Chromatography hyphenated techniques belong to a family of analytical methodologies that integrate the potential to separate with spectroscopic detection technologies offering structural elucidation [1,2]. Natural products continue to make up a significant fraction of the more than 0.5–1 million known organic compounds since they have been a key source of compounds with medicinal or nutritional value for generations [3]. An exponential growth of secondary metabolites is thought to occur, and many of these remain undiscovered. In the past 20 years, high throughput screening and combinatorial chemistry have emerged, which has led to a decrease in natural product research despite its significance [4]. The complexity of chemical analysis, especially in natural product research promoted the development of new analytical techniques. Chromatography-hyphenated techniques proved to be a breakthrough over the years, providing unsurpassed specificity and sensitivity [5].

Chromatography was first used by the Russian botanist Mikhail Tswett in 1903, who divided plant pigments with calcium carbonate and coined the word chromatography [6]. This work was the basis for chromatographic principles of separation by selective affinity. Although paper chromatography and thin-layer chromatography (TLC) were primitive, they made important contributions to separation science in the mid-20th century with their ability to easily separate complex mixtures. The emergence of gas chromatography (GC) in the 1950s, and high-performance

liquid chromatography (HPLC) in the 1960s kick-started an age of accuracy in separation science [7].

Some advanced chromatographic techniques include High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Affinity Chromatography, Ion Exchange Chromatography, and Size Exclusion Chromatography Ghandi, et al. 2022. The choice of chromatography technique depends on the properties of the analytes and the desired level of purity [8].

The hyphenated technique is a term that was introduced to define the combination of chromatographic separation with one or more spectroscopic detectors [9]. The motivation for this innovation stems from the necessity of a highly detailed characterization of chromatographically separated entities which was unattainable with traditional detection systems [10]. Hyphenated techniques have evolved with the advancement of technology. These methods have benefited from the miniaturization of components, advanced detector technologies, and computer-aided data analysis, which allow the valid and reliable analysis of more complex mixtures with great accuracy and precision [11]. The analysis of natural products usually involves complex mixtures of chemical compounds present at micro-concentration levels [12]. Separation of the different compounds present in a given natural product is the first analytical step required for the subsequent isolation and purification of the various components of interest, especially when chemically complex natural products are being studied [13].

Over the years, hyphenated techniques have revolutionized natural products analysis allowing unprecedented discovery of new compounds with more complex structures and improved knowledge of their intricate biological functions [14]. These techniques are essential in fields such as pharmacognosy, metabolomics, toxicology, quality control & assurance, environmental analysis, and much more [15]. This review paper deals with advances and possibilities in modern chromatography techniques used in natural product analysis.

Fundamentals and basic principles of chromatography

For the purpose of this review, chromatography is an investigative method in which the original ingredients are slightly changed to appear on an analytical column at various periods or locations [16]. A solvent (for example, organic solvents are frequently used in liquid chromatography), the gas phase, supercritical fluids (such as "SFC"), ion-pair solutions, micellar solutions, or even chromatography done directly on a support (such as solid-phase microextraction) can all be used as the mobile phase [17]. It is preferable to think of the chromatographic process as a two-stage procedure. The materials that need to be separated are first divided into the mobile and stationary phases. The substance to be separated is first divided into the stationary and mobile phases. The components of the sample are then carried along a percolating path—which is essentially an extended, thin sponge-like region—by a particular force caused by the movement of the mobile phase [18]. Time determines how the surface area that an analyte is adsorbed onto; this dependence on time is caused by analyte diffusion. In other words, since the solvent preferentially flows down the column in the direction of the detector, the analytes must first migrate from the sample counter-diffuse to the solvent and be able to seep through the porous media [19]. Phase balance is crucial as it determines how an analytical column function. To prevent interstitial capillaries, columns are filled with uniform particles; for example, a peak should be Gaussian to enable the equilibrium target distribution to be desorbed isothermally together with its absorptive fossil [20]. Reproducibility from one batch to another batch is equally crucial. When columns are packed so that mass transmission is the same between each column, equivalency across sheets is observed. That is to say that the diameter, particle size, and column packing all affect the mass transfer across the column [21]. The above stepwise flow pattern is determined by measuring and investigating the sources of solute retention. Below are key fundamentals of chromatographic techniques.

1. The equilibrium partitioning of an analyte between the mobile and stationary phases is described by the distribution, often known as the partition coefficient (K). Maintaining a constant K is crucial for reproducible separations [22].

2. Ensuring that, in order to prevent peak distortion from nonlinear influences, the separation takes place inside the linear area of the adsorption isotherm [23].

3. Maximizing solute diffusion rates and minimizing linear velocity to approach local equilibrium between the phases [24].

4. The use of different stationary phase chemistries (e.g. normal phase, reversed phase, ion exchange, and affinity) to selectively retain analytes based on their properties [25].

5. High pressures and small stationary phase particles are used in techniques like GC and HPLC to provide highly effective and high-resolution separations [26].

The following are parameters involved in chromatographic analysis [27].

Retention Time (tR): The time it takes for a component to pass through the system from the point of injection to detection.

Selectivity Factor (α): This is the ratio of the retention factors of two components. This is presented in Equation 1.

$$a = \frac{K2}{K1} \quad (1)\ [28]$$

where $k2$ and $k1$ represent the retention factors of the two components.

Retention factor (k): Indicates how much time a chemical is in the mobile phase as opposed to the stationary phase. Calculated as in Equation 2.

$$k = \frac{(tR - t0)}{t0} \quad (2)\ [29]$$

where t is the retention time of the compound, and $t0$ is the time it takes for the unretained solute to pass through the system.

Resolution (R): This is the measure for the degree of separation between two components. It is determined by Equation 3.

$$R = \frac{2(\mathrm{tR}2 - \mathrm{tR}1)}{\mathrm{W}1 + \mathrm{W}2} \quad (3)\ [30]$$

where $tR1$ & $tR2$ are the retention times of the two components, and $w1$ & $w2$ are the widths of the peaks at baseline.

Hyphenated techniques, advantages, limitations and their applications in natural product analysis

According to Awuchi and Twinomuhwezi [1], Chromatography hyphenated techniques commonly used for the analysis of natural products include:

1. Gas Chromatography-Mass Spectrometry (GC-MS)

The process begins with sample injection and culminates in the data system, comprising a computer and software, is vital for collecting, processing, and analyzing the data generated by the GC-MS [31] (Figure 1).

This technique is widely used in various fields, including environmental analysis, pharmaceuticals, food safety, and forensics [33].

Figure 1: GC – MS Representation [32].

Continuous advancements in GC-MS technology have expanded its applications and enhanced its performance, combining the separation power of gas chromatography (GC) with the molecular identification capabilities of mass spectrometry (MS). This combination allowed for the detailed analysis of volatile and semi-volatile compounds, revolutionizing the field of analytical chemistry. The coupling of GC to MS provided orthogonal selectivity chromatographic retention plus mass spectral fragmentation which significantly improved both qualitative and quantitative accuracy [34]. These dual-selection mechanisms minimized false positives and helped establish GC-MS as the "gold standard" for forensic toxicology, pesticide residue analysis, environmental pollutant detection, and natural product profiling [35]. The introduction of tandem mass spectrometry (MS-MS) and time-of-flight (TOF) detectors has broadened the scope of GC-MS applications, particularly in environmental chemistry [35].

Capillary gas chromatography has gained broad acceptance and application in the analysis of organic compounds, including those with complex compositions. This trust is founded on a number of laws [35]. 1) In comparing conditions in planar chromatography with atmospheric distillation, the separational capability of gas chromatography (GC) methods under optimal separation conditions can be provided with an efficiency level of 1012 equivalent theoretical plates [36]. This efficiency is achieved by the use of narrow-bore capillary columns covered with thin stationary phase films that reduce band widening and improve resolution. The separation process is governed by the partition coefficient (K), which determines how compounds are distributed between the stationary and mobile phases. According to Aguilar Meza, et al. [37], molecules with a higher affinity for the mobile phase elute faster, whereas those with a higher affinity for the stationary phase have longer retention times.

2) The ability of this method to separate decomposition products without decomposing the analytes provides almost non-existent probability for co-elution [38]. 3) Sample interaction with column material can also be minimized. Although being of infrequent occurrence, the design of a column with a film thickness equating to just a few micrometers can roughly determine the qualitative characteristic of certain hexadecyl poly (dialkylsiloxane) fillers, which boast thin films, pervaporation, and a specific level of phase domain formation characteristics [39]. 4) For the most part, the situation of substance groups giving categorical chromatography separates extremely well to the point where it is mostly reduced to simple optimization calibration analysis method, as well as efficiency being reduced to a set performance [40]. 5) This method also allows separation of single isomers for highly complex organic substance groups with longer separation time [41].

Recent advancements in GC-MS have resulted in enhanced sensitivity and selectivity. New ionization techniques and mass

analyzers have been developed to improve detection limits and selectivity. High-resolution mass spectrometry (HRMS) has become prevalent, utilizing TOF and Orbitrap analyzers for high-resolution and accurate mass measurements, thereby enabling better identification of compounds [42].

Comprehensive two-dimensional gas chromatography (GC×GC) represents another significant advancement. By coupling two GC columns with different stationary phases, this technique provides improved separation of complex mixtures [43]. Automated sample preparation systems have also been integrated into the workflow, streamlining sample extraction, purification, and derivatization processes, which reduces human error and increases efficiency [44].

Data analysis and machine learning have revolutionized the processing of GC-MS data. Advanced software and machine learning algorithms are now used for data processing, peak identification, and quantification. These tools also predict compound properties, enhancing the accuracy and reliability of the analysis [45].

Satyal [46] used GC-MS to determine the chemical makeup of essential oils isolated from different plants. The study demonstrated the capacity of GC-MS to detect and quantify main and minor components in complicated mixtures. The study successfully illustrates the excellent resolution and sensitivity of GC-MS in separating and detecting volatile chemicals.

Hu, et al. [47] used GC-MS to profile metabolites in medicinal plants, revealing a diverse spectrum of bioactive chemicals. The study underscored the technique's efficacy in uncovering novel natural compounds with potential medicinal applications. While the work demonstrates the flexibility of GC-MS in natural product identification, it also highlights the limitations of GC-MS in evaluating non-volatile, thermally unstable chemicals. Further study shows that the analysis can be enhanced by coupling GC-MS with derivatization techniques such as high-resolution mass spectrometry (HRMS) or tandem mass spectrometry (MS/MS).

Lee, et al. [48] investigated how environmental conditions impact plant metabolomic patterns using GC-MS. Changes in temperature and soil conditions caused considerable variances in secondary metabolites, according to the researchers. The study sheds light on how the environment changes the composition of natural products.

Patel [49] and his team used GC-MS to detect volatile chemicals in traditional herbal treatments and associate them with their medicinal capabilities. The investigation verified the existence of numerous known bioactive chemicals and discovered novel ones. This work highlights the importance of GC-MS in natural product research, particularly in the context of traditional medicine.

Garcia-Valverde, et al. [50] explored the application of comprehensive two-dimensional gas chromatography coupled with mass spectrometry (GCxGC-MS) to analyze complex natural product mixtures. The approach provided enhanced separation and improved identification of compounds in samples with a wide range of chemical diversity. The use of GCxGC-MS represents a significant improvement in the field, addressing the challenge of co-elution in one-dimensional GC-MS.

The advantages of GC-MS are numerous. From providing trace analysis where GC-MS excels at detecting and quantifying compounds present at very low concentrations, to early compound detection due to its sensitivity, which is crucial for applications such as disease diagnosis, food safety, and quality control [51]. GC-MS also provides unique/highly specific fingerprints, thereby minimizing the risk of false positives or negatives, which is attributed to the mass spectra generated by GC-MS [52].

GC-MS can distinguish between isomers and structurally similar compounds, making it ideal for analyzing complex mixtures [53].

By comparing experimental mass spectra to spectral libraries, researchers can identify unknown compounds, which can help in drug discovery, environmental research, and forensic investigations Brown, et al. 2020.

GC-MS equally offers precise and accurate quantification of analytes through calibration curves and internal standards, also the combination of chromatographic separation and mass spectral data provides robust data for quality control and regulatory compliance [51].

In the analysis of natural products, GC-MS is preferred over HPLC-MS and UHPLC-MS when analyzing volatile and semi-volatile natural products e.g., essential oils, terpenes, alkaloids with low polarity, and when high chromatographic resolution is needed for small, thermally stable compounds [54,55]. GC-MS is also preferred when extensive spectral libraries for compound identification are required [55].

Like every other man-made thing, GC-MS has some limitations which stem from the physicochemical properties of natural compounds, technical constraints of the technique and the complexity of natural matrices.

1. GC-MS is limited to the analysis of volatile and thermally stable compounds. Natural products such as high-molecular-weight polysaccharides, proteins, and thermally labile compounds (e.g., certain alkaloids and glycosides), cannot be subjected to GC-MS analysis without derivatization. Derivatization, also known as chemical structure modification [56], is the process of chemically altering a compound to enhance its volatility, thermal stability, or detectability. This is made possible by adding a functional group or modifying already existing ones, making the compound more suitable

for GC-MS analysis. Common derivatization reactions include silylation, acylation, alkylation, and esterification [57].

Derivatization, although useful, complicates sample preparation and may degrade sensitive compounds [38,58].

2. Thermally labile compounds can decompose at the high temperatures required for GC separation, resulting in false identification and quantification. For example, some flavonoids and terpenoids degrade easily under GC conditions, resulting in the loss of structural integrity and the formation of decomposition products that interfere with data interpretation [52].

3. Polar compounds, like organic acids, sugars, and amino acids are not suited for GC-MS analysis due to their low volatility and strong interactions with the stationary phase [59].

4. Complex mixtures of compounds are often found in natural product extracts with similar physicochemical properties, which can cause co-elution and overlapping peaks in GC-MS chromatograms [14].

5. Though GC-MS offers molecular weight and fragmentation patterns, it offers limited structural information compared to techniques like NMR or LC-MS/MS. For instance, distinguishing between isomers or resolving complex stereochemistry can be challenging with just GC-MS, necessitating the use of complementary techniques for complete structural characterization [53].

6. Sample preparation for GC-MS analysis, especially for natural products, can be laborious, time consuming and require major cleanup to remove interfering matrix components. Solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are commonly used extraction procedures, but these methods can lead to the loss of target analytes or contamination [44].

7. Matrix effects, such as ion suppression or amplification, can significantly affect the accuracy and precision of GC-MS data. Natural product matrices, which often contain high levels of co-extractives like pigments and lipids, can interfere with ionization and detection, leading to biased quantification [51].

Because of a number of significant technological advancements that significantly improved the precision of chemical analysis, GC-MS is regarded as revolutionary in analytical chemistry [55]. With the introduction of electron-ionization (EI) and chemical-ionization (CI) sources, reproducible fragmentation patterns that could be utilized to create large mass spectral libraries were made possible, making GC-MS the first method that could quickly and accurately identify unknown compounds [60,61].

Also, the switch from packed columns to high-efficiency capillary columns, which increased separation efficiency by several orders of magnitude, was another significant development [62]. Under ideal circumstances, their theoretical plate counts (up to 10^{12}) significantly decreased co-elution, enabling GC-MS to resolve complex mixtures that were previously impossible to separate using conventional GC or TLC techniques [63] (Figure 2).

2. High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

HPLC-MS is a standard technology for both researchers and industry experts, providing uncommon capabilities to analyze complex mixtures [64]. It is a versatile instrument that can handle a wide range of chemicals, from small molecules like pharmaceuticals to big macromolecules like proteins, making it useful in fields such as drug development, environmental studies, and food science [65]. HPLC separates chemicals based on their interactions with the stationary and mobile phase, enabling the separation, identification and quantification of individual components in complex samples [25]. Modern MS detectors, particularly tandem MS (MS/MS), are extremely sensitive, allowing the identification of trace-level analytes even in challenging matrices [66]. MS extracts structural information from molecules based on their fragmentation patterns. This is critical for identifying new compounds and understanding their characteristics. The technique has become more accessible to a larger variety of academics and aiding high-throughput analysis with the development of smaller, more automated HPLC-MS equipment [2] (Figure 3).

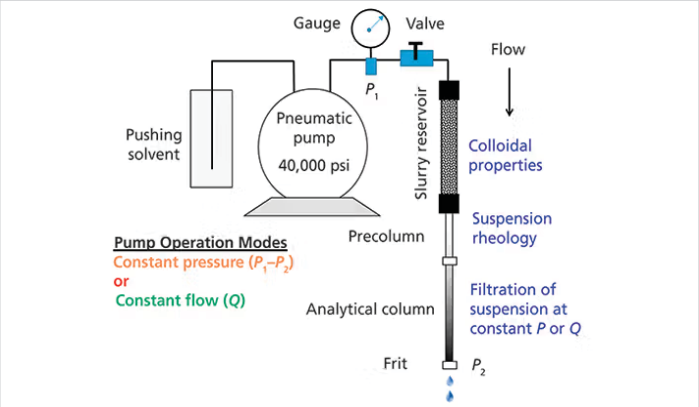

Figure 2: Schematic diagram showing analytical packing system showing major components required for high efficiency packing [62].

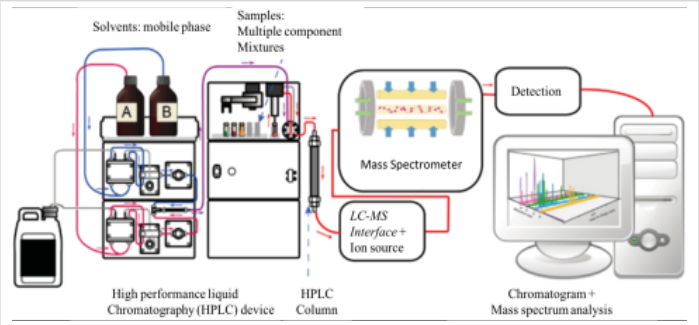

Figure 3: Flow Representation of HPLC-MS [67].

By pushing the limits of separation speed and resolution, Ultra-High-Performance Liquid Chromatography (UHPLC) technology enables even quicker analysis times and greater peak capacity for complex samples [68]. Mass spectrometry has advanced recently, providing excellent mass accuracy and resolution, direct sample analysis without prior preparation, workflow simplification, and increased applicability of HPLC-MS, all of which contribute to more reliable compound identification and isomer differentiation [2].

HPLC-MS is widely used in the analysis of medicinal plants to identify and quantify bioactive compounds such as alkaloids, flavonoids, terpenes, and acids [69]. The technique provides detailed insights into the chemical composition of plant extracts, thereby aiding the discovery of new therapeutic agents [70].

In recent years, the development of green chromatography methods has gained attention in natural products analysis. Researchers are increasingly focused on minimizing the environmental impact of analytical methods [71]. Innovations such as supercritical fluid chromatography (SFC) and the use of green solvents in HPLC-MS provide more sustainable alternatives while maintaining high analytical performance [72]. These environmentally friendly techniques support the principles of green chemistry and sustainability, promoting environmentally responsible research practices [73]. Due to the inherent complexity of bioanalytical difficulties, high-performance liquid chromatography-mass spectrometry (HPLC-MS), frequently referred to as the "gold standard" of bioanalysis, has survived more than 30 years of continuous technology innovation and process advancements [74]. The continuous improvement of HPLC-MS, which pushes the boundaries of science by measuring numerous biomolecules in difficult and diverse matrices, is linked to overcoming matrix interference and improving assay selectivity, accuracy, sensitivity, robustness, and assay speed [75]. Industry-setting assay requirements in the low pg/mL/performance liquid chromatography-tandem mass spectrometry multiple reaction monitoring (LC-MS/MS MRM) range through the administration of biologically relevant mixtures have made it even more difficult to monitor exogenous drugs, their metabolites, and endogenous biomarkers via HPLC-MS at sub-pg/mL to pg/mL levels in complex matrices [76]. Liquid chromatographic columns, gradient behavior, mobile phases and mobile phase additives, ionization techniques, mass analyzers, and acquisition modes are only a few of the areas of HPLC-MS theory and practice where advances have been made due to uncompromising demands in technological requirements [77]. The Strengths and Advantages associated with the use of HPLC-MS in natural products analysis include:

Enhanced sensitivity: Trace levels of bioactive substances can be detected by modern MS detectors, especially tandem MS (MS/MS), which is essential for researching the distribution and possible bioactivity of these compounds [78].

Unparalleled Power for Complex Mixtures: Natural products often consist of complex mixtures of several substances. These may be separated according to polarity with great success using HPLC-MS, enabling individual characterization [11,79].

Structural elucidation: MS fragmentation patterns provide helpful insights regarding the structure of a molecule. This is helpful with the identification of unidentified natural products, a common problem in this sector [2].

Both qualitative and quantitative analyses are possible with HPLC-MS because of its dual functionality, which can be used to determine both "how much" and "what's there" in terms of information. Understanding the chemical makeup of natural products and their possible therapeutic benefits is crucial [80].

Data rich in information: A multitude of data regarding mass, retention duration, and fragmentation are produced by HPLC-MS [44].

In a recent study, Smith, et al. [81] used HPLC-MS to identify and quantify bioactive chemicals in medicinal plants. To evaluate extracts of Panax ginseng, they used UHPLC in conjunction with a quadrupole time-of-flight (QTOF) mass spectrometer. The main bioactive ingredients of Panax ginseng, approximately 50 ginsenosides, were effectively discovered by the study. Accurate molecular weight and structural identification of these substances was made possible by the use of high-resolution mass spectrometry (HRMS). Significant differences in ginsenoside content were found by quantitative investigation across various plant sections and development phases.

Likewise, Jones, et al. [82] used a thorough HPLC-MS technique, combining reversed-phase chromatography with an Orbitrap mass spectrometer, in research to characterize the metabolome of the marine species. The investigation used both focused and non-focused metabolomics to offer a comprehensive understanding of the chemical makeup of the sponge. In the conclusion, the study demonstrated the efficacy of HPLC-MS in thorough metabolomic profiling in addition to highlighting the existence of new compounds with possible medicinal benefits. Accurate mass measurements and high-resolution detection made possible by the use of an Orbitrap MS are crucial for untargeted analysis.

In another study, Lee, et al. [83] used HPLC-MS to evaluate the phytochemical composition of many dietary supplements. The samples were examined by utilizing a triple quadrupole mass spectrometer in conjunction with HPLC. Multiple reaction monitoring (MRM) was used in the investigation to accurately quantify the desired phytochemicals. Significant differences in the phytochemical composition of several brands of dietary supplements were found by the investigation. Important bioactive substances, including flavonoids and polyphenols,

were also found, and their amounts were measured in the study.

HPLC-MS and UHPLC-MS are preferred techniques when analyzing non-volatile, thermally labile, or polar natural products like flavonoids, alkaloids, and glycosides [84]. Both techniques are also preferred in cases where there is no need for derivatization (as required in GC-MS for non-volatile compounds), and when higher sensitivity is required for trace-level detection [79].

The limitations of HPLC-MS are similar to those of UHPLC-MS. HPLC-MS requires expensive instrumentation and frequent maintenance. It also requires careful optimization of mobile phase, ionization conditions, and mass analyzer selection [85]. Complex sample matrices can interfere with ionization, affecting accuracy and reproducibility.

HPLC-MS provides molecular weight but requires additional techniques like MS and NMR for full structural analysis [86]. Additionally, Poor ionization of non-polar or neutral compounds may necessitate derivatization, increasing complexity [87].

3. Liquid Chromatography-Fourier Transform Infrared Spectroscopy (LC-FTIR)

An effective analytical method that has been widely applied in the study of natural products is liquid chromatography-Fourier transform infrared spectroscopy [88]. The integration of liquid chromatography's separation skills with Fourier transform infrared spectroscopy's extensive structural elucidation and identification capabilities results in this coupling [89]. Since the technique enables the simultaneous separation and structural characterization of the different components, LC-FTIR is especially helpful for the investigation of complex mixtures, such as those seen in natural product extracts [90]. The molecular composition and structure of the substances present are ascertained using FTIR spectroscopy, which is based on the sample's absorption of infrared light [91] (Figure 4).

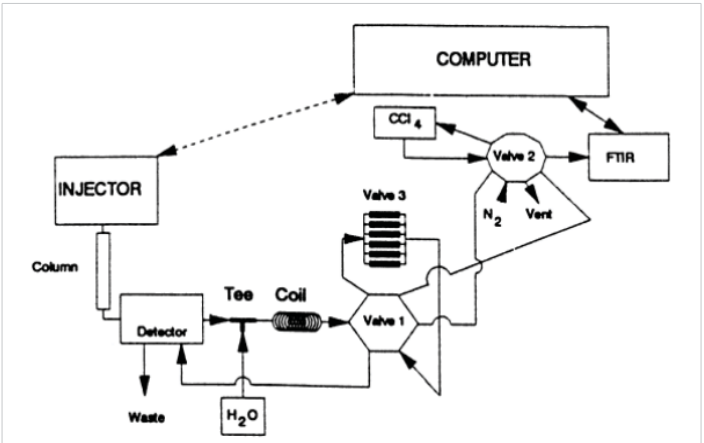

Figure 4: Flow layout of LC-FTIR [92].

The quantitative capabilities of FTIR spectroscopy have been further improved by recent developments in chemometrics and software algorithms, enabling the precise and trustworthy measurement of natural product ingredients [93]. This has been shown to be quite helpful in fields like biomedicine and pharmaceuticals, where accurate measurement of active ingredients is crucial [94].

Chemists who work on the identification and biosynthesis of secondary metabolites have been paying more and more attention to natural products [95]. Particularly, compounds such as lignins, terpenoids, and flavonoids are significant in how plants react to a variety of stresses, such as pathogenic and physical assaults. Their research has started to reveal comparable functions for a few of the essential oils [96].

Typically, chemists have combined thin-layer chromatography (TLC) with sensitive chemical or biochemical examinations. These procedures require a lot of effort and time, and in certain cases, they depend on feeding radioactive precursors [97]. For example, it has long been hypothesized that meillonin biosynthesis targets the enzyme system that directly generates the 3-hydroxy-3-methylglutaryl intermediate from acetate; this idea has recently been thoroughly validated [98]. The development of Fourier Transform Infrared Spectroscopy (FTIR) has made it feasible to study biological extracts without the need for sensitive staining methods or radioactive precursors. Since infrared spectroscopy (IR) is a destructive method, the only way to link the flow of the sample to the result is by comparing the relevant chromatograms [99]. It is now feasible to directly connect the infrared (IR) of the product to discrete parts of the chromatogram by tying liquid chromatography (LC) to this extremely precise detection technology. This method provides a number of strong benefits for the analysis of complex samples [100].

The ability of LC to separate complicated mixtures is a major benefit. Analysis can be hampered when certain components of a mixture are frequently obscured by others. By dividing the mixture according to particular physicochemical characteristics, LC solves this difficulty [101]. This enables the separate FTIR examination of every component, guaranteeing a more thorough comprehension of the sample's makeup. Besides separation, LC-FTIR also provides insightful information on the molecular structure of the separated components [77]. The ability of FTIR spectroscopy to disclose a molecule's functional groups is excellent. By serving as a kind of fingerprint, this data makes it easier to identify components that are unknown and gives us more insight into their chemical makeup [102].

The complementary character of LC-FTIR in relation to other analytical techniques is another benefit [103]. While methods such as LC-MS are excellent at identifying particular compounds, LC-FTIR provides a more comprehensive view of the functional groups that are present. Because of their

complementary nature, researchers may make use of each technique's advantages to provide a more complete view of a sample's composition [104]. In addition, LC-FTIR can often be non-destructive. After analysis, the sample is frequently not altered. This provides opportunities for additional research or even the possible recovery of the separated components for use in subsequent analyses, which is a big benefit for rare or valuable materials [105].

Liquid chromatography was used by Zhang, et al. [106] to isolate the constituents of essential oils, and FTIR spectroscopy was then utilized for structural analysis. Important ingredients in the essential oils, including linalool and menthol, were effectively identified by the study. FTIR spectra confirmed the existence of several phenolic compounds and terpenes by providing precise information on functional groups. The results of comparative research showed that the chemical makeup of oils from various regions varied.

Zhang's research demonstrated how well LC-FTIR performs when assessing complicated mixtures, such as essential oils. Precise identification and structural clarification of constituents were made possible by the combination of infrared spectroscopy and chromatography.

Kim, et al. [107] separated and analyzed the polyphenols in green tea using high-performance liquid chromatography (HPLC) coupled with FTIR. A thorough spectrum analysis was part of the investigation to pinpoint important functional groups. Major polyphenols, including epicatechins and catechins, as well as their derivatives, were found by the investigation. FTIR spectra shed light on the carbonyl and hydroxyl groups that define polyphenols. The work demonstrated how spectral fingerprints may be used by LC-FTIR to differentiate between different polyphenolic substances. Kim, et al. skillfully illustrated how to use LC-FTIR for polyphenol profiling, highlighting the method's potential to yield structural details. Although the spectral analysis of the study was extensive, quantitative information on the concentration of polyphenols was absent. Quantitative LC-MS analysis might improve the results and present a more comprehensive picture.

Gonzalez, et al. [23] employed LC-FTIR to examine herbal supplement samples that were suspected of being adulterated in a related investigation. The goal of the study was to identify medicinal compounds that were not reported and synthetic additives. Several undisclosed substances, such as steroids and synthetic stimulants, were found in the herbal supplements, according to the research. FTIR spectra showed distinctive peaks that matched the adulterant's functional groups. The study highlighted the possible health hazards linked to contaminated herbal items. Gonzalez demonstrated the value of LC-FTIR in assuring product safety by offering important insights into the identification of adulterants in herbal supplements. The capacity of the research to recognize a wide variety of adulterants is one of its strongest points.

Additionally, Singh, et al. [108] isolated alkaloids from plant extracts using liquid chromatography and then employed FTIR analysis to characterize the structural features of the mixture. The study concentrated on medicinal plants rich in alkaloids, including Rauvolfia serpentina and Catharanthus roseus. The investigation found vincristine and reserpine to be two important alkaloids. FTIR spectra confirmed the existence of quinoline and indole alkaloids by providing extensive information on the functional groups. Based on these alkaloids' structural properties, the study emphasized the medicinal uses they may have. Singh, et al. successfully characterized alkaloids using LC-FTIR, offering insightful information on their structural makeup. Although the study's comprehensive spectrum analysis was impressive, it lacked quantitative information on the quantities of alkaloids. Combining LC-FTIR with quantitative methods like HPLC-UV or LC-MS might help future studies give a more thorough understanding.

For functional group identification, LC-FTIR is preferred to LC-NMR-MS, for instance, when distinguishing between isomers according to carbonyl or hydroxyl functional groups [109]. LC-FTIR is equally preferable when characterizing new compounds in plant extracts without the use of spectral libraries and when the sample needs to be preserved for further testing or when non-destructive analysis is required [110].

1. LC-FTIR has some limitations that can make other techniques preferred for natural products analysis. The limitations are as followsIn aqueous-based LC separations, the identification of analytes may be hampered by the high infrared absorption bands of water. On account of this, IR-transparent solvents or post-column solvent removal methods are required [111].

2. While FTIR offers useful functional group information, it is not as capable as methods such as LC-MS or LC-NMR for elucidating high-resolution molecular structures. Compound identification may become less certain as a result [112].

3. The overlapping IR absorption bands of complex mixtures can make spectral interpretation challenging, requiring advanced software and spectral libraries for accurate analysis [113].

4. FTIR detectors need specialized optics and attenuated total reflectance (ATR) accessories, which increase the system's complexity and cost in contrast to traditional UV or MS detectors [114].

5. A good number of LC-FTIR systems require post-column solvent evaporation or solvent exchange before IR detection to minimize solvent interference. This extra step may increase analysis time and reduce throughput [77].

4. Liquid Chromatography-Nuclear Magnetic Resonance Spectroscopy-Mass Spectrometry (LC-NMR-MS)

This recently developed hyphenated technique combines the sensitivity and molecular weight information from MS with the separation power of LC and the structural elucidation capabilities of NMR [112]. The use of the analytical approach in the field of natural products has been documented recently, owing to the exceptional benefit of LC-NMR-MS. Complex mixtures must first be divided into their parts according to their respective physicochemical characteristics, such as polarity and affinity for the stationary phase, using liquid chromatography (LC) [115]. The separated components enter the NMR spectrometer when they elute from the LC column. This is where the molecules' detailed structural information is found, which offers important details regarding connectivity, stereochemistry, and functional groups [116]. The mass spectrometer, which measures the compounds' molecular weight, receives the LC effluent as well. Furthermore, MS fragmentation patterns can supplement NMR with additional structural information [117].

There are various benefits associated with LC-NMR-MS. This hyphenated method totally provides quantitative and structural information for compound characterization [112]. It can be difficult to differentiate between isomers using conventional methods, but LC-NMR-MS does a great job at it [112]. In contrast to some other analytical techniques, LC-NMR-MS is often non-destructive, enabling further analysis or sample recovery [118]. This method works especially well for examining complex mixtures, such as natural products, where it might be difficult to distinguish several components that have similar characteristics [119].

The use of LC-NMR-MS in the examination of natural products is revolutionary [120]. Complex mixtures may now be quickly characterized by researchers without the need for time-consuming separation and purification procedures [121]. This effectiveness makes it possible to find compounds that could otherwise go unnoticed [14]. Furthermore, the method's capacity to distinguish between isomers—which present major challenges for conventional analytical techniques—is crucial in the field of natural product chemistry [119].

Researchers like Tan, et al. [122] used LC-NMR-MS to comprehensively profile secondary metabolites in herbal extracts. While molecular weight information and further structural insights were obtained by MS, NMR was utilized to get precise structural information. Numerous secondary metabolites, including flavonoids, alkaloids, and terpenoids, were effectively discovered by the study. The NMR spectra provided comprehensive structural details, including stereochemistry and connectivity, whereas the MS verified the molecular weights and fragmentation patterns. The combination of methods made it possible to identify a number of unique compounds that had not been documented before in any literature.

LC was used by Wang, et al. [123] to isolate the antioxidant components found in fruit extracts. While MS revealed fragmentation patterns and molecular weight information, NMR provided structural information. Many antioxidants, such as vitamin C, flavonoids, and polyphenolic substances, were found in the research. NMR spectra provided comprehensive structural details, such as stereochemistry and functional groups. The identification of the antioxidants was aided by the confirmation of the molecular weights and fragmentation patterns by MS data. A thorough comprehension of the antioxidant chemicals was made possible by the combination of approaches.

To isolate the complex mixture of bioactive chemicals present in marine algal preparations, Kim, et al. [124] employed liquid chromatography (LC). Molecular weight information and fragmentation patterns were provided by MS to assist in compound identification, while NMR supplied comprehensive structural details. For the integration of LC, NMR, and MS data, the study offered a clear process. Polysaccharides, peptides, and small-molecule metabolites were among the bioactive substances that the investigation also found. The three-dimensional structures and functional groups of the compounds that were found were described in detail by the NMR spectra. Clarifying the structure was made easier by the confirmation of fragmentation patterns and molecular weights by MS data.

Singh, et al. [125] also used LC-NMR-MS to analyze phytochemicals in traditional medicinal plants. Numerous phytochemicals were found in the study, such as glycosides, phenolic acids, and flavonoids. While MS data validated the molecular weights and fragmentation patterns, supporting the identification procedure, NMR spectra offered comprehensive structural information, including stereochemistry and connectivity.

LC-NMR-MS is preferred over LC-MS when full structural elucidation is required, especially for novel compounds without spectral library matches [126]. It is also favored in cases where differentiating positional isomers that have identical mass spectra is necessary and in analyzing complex mixtures where compounds co-elute in LC-MS, making mass spectra alone insufficient [127].

In spite of its many advantages, LC-NMR-MS has some limitations. The technique requires sophisticated instrumentation and expertise, which can be expensive and time-consuming [128]. Also, NMR's sensitivity is generally lower than MS, which can limit its use for trace analysis [129]. However, in recent years, NMR sensitivity has greatly improved due to advancements in cryogenic technology and flow probes [130].

5. Ultra-High-Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS)

UHPLC operates on the same fundamental principles as High-Performance Liquid Chromatography (HPLC) but utilizes smaller particle sizes (typically sub-2 µm) in its stationary phase [131]. This is crucial for operating at greater pressures (up to 100 MPa), which will lead to quicker and more effective separations [132]. By utilizing mass spectrometry integration, molecules may be identified and quantified according to their mass-to-charge ratio, yielding comprehensive insights into the chemical composition and structure of the analytes [2]. Although both UHPLC and HPLC use comparable operating procedures, UHPLC offers superior chemical separation because of smaller particle sizes and considerably shorter analytical times Nahar, et al. 2020. Figure 5 presents the flow diagram for UHPLC-MS.

The capacity of UHPLC-MS to handle a wide variety of analytes is a unique benefit. This method shows remarkable adaptability in handling small molecules as well as biomacromolecules [134]. Due to its sensitivity, it can identify and measure molecules at the trace level. Additionally, high-throughput analysis is made possible by UHPLC-MS's speed, which speeds up the screening and quality control processes [135]. Other advantages include: Improved Resolution and Sensitivity: The instrument can identify and measure minute components in intricate matrices because of UPLC's much better peak resolution and sensitivity, particularly caused by its smaller particle size columns and higher working pressures [136] (Figure 6).

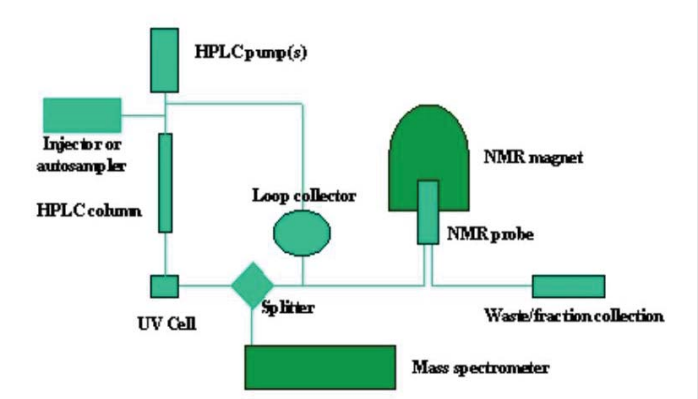

Figure 5: Flow Representation of LC-NMR-MS [133].

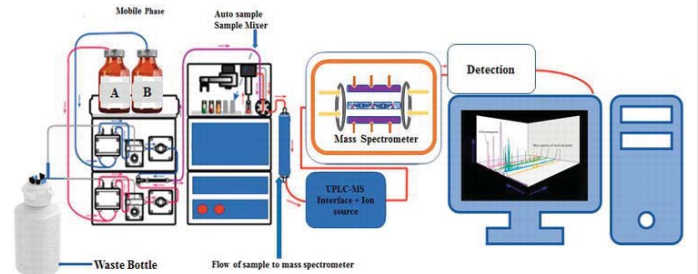

Figure 6: Flow diagram of UHPLC-MS [137].

Quick analysis: UHPLC-MS provides significantly quicker analysis times than conventional HPLC-MS, thereby boosting the volume of samples and effectiveness [138].

Better data quality: Cleaner spectra and more accurate data are produced by the combination of sensitive mass detection and high-resolution separations, which makes compound identification and quantification easier Guo, et al. 2020.

In a study by Chen, et al. [139], researchers looked into the chemical composition of traditional Chinese medicinal herbs using UHPLC-MS. Identifying and quantifying the bioactive substances that give these plants their medicinal properties was the main goal. The MS component supplied comprehensive molecular information, such as molecular weights and fragmentation patterns, whereas the UPLC component was employed for its effectiveness in separating complex mixtures. They pointed out that compounds present at low concentrations that could have gone undetected with less sophisticated methods have been found thanks to the excellent sensitivity and resolution of UHPLC-MS. The study emphasized the use of UPLC-MS in the thorough examination of medicinal plants, which aids in the identification of possible novel therapeutics. Chen, et al. did, however, draw attention to the necessity of additional in vitro and *in vivo* research to confirm the biological activities of the discovered compounds and support their therapeutic potential.

Similarly, Johnson, et al. [140] utilized UHPLC-MS to examine marine sponges in particular and other secondary metabolites in marine species. The objective of the study was to investigate the chemical variety of marine sponges and find substances that could have therapeutic uses. The exact mass analysis and effective separation of the metabolites were made possible using the UHPLC-MS technology. A number of new polyketides and peptides with noteworthy bioactivity, such as antibacterial and anticancer capabilities, were discovered by Johnson, et al. Precise molecular weight measurements and the ability to differentiate between structurally identical molecules were made possible by the high-resolution capabilities of UHPLC-MS. The study brought attention to the marine sponges' unrealized potential as a source of new bioactive chemicals.

In another recent study, García, et al. [141] utilized UHPLC-MS to investigate the polyphenolic content of various fruit extracts. The research aimed to identify and quantify polyphenolic compounds, which are known for their antioxidant properties. The use of UHPLC-MS enabled the separation of complex polyphenolic mixtures and provided accurate mass data for compound identification. García, et al. identified a wide range of polyphenols, including flavonoids, phenolic acids, and tannins, and quantified their concentrations in different fruit extracts. The researchers highlighted the

health benefits associated with these polyphenols, such as reducing the risk of chronic diseases due to their antioxidant activity. The study demonstrated the effectiveness of UHPLC-MS in the detailed profiling of polyphenolic compounds, contributing valuable information to the field of nutritional science.

UHPLC-MS is preferred over HPLC-MS when there is a need for faster analysis with better resolution and peak capacity. Also, when there is limited sample volume, a need for high-throughput screening of complex plant extracts, and efficiency in solvent usage is important, UHPLC is the technique of choice [142].

1. The major limitations of UHPLC-MS are underlisted. UHPLC-MS systems are expensive to purchase and maintain, requiring specialized columns, high-pressure pumps, and advanced mass spectrometers. Operational costs, including ultra-pure solvents and maintenance, are also higher compared to traditional HPLC-MS [143,144].

2. Optimizing UHPLC-MS methods is time-consuming and requires experience. High pressures and rapid flow rates demand careful adjustment of parameters like column chemistry, particle size, and gradient conditions [52].

3. UHPLC requires specialized columns with small particle sizes (<2 µm), which are more susceptible to clogging and degradation. Older HPLC columns are often incompatible, limiting flexibility [145].

4. High-resolution UHPLC-MS data is complex to interpret and generates large file sizes, requiring advanced software and significant storage capacity. This can complicate data management and analysis [52].

5. UHPLC-MS is prone to matrix effects, such as ion suppression or enhancement, which can affect quantification [146].

6. Liquid Chromatography- Ultra Violet- Mass Spectrometry (LC-UV-MS)

This technique combines the separation capabilities of liquid chromatography with the detection and quantification abilities of ultraviolet (UV) and mass spectrometry (MS) for the separation and identification of complex mixtures [112]. The combination of LC-UV with MS provides a comprehensive approach to understanding the structural and kinetic characteristics of natural products. LC separates compounds based on their chemical properties, while UV provides a preliminary identification based on absorbance, and MS offers detailed molecular information [147]. This combination is effective for detecting and characterizing small molecules in complex mixtures [148].

LC-UV-MS is widely applied in various natural product analyses, including:

Metabolite profiling: Identification of secondary metabolites such as alkaloids, flavonoids, and terpenoids in medicinal plants [149].

Quality control of herbal medicines: Standardization and authentication of herbal formulations based on characteristic UV and MS fingerprints [150].

Pharmacokinetic studies: Determination of bioavailability and metabolic pathways of natural products in biological samples [151].

Food and nutraceutical analysis: Detection of bioactive polyphenols, carotenoids, and vitamins in food products [152].

Advantages of LC-UV-MS abound, and a few of them are underlined.

1. LC-UV-MS offers a robust method for initial screening and quantification of compounds with UV-active chromophores. It is widely used due to its simplicity and cost-effectiveness.

2. LC-UV-MS is versatile as it is suitable for a wide range of natural products, including polar and non-polar compounds.

3. This technique is highly Sensitive as the MS enhances detection limits compared to UV alone.

4. The non-destructive UV Detection allows for real-time monitoring of chromatographic elution.

Limitations of LC-UV-MS [112,151,153,154]

- LC-UV-MS is limited by its reliance on UV-active compounds, which may not be present in all natural products. It cannot differentiate isomers effectively
- Non-chromophoric compounds like some terpenoids remain undetected by UV.
- The combined UV and MS spectra require advanced spectral deconvolution techniques.
- Co-eluting compounds may affect UV absorbance and ionization efficiency in MS due to matrix interference.
- Matrix effects may lead to variable ionization efficiency, affecting quantification accuracy.

Advancements in LC-UV-MS aim to enhance analytical capabilities in the areas of improved UV detectors with higher sensitivity, High-Resolution Mass Spectrometry (HRMS), miniaturization of LC-UV-MS systems for field analysis, and integration with Artificial Intelligence for automated peak identification and data processing [155].

LC-UV-MS is preferred over stand-alone UV or MS techniques for its ability to provide both qualitative and quantitative data in a single run [156]. It is also preferred when a combination of UV and MS detection can enhance compound

characterization, especially for UV-active compounds such as polyphenols, carotenoids, and flavonoids [157]. LC-UV-MS is equally the ideal technique when rapid screening of samples is needed using UV fingerprints [158].

In a recent study by Saidi, et al. [159], the phytochemical composition, biological activities, and pharmacological properties of Ajuga iva (L.) leaf extracts were investigated to confirm its traditional use in Moroccan ethnomedicine for treating diabetes, microbial infections, and oxidative stress. The study focused on analyzing the plant's chemical composition, antioxidant, antimicrobial, and antidiabetic properties using various in vitro and *in vivo* methods.

In the study, LC-UV-MS played a crucial role in the phytochemical analysis of Ajuga iva by identifying and characterizing its polyphenolic compounds. Specifically, the aqueous extract of Ajuga iva was analyzed using LC-UV-MS, which led to the identification of 32 polyphenolic compounds, including ferulic acid (19.06%), quercetin (10.19%), and coumaric acid (9.63%) and apigenin-7-(2-O-apiosylglucoside) (6.8%). LC-UV-MS provided a comprehensive chemical profile, which correlated with the plant's antioxidant, antimicrobial, and antidiabetic activities, demonstrating that LC-UV-MS is a powerful tool for metabolite profiling in natural product research.

In another study by Tinnirello, et al. [160], the protective effects of industrially produced lemon-derived nanovesicles (iLNVs) in inflammatory bowel disease (IBD) using a rat model were analysed. The key findings in the study were a) Characterization of iLNVs, b) In Vitro Studies (on Human Macrophages), c) *in vivo* Effects (Rat Model of Colitis).

Qualitative and quantitative characterization of iLNV metabolites was carried out. Qualitative analysis of iLNVs was performed by UHPLC-UV-ESI-MS. Q-Exactive Plus mass spectrometer equipped with an electrospray source (ESI) and coupled to an Ultimate 3000 SD UHPLC. Citric acid and isocitric acid content were also quantified by UHPLC-UV-ESI-MS, consisting of a Q-Exactive Plus mass spectrometer equipped with an electrospray source (ESI) and coupled to an Ultimate 3000 SD UHPLC. This step provided a comprehensive chemical profile of LNVs, highlighting the specific compounds responsible for their therapeutic effects. It also helped support the study's findings on the antioxidant activity of LNVs and their role in reducing oxidative stress in colitis.

7. LC-Ion-Mobility/LC-Ion-Mobility-MS

For this review, liquid chromatography ion mobility spectrometry (LC-Ion-Mobility) and liquid chromatography ion mobility mass spectrometry (LC-Ion-Mobility-MS) will be discussed together.

Liquid Chromatography-Ion Mobility Spectrometry (LC-IMS) integrates liquid chromatography (LC) with ion mobility spectrometry (IMS) to enhance the separation and analysis of complex mixtures, especially in the study of natural products [161]. This combination offers enhanced resolution and structural elucidation capabilities by utilizing the unique separation processes of both approaches [68].

In LC-IMS, liquid chromatography is used to first separate substances according to their physicochemical characteristics. After that, the eluted chemicals are ionized and put into the IMS cell, where an electric field separates the ions according to how easily they can move through a neutral gas. Finally, the separated ions are fed into the mass spectrometer, where they undergo additional analysis based on their mass-to-charge ratio, allowing for structural elucidation and precise mass determination [162]. Several variables, including the ion's size, shape, charge, and collision cross-section, affect its mobility. Ions that are smaller or more compact usually go through the IMS cell more quickly than those that are longer or bigger [163]. Combining LC and IMS improves the study of complicated mixtures by offering a multidimensional separation strategy [164].

In natural product research, LC-IM-MS helps to characterize bioactive substances with structural similarities. Many natural products have isomeric or identical structures, complicating analysis [165]. The inclusion of ion mobility makes it possible to distinguish between these compounds, making it easier to identify and structurally elucidate novel bioactive molecules with pharmacological or nutraceutical potential [78].

A key advantage of LC-IMS is its enhanced separation efficiency. The combination of LC and IMS provides orthogonal separation processes; this means that compounds are separated according to both their chromatographic retention time and gas-phase ion mobility [166]. This makes it possible to resolve compounds that could otherwise co-elute in conventional LC methods, leading to improved identification and quantification of complex mixtures [167].

Another benefit is the ability of IMS to provide structural information about analytes. The IMS component provides insights into the gas-phase conformations of ions, which is particularly useful for distinguishing between isomers and conformers [168]. This structural differentiation is crucial in applications such as natural product analysis and metabolomics, where many compounds share similar molecular weights but differ in their three-dimensional structure [169].

LC-IMS also allows for quick analysis, as IMS operates on a millisecond timescale. This fast separation capability enables high-throughput screening and real-time analysis, making the approach appropriate for applications requiring fast and efficient sample processing [123]. The speed of IMS does not affect the quality of the results, ensuring both rapid data capture and high-resolution separation [170].

Also, LC-IMS improves sensitivity by increasing the signal-to-noise ratio. The additional ion mobility separation stage reduces chemical noise, resulting in better detection of low-abundance compounds. This benefit is especially useful in trace analysis, where detecting minute amounts of bioactive chemicals can be difficult [171]. With the inclusion of mass spectrometry MS, the system provides superior evaluation of complex samples by utilizing different separation mechanisms [166].

Limitations of LC-Ion Mobility Spectrometry/LC-Ion-Mobility-MS

1. Differences in ionization efficiencies between compounds can adversely affect the sensitivity and quantitative accuracy of the assay [172].

2. The multidimensional data generated require advanced computational tools and skills for proper interpretation [173].

3. Integrating IMS with LC and mass spectrometry (MS) adds to the system's complexity, potentially increasing maintenance requirements and operational costs [174].

4. The integration of LC, IMS, and MS results in a high-cost, complex instrument requiring regular calibration and maintenance [175].

5. The multidimensional data generated in LC-IMS-MS requires advanced bioinformatics tools for interpretation [176].

6. Unlike traditional MS databases, IMS spectral libraries are still under development, which makes the identification of unknown compounds more challenging [177].

LC-IMS is particularly preferred in cases where separation based on molecular shape, size, and charge is the primary objective [178]. Since IMS differentiates molecules based on their gas-phase mobility, it is especially useful for resolving isomeric compounds that may exhibit similar mass spectra but differ in structural conformation [179]. This makes LC-IMS a valuable instrument for researching compounds with small structural differences that typical LC techniques may find difficult to separate [166].

The integration of mass spectrometry and LC-IMS increases analytical power, making it the preferred approach in more complex studies [166]. LC-IMS-MS is especially useful for evaluating complex natural product mixtures containing isobaric or structurally related compounds, as it adds a dimension of separation beyond mass-to-charge ratio alone [180]. Furthermore, where the investigation requires separation based on gas-phase conformation, LC-IMS-MS increases resolution and aids in the identification of compounds that would otherwise be indistinguishable [181]. This approach is also very useful for studying conformational differences in biomolecules like peptides and flavonoid glycosides, where structural variations play an important role in biological function and activity [182].

In a study by Venter, et al. 2018, the power of combining multiple chromatographic and spectrometric techniques for comprehensive chemical analysis was leveraged.

This approach greatly enhanced the resolution of complex phenolic mixtures. HILIC successfully separated highly polar phenolic compounds, but RP-LC proved more effective for non-polar and moderately polar compounds. IMS provided an additional layer of separation based on molecule size, shape, and charge to help differentiate structurally related substances.

By taking advantage of the orthogonality of these approaches, the study produced greater phenolic compound characterization, allowing for better distinction of isomers and structurally similar species. The use of IMS assisted in resolving co-eluting substances that would otherwise be indistinguishable in conventional LC-MS analysis.

In another article by Zheng, et al. [183], the application of modern analytical techniques to characterize the chemical composition of diverse portions of the medicinal plant Gynostemma longipes was investigated. The authors used two-dimensional liquid chromatography (2D-LC) combined with ion mobility-mass spectrometry (IM-MS) to accomplish high-resolution separation and identification of multicomponent mixtures in the plant. The study discovered a variety of bioactive substances, including saponins, flavonoids, and phenolic acids, which are renowned for their therapeutic qualities. The use of 2D-LC-IM-MS revealed its potential to do thorough multicomponent analysis, paving the way for future medicinal plant studies.

Carnevale Neto, et al. [184], in a study, investigated the use of ion mobility spectrometry (IMS) in the investigation of complex natural product mixtures. The study looked at how IMS, paired with mass spectrometry (MS), can improve the structural characterization and constitutional assignment of compounds in natural product extracts. The study emphasized the limitations of evaluating complex mixtures, such as overlapping peaks and structural similarities, and showed how IMS can increase resolution and provide new structural insights. The key findings in the study are summarized below:

Ion mobility spectrometry (IMS), which adds a dimension of separation based on size, shape, and charge, greatly enhances the separation of chemicals in complex natural product mixtures. As a result, the resolution of structurally related molecules is improved, and peak overlap is decreased. The study shows how well IMS-MS analyzes complex natural product extracts, including those from microbes and plants. It highlights how IMS can distinguish between isomers and

structurally similar molecules, which are frequently difficult to resolve with traditional MS alone.

Current trends and future directions

Current trends and prospects in chromatography hyphenated techniques reflect significant advancements in analytical chemistry, particularly in enhancing the sensitivity, speed, and resolution of complex sample analyses [164,185]. These techniques combine chromatographic processes with spectroscopic detection to offer both comprehensive qualitative and quantitative data. Here's an overview of the existing climate and anticipated developments in this field [186] (Tables 1,2).

Recent advancements have resulted in a rise in the use of triple hyphenated techniques, where multiple analytical methods are combined [1]. This approach enables more detailed analysis by integrating separation methods, such as liquid chromatography (LC) or gas chromatography (GC), with various detection techniques, such as mass spectrometry (MS), nuclear magnetic resonance (NMR), and Fourier-transform infrared spectroscopy (FTIR) [190]. For instance, combining LC-MS-MS or LC-NMR-MS facilitates the identification and quantification of compounds in complex matrices, benefiting pharmaceutical and environmental analyses [191]. Hyphenated techniques are becoming increasingly important in natural products analysis, enabling researchers to isolate and identify compounds from complex mixtures efficiently. LC-MS and LC-FTIR techniques are increasingly used for chemotaxonomic studies, chemical fingerprinting, and quality control of herbal products [192]. This trend is motivated by the need for fast and reliable analysis in natural

Table 1: Table comparing the different chromatography hyphenated techniques [187-189].

Hyphenated Technique	Principle	Sensitivity	Detection Limit	Pre-cision	Sample Preparation Impact	Advantage	Disadvantage	Best Applications	Resolution	Throughput	Instrument Cost And Maintenance	Suitable Sample Types
GC-MS	Combines gas chromatography (separation) with mass spectrometry (detection).	High	Picomolar (extremely low concentrations)	High	High. Often requires derivatization for polar/non-volatile compounds.	-High chromatographic resolution. -extensive spectral libraries	-Requires derivatization for non-volatile compounds. -Limited to thermally stable analytes	Analysis of essential oils, terpenes, and volatile organic compounds	High	High	Moderate	Volatile and semi-volatile compounds
HPLC-MS	Combines liquid chromatography (separation) with mass spectrometry (detection)	High	Nanomolar (very low concentrations)	High	Moderate. Requires extraction, filtration, and sometimes derivatization.	-Broad applicability suitable for thermally labile/ non-volatile compounds	-Lower chromatographic resolution than GC -Matrix effects may influence ionization	Alkaloids, flavonoids, and polar compounds in plant extracts	Moderate	Moderate	High	Polar and non-volatile compounds
LC-FTIR	Combines liquid chromatography (separation) with Fourier transform infrared spectroscopy (detection).	Moderate	Nanomolar	Mode rate	Moderate. Requires solvent compatibility with FTIR detection.	-Provides structural information -Non-destructive analysis	-Lower sensitivity -water interference in IR spectra -Requires specialized detectors	-Identification of functional groups in complex mixtures	Moderate	Low	High	Samples with functional groups amenable to IR detection
LC-NMR-MS	Combines liquid chromatography (separation) with NMR & MS (detection).	Moderate	Micromolar (low concentrations)	High	High. Requires high sample purity, deuterated solvents for NMR, and oncentration optimization for sensitivity. The challenge to maintain compatibility with the three techniques while maintaining sample integrity and achieving good signal strength is high.	-\Comprehensive structural elucidation -Combines molecular weight and structural information	-High cost -Requires large sample amounts -Complex data interpretation	-Structure determination of unknown natural products -Metabolite identification	High	Low	Very High	Samples available in sufficient quantities and complex mixtures requiring detailed analysis
UHPLC-MS	Uses ultra-high-pressure liquid chromatography for faster separations with MS.	Very High	Nanomolar	High	Moderate; similar to HPLC-MS but may require finer particle filtration.	Higher resolution and speed than HPLC, reduced solvent consumption, and suitable for high-throughput analysis	-High backpressure -Requires specialized equipment	-Rapid profiling of natural product extracts -Analysis of complex mixtures with improved resolution	Very High	Very High	High	Complex mixtures, samples requiring high-throughput analysis

LC-UV-MS	Combines liquid chromatography with UV detection and mass spectrometry.	High	Nanomolar	High	Moderate. Requires UV-active compounds for detection.	Dual detection enhances compound identification -UV provides structural information	-UV detection limited to chromophoric compounds -Increased complexity in data analysis	Polyphenols, UV-absorbing compounds, quality control of herbal products	Moderate	Moderate	Moderate	Samples containing UV-active compounds
LC-ION-MOBILITY	Combines liquid chromatography with ion mobility spectrometry.	Very High	Nanomolar to picomolar, depending on the ionization source and the detector used	High	Moderate. Less complex than LC-ION-MOBILITY-MS but still requires optimization.	- Separates compounds based on size, shape, and charge in addition to mass-to-charge ratio. - Resolves isobaric and isomeric compounds that conventional LC-MS may struggle with. - Provides additional structural information through collision cross-section (CCS) values. - Reduces background noise and enhances signal clarity.	- Requires specialized instrumentation with an additional ion mobility separation module. - Increased complexity in data interpretation. - Higher cost compared to traditional LC-MS.	- Separation and characterization of isomeric and structurally similar compounds in plant extracts. - study of small molecules, fat, and other related molecules in living organisms where structural differentiation is crucial. - Detection of trace-level secondary metabolites in complex mixtures.	Very High	Moderate	Very High	Complex natural product extracts containing isomers, samples with interfering matrix effects that require enhanced separation, metabolite profiling in biological and environmental samples.
LC-ION-MOBILITY-MS	Combines liquid chromatography with ion mobility separation and MS.	Very High	NANOMOLAR	High	Moderate to high. Requires optimization for ion mobility separation.	Separation based on size, shape, charge -Resolves isobaric compounds -Provides collision cross-section data	-Requires specialized instrumentation -Complex data interpretation	-Isomer separation -Complex mixture analysis	Very High	Moderate	Very High	Complex mixtures with isobaric/ isomeric compounds, conformational analysis

Table 2: First Glance Summary of Reviewed Literatures Showing Techniques used, Country of Study, and Research Focus.

Technique	Study	Country / Region	Main Research Focus
GC-MS	Satyal [46]	USA / Nepal	Essential oils, volatile profiling
HPLC-MS	Smith, et al. [81]	USA	Natural product metabolite profiling
UHPLC-MS	Johnson, et al. [140]	USA	High-throughput phytochemical analysis
GC-MS	Hu, et al. [47]	China	Plant metabolite characterization
LC-FTIR	Zhang, et al. [106]	China	Structural elucidation via IR spectra
LC-NMR-MS	Wang, et al. [123]	China	Multi-hyphenated structural analysis
UHPLC-MS	Chen, et al. [139]	China	High-resolution profiling
LC-IM-MS	Zheng, et al. [183]	China	Ion-mobility-based compound separation
GC-MS	Lee, et al. [48]	South Korea	Volatile compounds & functional components
HPLC-MS	Lee, et al. [83]	South Korea	Bioactive compound identification
LC-FTIR	Kim, et al. [107]	South Korea	IR-based structural confirmation
LC-NMR-MS	Kim, et al. [124]	South Korea	Comprehensive metabolite structure profiling
GC-MS	Patel, et al. [49]	India	Plant extract chemical composition
LC-FTIR	Singh, et al. [108]	India	Functional group analysis
LC-NMR-MS	Singh, et al. [125]	India	NMR-based metabolite elucidation
GC×GC-MS	Garcia-Valverde, et al. 2020	Spain	Multidimensional volatile analysis
UHPLC-MS	García, et al. [118]	Spain	High-resolution metabolomics
HPLC-MS	Jones, et al. [82]	UK	Authentication and metabolomics
LC-FTIR	Gonzalez, et al. [23]	Mexico	Structural fingerprinting
LC-NMR-MS	Tan, et al. [122]	Singapore	Advanced 3D structure elucidation
LC-UV-MS	Saidi, et al. [159]	Morocco	UV-based profiling
LC-IM / LC-IM-MS	Venter, et al. 2018	South Africa	Ion mobility separation and profiling
LC-IM-MS	Carnevale Neto, et al. [184]	Brazil	Complex metabolite separation

product research, which often involves complex matrices. In addition, innovative sample preparation techniques, such as solid-phase extraction (SPE) and large volume injection (LVI), are being incorporated into hyphenated systems to improve sensitivity and reduce analysis time [193]. These methods help concentrate analytes and remove interfering substances, thereby enhancing the overall performance of the analytical techniques [138]. In the fields of environmental monitoring and food safety, where accurate contaminant detection is crucial, hyphenated techniques are being used with increasing frequency. Research and application in the field of analyzing trace levels of contaminants in environmental samples and monitoring food products for safety and quality are growing [194].

Future developments in hyphenated chromatography techniques will focus on creating increasingly selective and sensitive processes [195]. Mass spectrometry advances like tandem mass spectrometry (MS/MS) and high-resolution mass spectrometry (HRMS) will improve the capacity to evaluate complex samples with lower detection limits and higher accuracy [196]. Also, hyphenated technique automation and instrument miniaturization are trending toward greater accessibility and user-friendliness. This shift is anticipated to speed up sample processing for a variety of applications, including drug development and clinical diagnostics, by enabling high-throughput analysis [197]. Combining cutting-edge technologies like ion mobility spectrometry (IMS) with conventional hyphenated procedures may enhance the study of intricate biological and environmental materials by adding new dimensions of separation and identification [198]. It is anticipated that this hybrid technique would improve analysis speed and resolution. Applications for hyphenated techniques will likely increase in a number of fields, such as metabolomics, forensic science, and biological research, as they continue to advance [199]. It will be easier to find novel compounds and their biological functions and to promote multidisciplinary research efforts if comprehensive analytical data is available [200].

The role of green chromatography approaches in reducing environmental impact

Traditional chromatography methods usually involve the use of large volumes of organic solvents, generate significant laboratory waste, and consume large amounts of energy. To address these challenges, green chromatography approaches are employed [201]. The aim of green chromatography and green chemistry approaches is to enhance biodiversity through eco-friendly sample collection/preparation, enhance safety for laboratory personnel by minimizing exposure to harmful chemicals/substances, reduce solvent dependency, and minimize environmental impact while maintaining analytical performance [202]. Below are a few strategies in green chromatography for hyphenated techniques:

-Use of eco-friendly Solvents [203]

Traditional solvents like acetonitrile and methanol, which are widely used in liquid chromatography, pose environmental and health hazards. To cut down on waste and toxicity, green alternatives such as ethanol, water, and supercritical CO_2 are being explored. CO_2 is used as the mobile phase in supercritical fluid chromatography (SFC), a potent green technology that greatly reduces the use of organic solvents and is a great substitute for natural product analysis.

-Cutting Down on the Use of Solvents and Reagents [203]

Ultra-high-performance liquid chromatography (UHPLC-MS) operates at higher pressures, allowing for shorter columns, quicker run time, and lower solvent consumption while preserving excellent separation efficiency.

-Energy-Efficient Instrumentation

Reduced energy consumption in hyphenated chromatography systems has been facilitated by the development of more effective mass spectrometry (MS) detectors and ionization techniques [204]. Compact and portable chromatography-MS equipment reduces power needs, making field analysis of natural products more feasible [205].

-Recyclable and Biodegradable Materials

Compared to conventional bonded phases, the creation of biodegradable stationary phases, such as silica-based or polymeric columns, reduces the environmental impact. Reusable and recyclable parts for chromatography instruments help minimize laboratory waste [206].

-Solvent Recycling and Waste Management

Solvent recycling methods can recover and purify solvents for reuse in chromatography, thereby significantly reducing waste and operation cost [207].

Advanced waste treatment technologies, such as enzymatic degradation and adsorption-based removal, are currently being integrated into analytical processes to efficiently handle hazardous waste [208].

-Greener Methods for Preparing Samples

Traditional sample preparation methods often require toxic solvents and produce hazardous waste [209]. Green alternatives include Solid-phase micro extraction (SPME) and stir-bar sorptive extraction (SBSE), which use minimal solvents [210]. Also, microwave-assisted and ultrasound-assisted extraction, which enhance extraction efficiency with reduced solvent and energy consumption [211-213].

Novel contributions and advancement of knowledge

This review advances the field of natural product analysis by providing a holistic synthesis of modern hyphenated

chromatographic techniques, including LC-NMR-MS, LC-FTIR, GC×GC-MS, UHPLC-MS, and LC-IM-MS, framed not as isolated tools but as an interconnected analytical platform. It emphasizes the complementary qualities of these approaches and their combined capability for handling challenging analytical problems by going beyond single-technique assessments.

This work's multidisciplinary approach, which compares the usefulness, effectiveness, and limitations of various methods across natural product isolation, environmental monitoring, food safety, and pharmaceutical quality control, is one of its unique features. Making method selection criteria clearer gives researchers a more sophisticated knowledge of which tools are most appropriate for particular applications.

The review also presents an organized framework for green chromatography in hyphenated systems that systematically connects biodegradable stationary phases, energy-efficient detection techniques, eco-friendly solvents, and solvent-recycling methods. It closes a crucial gap between scientific rigor and environmental responsibility by incorporating sustainability into high-performance analytical operations.

This work identifies important directions for future research and technological development by highlighting persistent issues, such as the absence of standardized multi-hyphenated workflows, inadequate spectral libraries for multidimensional datasets, constraints in miniaturization and field deployment, and the conflict between green practices and high-sensitivity detection.

Lastly, the review presents itself as a forward-looking manual that charts the development of hyphenated chromatography toward integrated, high-throughput, and sustainable analytical platforms by examining new trends like IMS-MS, HRMS-enabled workflows, automation, and machine-learning-assisted peak analysis.

Conclusion

Chromatography hyphenated techniques significantly accelerate natural product analysis by providing detailed information about chemical constituents and properties. The future potential of natural product analysis lies in the synergistic combination of various methodologies. This integration will enable scientists to unravel the complex chemical networks found in nature, leading to groundbreaking discoveries in industries such as environmental protection, agriculture, and medicine. GC-MS, HPLC-MS, UHPLC-MS, LC-FTIR, LC-NMR-MS, LC-UV-MS, LC-ION-MOBILITY, and LC-ION MOBILITY each bring unique benefits to the table, from high-resolution separation to detailed molecular characterization. Simultaneously, the shift toward green chromatography ensures that these technological advancements are in line with environmental sustainability goals. Green methods promote safer, more effective, and environmentally friendly analytical procedures by lowering solvent use, energy consumption, and laboratory waste. Together, these trends position hyphenated chromatography as a cornerstone of future analytical research and innovation.

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