MODERN ANALYTICAL TECHNIQUES USED FOR THE IDENTIFICATION OF BIOACTIVE MOLECULES

Abstract

Bioactive molecules are a group of compounds that have been isolated from various parts of plants. The identification and quantification of these molecules are crucial steps in the discovery of new biomolecules and can be utilized as a lead molecule to synthesise increased efficacious molecules. This chapter aims at the current trends and best methods for separation, identification, characterization, and quantitative estimation of bioactive molecules using various analytical approaches with specific emphasis on advanced chromatographic, spectroscopic, and spectrometric methods. An overview of the characterization methods available for bioactive molecules such as TLC, HPTLC, HPLC, GC, multidimensional chromatography, FTIR, NMR, and Mass Spectrometry, along with the hyphenated techniques, is presented in this chapter, with a focus on the current limitations of the various methods and a look ahead to potential future advancements. The methodology that has been outlined here can provide valuable insight for a comprehensive investigation of plant raw components as well as the bio-actives.

Keywords: Analytical techniques, Bioactive molecules, Chromatographic methods, Spectroscopic methods, Hyphenated techniques.

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I. INTRODUCTION

Plants serve as a repository for thousands of structurally diverse bioactive molecules. The abundant secondary metabolites present in plant show interesting bioactivities because of their diverse range of structural arrangements and characteristics. Natural compounds contained in various portions of plants are an attractive subject of research because of several bioactivities of these molecules such as antioxidant, antiinflammatory, immunomodulatory, and so on. The unique properties of bioactive molecules have compelled researchers to characterize these systems both by experimental and theoretical methods. The separation of bioactive molecules from plants, as well as their qualitative and quantitative estimation are crucial steps in the discovery of new biomolecules and can be utilized as a lead molecule to synthesise increased efficacious molecules. However, we all know that natural products are complex mixtures made up of numerous chemical constituents and have synergistic effects [1]. Therefore, it is challenging to assess and pinpoint the bioactive components which might be responsible for their efficacy [2]. There is a need for the creation of trustworthy techniques for natural product's bioactive components to be screened for potential novel therapeutic leads [3]. Building natural product libraries will benefit greatly from the discovery of novel bioactive molecules [4]. Because different plant materials include a variety of bioactive compounds, practical research demands the selection and optimization of each step of the entire analytical procedure. In plants, bioactive substances can be classified into a number of types based on their metabolic processes: glycosides, phenolic, flavonoids, stilbenes, anthocyanins, tannins, terpenoids, lignans, resins, alkaloids, proteins and peptides *etc*. [5, 6]. All of these classes have unique chemical compositions and physical-chemical characteristics, necessitating the use of various analytical techniques to ascertain them. Additionally, the matrix itself heavily influences the selection of the right analytical method, particularly the extraction protocol. An extensive variety of plant matrices, including medicinal plants, are used to extract bioactive chemicals [7, 8], fruits and vegetables [9], cereals [10, 11], nuts [12], edible flowers [13], *etc*. Further, the selection of plants and compounds from among many potentially valuable natural bioactive compounds for commercialization-oriented research is a very laborious process that requires considerable knowledge and careful examination of numerous market-related issues, such as the thorough extraction and product development processes [14].

Although the classical applications of medicinal plants are well known, it is hoped that thorough scientific research into the discovery of bioactive plant components and their properties will lead to novel therapeutic treatments as well as food and cosmetic products that are derived from nature. The method devised for the identification, separation, and quantification these compounds at varied concentration levels are critical steps in any analytical approach. Further, the development of effective and economically productive technologies for identifying bioactive molecules remains a challenging task. Therefore, a thorough validation and characterization of these bioactive molecules is advantageous in revealing new sources of therapeutic agents and in determining the true significance of traditional medicines [15]. Over the past two decades, measurement techniques have grown at an unprecedented rate. Analysis of bioactive compounds is now easier than before because of incredible advancements in sophisticated analytical methods like spectroscopy, chromatography, and microscopy along with other micro-devices and sensors. General approaches in identification and characterization of bioactive molecules are depicted in Figure 1. This chapter is therefore would be of significant value for the researchers in order to

create acceptable methods and techniques for chemical extraction and purification as well as to increase their yield for potential applications. The screening methods are thoroughly described, their approach is reviewed, and the existing limitations of the various methods and a look ahead to potential future advancements in characterization techniques are discussed. A range of advanced technologies along with the present methods for extracting and characterizing the bioactive molecules from certain commercial medicinal plants are also included in this chapter. The use of high resolution analytical techniques is taken into account for the characterization, quantification, and identification of bioactive compounds.

This chapter aims at the current trends and best methods for separation, identification, characterization, and quantification of bioactive molecules using various analytical approaches with specific emphasis on advanced chromatographic, spectroscopic, and spectrometric methods. The chapter highlights the analytical techniques, which includes the identification and characterization of bioactive molecules available in plants through various chromatographic techniques such as TLC (Thin Layer Chromatography), HPTLC (High Performance Thin Layer Chromatography), HPLC (High Performance Liquid Chromatography), GC (Gas Chromatography), multidimensional chromatography and spectroscopic techniques such as UV-Visible, Fourier Transform Infra-Red spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), and Mass Spectrometry (MS) along with the hyphenated techniques[16-20].

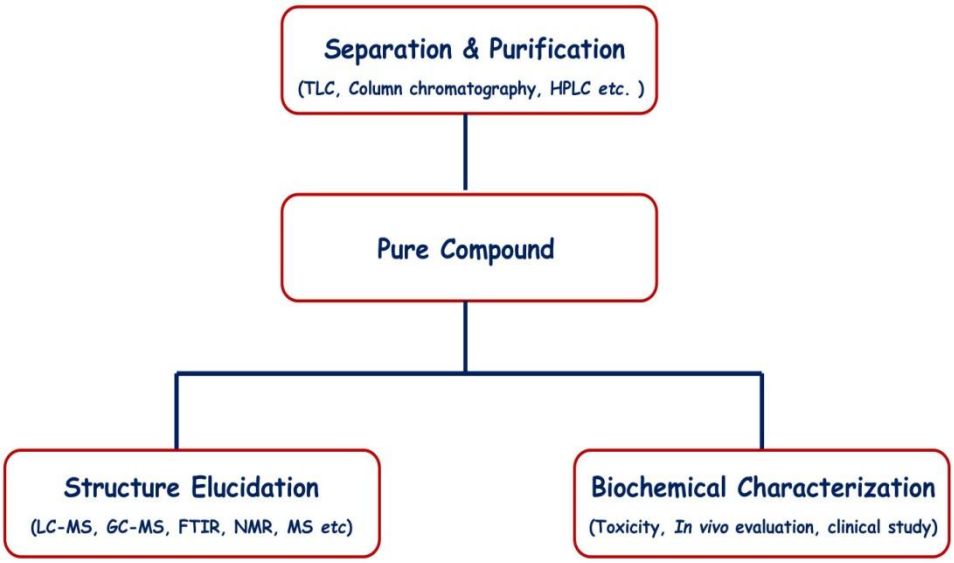

Figure 1: General approaches in identification and characterization of bioactive molecules.

II. DIFFERENT CHROMATOGRAPHIC METHODS

Chromatography is known as a separation method that separate the molecules based on their size, shape, and charge by moving a mixture containing mobile phase in contact with a selectively absorbent stationary phase [21]. It is also essential as an analytical approach for

quality assurance and standardized bioactive molecule production. Current applications for the separation and identification of bioactive molecules use chromatographic techniques, which exhibit great sensitivity, selectivity, and adaptability [22-24]. The chromatographic techniques have been classified in various categories based on the physical state of mobile phases (Liquid/Gas), stationary phase (Paper & Thin Layer/Column), Separation mechanism (Affinity/Partition/Size exclusion or Gel filtration /Ion exchange/) *etc.* While chromatographic methods can be used for both the qualitative and quantitative studies, TLC provides a preliminary evaluation of the qualitative analysis of complex mixture.

1. Thin Layer Chromatography (TLC) and Preparative TLC

TLC, an adsorption chromatography separates the compounds based on the interaction of a thin layer adsorbent adhered to the surface [25, 26]. The silicon atoms present in the silica gel have binding property through hydrogen bonding via hydroxyl groups and considered to be responsible for the separation processes. The mixture's components get separated according to the variation in the molecular functional groups' binding efficiencies which cause the mixture to attach to the silica gel. Higher polarity compounds are easily adsorbed by the silica gel, but lower polarity compounds are travel with mobile phase and appear as spot on the plate. A modification of this technique results in preparative thin layer chromatography (prep-TLC). This technique separate components in higher amount by loading more amount of sample on thick layer (0.5-5 mm) of the stationary phase.

2. High Performance Thin Layer Chromatography (HPTLC)

HPTLC, a planar chromatography technique separates the compounds through high performance layers along with data acquisition and detection. Pre-coated sorbent plates of 5-7 microns particle size and 150–200 thickness make up these high-performance layers. The separation efficiency increases with reduction in particle size and thickness of layer. When using HPTLC, the separated samples obtained through chromatography are visually viewed as a chromatogram. Pore and particle size of sorbents are the primary distinction between TLC and HPTLC.

3. Liquid Chromatography (LC) and Preparative LC

For complex mixtures separation, liquid chromatography is a proven, dependable method which frequently utilized in quality control and quality analysis. In addition to offering high resolution similar to LC, preparative liquid chromatography (PLC) also significantly increases the sample loading volume. High productivities can be achieved by severely loading columns, which is frequently done by testing solubility limits, injecting big sample volumes, or injecting large sample concentrations. As a result, it is possible to obtain good quantity of bioactive molecules from natural sources.

4. Flash Chromatography

This technique for the separation and purification of chemicals is also referred to as medium pressure liquid chromatography. Mixture of bioactive molecules in large sample volumes can be separated with a controlled moderate pressure, producing highly pure molecules. The recent development of fully automated fraction collectors and real time

detection has enhanced the effectiveness of separation and isolation along with purification and identification of molecules in a crude extract [27].

5. High Performance Liquid Chromatography (HPLC)

By using HPLC, compounds are separated depending on their interaction with the mobile phase's solvent and the particles of a tightly packed column. In HPLC, combination of a polar liquid phase and a non-polar solid phase are both employed. High pressure must be used to elute the analyte through a column, after which it passes via a diode array detector (DAD). A DAD evaluates the analytes' absorption spectra to assist in identifying them. HPLC is helpful and works well in conjunction with gas chromatography to detect bioactive molecules for substances which do not vaporize or break down at high temperatures, [28]. UHPLC uses further high pressures which results in lesser run time and solvent consumption with better analytical separation [29].

6. Preparative High-Performance Liquid Chromatography (Prep-HPLC)

Prep-HPLC is used on industrial scale with kilogram quantities for separartion and purification purpose. Large columns which run at higher flow of the eluting solvents are used for this purpose. In normal HPLC, sample moves to trash after the evaluation but in Prep-HPLC, sample leaves detector and enters the fraction collector. Prep-HPLC offers an advantage over traditional HPLC because it uses more pressure and increasing throughput while ensuring adequate separation power. In comparison to previous purification methods, fully automated prep-HPLC is currently able to quickly and easily purify a significant number of important bioactive molecules. The isolation and purification process are made much more effective using an UV-Visible detector with prep-HPLC [30].

7. Reverse-Phase High-Performance Liquid Chromatography

This often referred as hydrophobic chromatography because it employs a stationary phase which is hydrophobic in nature. This is distinct from normal phase chromatography that uses silica/alumina resins which are hydrophilic in nature. In reverse-phase HPLC, the hydrophobic stationary phase is made up of covalently linked alkyl groups that attach to the less polar (hydrophobic) chemicals and transported by mobile phase. Reversed-phase chromatography offers the extra benefit of separating charged molecules utilizing ion interactions in modern methods.

8. Gas Chromatography (GC)

Gas Chromatography is utilized for the isolation and identification of complex mixtures of volatile compounds [31]. This technique includes sample vaporization and injection in a column's head that comprises of liquid stationary phase. The sample is carried by the flow of an inert, gaseous mobile phase. For example, molecules that completely disperse into the gas phase will travel at the similar speed of the gas, but molecules that completely disperse into liquid stationary phase remains behind. Species that split their distribution between the two stages will migrate at a moderate pace [32]. To distinguish between chemicals that act similarly throughout the GC process, different temperature programs may be employed to improve the analysis [15]. Due to their great sensitivity,

selectivity, and versatility, mass spectrometers (MS) are one of the detectors employed most frequently today for the detection of bioactive molecules. However, due to various tactical advantages, such as linearity for broad concentration ranges, reduced prices, and low maintenance needs compared with MS, FID, ECD detectors are also frequently employed in gas chromatography. The most popular method for identifying and measuring phytochemicals is GC-MS, which is both a strong instrument and a reliable methodology. The interpretation of the spectrum and comparison with National Institute of Standards and Technology (NIST) allowed identification of bioactive species [33]. In a recent work, Mani Ganesh and Murugan Mohankumar used GC-MS to identify the bioactive substances in ethanol extracts of *S. cordata* whole plant [34]. Amudha et al. have also identified potential phytocompounds in ethyl acetate extract of *E. acoroides* using GC-MS [35].

9. Size-Exclusion Chromatography (SEC)

In this technique, molecule is separated based on its size, that further determines its molecular weight, in a process known as molecular sieve chromatography. Large molecules having complicated structures are best separated with this approach. The columns used for this process are tiny, sieve-like reticular beads made by dextran polymers, agarose (Sepharose), or polyacrylamide (Sephacryl). According to their size, the components in the extracts are caught in the beads, making it easier to estimate the sizes of macromolecules and achieve a fair molar mass distribution [36]. When an aqueous solution is used, it is called gel-filtration chromatography, and when an organic solvent is used, it is referred to as gel permeation chromatography.

10. Counter-Current Chromatography (CCC)

Traditional chromatographic techniques with lower pressure are time/solvent intensive and need numerous chromatographic steps. In some cases, CCC might be a perfect replacement for these traditional purifying techniques. CCC has some advantages over conventional liquid-solid separation techniques, including high injection sample recovery, minimal peak tailing, low sample denaturation risk, compatibility with particulate samples, and ease of switching solvents for fresh samples. CCC is divided into hydrophobic and hydrophilic equilibrium systems depending on the various fluid mechanics concepts that apply to them. Pauli, Pro, & Friesen (2008) have written a thorough review that provides explicit details of such systems along with their key features [37]. By using different gradient elution processes, this method can be used to improve the removal of contaminants from both the raw extract/finished product and pure molecule can be isolated from raw extract in single step without the need for sample pre-treatment [38, 39].

11. Bio-Affinity Chromatography

Bioactive molecules from natural products have already been successfully screened using the significant and well-liked technology of bio-affinity chromatography, which depends on interaction between bioactive molecules and immobilized targets [40]. Targets such as cell membrane, protein, liposome *etc*. are utilized as a stationary phase along with a carrier to keep the bioactive molecules which precisely attach to the target. Natural products extract is first injected in affinity column filled by carriers that are masked with the targets. An elution buffer is subsequently used to separate the bound bioactive molecules from the

targets, which are then subjected to detector analysis. Cellular membrane affinity chromatography (CMAC) [41], cellular membrane chromatography (CMC) [42-44], immobilized liposome chromatography (ILC) [45], and biological molecule chromatography [46] are the type of bio-affinity chromatography that have received the most attention to date.

Modern pharmacological research has shown that the first step in predicting a drug's behavior in an organism is its capacity to connect to the receptors which is crucial in their action [47]. The concept of CMC, first introduced by He et al. gained popularity as standard cell membrane dependent model for bioactive molecule's screening [48, 49]. Although this CMC technique is quicker and easier than traditional screening and analysis, its implementation is restricted by limited column and peak efficacy. These drawbacks have been significantly minimized by CMC and multidimensional chromatography (2D CMC-HPLC) which will be discussed in the following section of multidimensional and hyphenated chromatography techniques.

12. Multidimensional liquid chromatography (MD-LC)

Because of their unquestionably greater separation ability compared to the traditional 1D chromatographic method in deciphering complicated samples, multidimensional chromatography techniques continue to occupy a growing niche in the field of separation science. Even with careful method optimization, a single chromatographic process may fall short of providing a suitable level of resolving power when dealing with extremely complex samples, necessitating the use of more potent analytical techniques. The evaluation of complicated samples using MD-LC has become a compelling option. Natural products are not an exception, and due to their selectivity and sensitivity, multidimensional approaches can be used to analyse bioactive compounds as well as identify minor components. To greatly improve the isolation potential of related 1D-LC methods, MD-LC enables the combining of two or more separation processes. General aspects such as basic principles, models, and utility of the various MD-LC approaches in natural product investigation have already been covered in several fascinating reviews [50-58]. In this section an overview of comprehensive MD-LC methods employed in offline/online mode is discussed that is used as effective tool in investigation of the natural products.

13. Offline L×C

When performing *off-line LC×LC*, the fractions of particular interest eluted from the initial 1D separation are collected manually, subjected to evaporation, and subsequently injected into the 2D. Consequently, this approach yields a final outcome comprising multiple two-dimensional separations, corresponding in number to the fractions collected from the one-dimensional separation. The primary advantages include the ease of execution, without the necessity of alternate valves. This compatibility allows for the integration of a wide range of LC-modes.

14. Online L×C

The interfaces used in *online L×C* can automatically transfer 1D fraction into 2D. In contrast to the offline mode, the online approach offers several notable advantages: it can be easily automated, enhancing sample throughput, while reducing the risk of sample

contamination, and it results in a shorter overall run time. Furthermore, it provides greater reproducibility, and there is a significant potential for discovering "unknowns" due to the emergence of distinct group type patterns on the 2D plane.

15. Hyphenated (Chromatographic-Spectroscopic/ Spectrometric) Techniques

The analytical methods in which a chromatographic technique and spectroscopic or spectrometric technique are coupled to analyse a variety of samples are known as "hyphenated techniques". These are based on the target's initial extraction and/or separation using LC, HPLC, GC, *etc.*, and their subsequent characterisation using spectroscopic techniques like FTIR, MS, NMR, *etc*. All efforts made over the last few decades by scientists and engineers resulted in the creation of high-tech hyphenated equipment such as LC-MS, LC-NMR, HPLC-MS, and GC-MS. As shown in Figure 2, GC-MS and LC-MS (in hydrophilic interaction/HILIC and C18 mode) are widely used in the characterization of a range of bioactive molecules. Table 1 provides a comprehensive overview of the strengths and weaknesses associated with both of these methodologies.

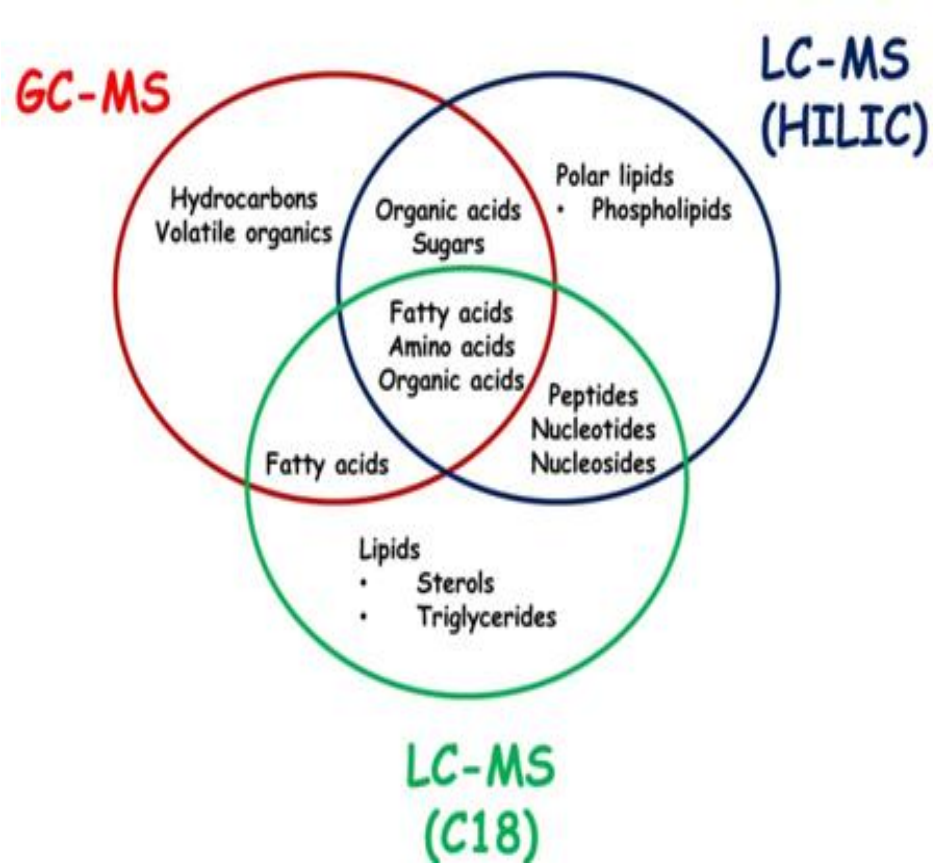

Figure 2: Selection of the characterization methods for bioactive molecules based on their nature and polarity.

Table 1: Advantages and limitations of GC-MS and LC-MS methods

Techniques	Advantages	Disadvantages
GC-MS	Volatile components and low molecular mass hydrophobic compounds are easily analysed.	Derivatisation is required for non-volatile and thermally unstable compounds which sometime mask the results.
LC-MS	Suitable for characterization of relatively polar as well as thermally unstable compounds without any derivatization. By using different ionisation approaches, full range of molecular weights (low, intermediate, and high) can be detected.	Poorly reproducible fragmentation patterns and formation of adducts are the two major problems.

Some more advanced techniques have been introduced in the recent years by coupling between chromatography and spectroscopy such as LC-MS/MS, LC-NMR-MS, GC-MS/MS *etc*. In this section some recent studies in the field of bioactive molecules characterization using hyphenated techniques have been discussed. The development of increasingly advanced high resolution analytical methods with enhanced sensitivity, effectiveness, and selectivity is of paramount importance. The use of LC in conjunction with various detectors, such as the photodiode array (PDA), fluorescence detector (FD), and mass spectrometry (MS), enables the structural elucidation and identification of bioactive compounds at a comprehensive level. PDA is frequently used because of its dependability and affordability, and it can be used to identify wide range of secondary metabolites. Overall, electrospray ionization-based MS detectors deliver the best performance. When paired with Mass Spectrometry (MS), chromatographic techniques have shown promise as separation and characterisation of bioactive compounds simultaneously [59, 60]. These platforms are highly selective, sensitive, quick, and high-resolution. Certain ambient MS techniques' direct analysis capabilities have proven to be particularly beneficial for the analysis, identification, and characterisation [61]. An integrated identification method incorporates various elements such as HRMS, ion source and MS/MS fragmentation, determination of molecular formulas, and data base searches. Advanced hyphenated techniques, specifically the coupling of liquid/gas chromatography (GC) with HRMS, are potent tools to tackle the intricate analysis of botanicals [62, 63]. Silylation is a derivatization method that makes the derivatives more flammable and enabling them direct injection into the GC system. Silyl group insertion can also result in a more advantageous ion fragmentation pattern that is employed for structural analysis [64]. The most common analyzers are orbitrap based hybrid mass spectrometry (LTQ orbitrap) and quadrupole time of flight (QTOF). Due of its capability to do multiple reactions monitoring (MRM), QqQ is frequently utilised for quantitative reasons. The best resolution for identifying bioactive compounds is provided by Q-TOFMS, which enables one to carry out MS/MS investigations with enhanced structural details and selectivity. Using HPLC-ESI-QTOF/MS, phenolic compounds from several matrices have been successfully identified [65-69]. Despite the widespread use of these methods, MS-based procedures produce enormous, complex datasets with hundreds of MS characteristics, leads to difficulties in post-acquisition data processing and higher time consumption [70]. To

correctly recognize the possible bioactive components, raw MS data must be subjected to an integrated identification and elucidation technique. The mass-defect filtering, fragment-ion filtering, and neutral-loss filtering are a few recent research that have suggested useful methods to simplify the post-acquisition data processing [71-73]. Recently, LC-MS is used to characterise a variety of natural products, in the investigation of various plants and derived products [74-76]. The primary bioactive constituents in these extracts, as established by the most comprehensive published findings on characterizing *M. oleifera* leaf extract, identified a total of 59 chemical components [75]. Additionally, depending on the settings utilised, LC-MS is a promising alternate for recognizing a variety of bioactive chemicals as it may be able to distinguish between isomeric molecules [77, 78]. Using HPLC in conjunction with electrospray ionisation quadrupole-time of flight mass spectrometry (HPLC-ESI-QTOF-MS), chemical molecules can be identified by analysing MS spectra, and comparison with mass spectra database and scientific literature. These findings support previously reported qualitative findings indicating this method can be used to isolate at least 50 bioactive components from *M. oleifera* leaf optimised extracts [79]. Additionally, *M. oleifera* and *M. ovalifolia's* flavonoid contents were compared using ultra-HPLC in conjunction with QTOF-MS [76]. These results are supporting the similar recent study carried out by Castro-López and co-workers on *M. oleifera* leaf extracts [80]. They used UPLC-ESI-QTOF-MS2 to identify these bioactive compounds. In their investigation of the combined effects of pulsed electric field (PEF) and ultrasound (US), Manzoor and co-workers observed the stability of volatile components to assess the physico-chemical properties of bioactive substances along with structural assignments of almond extract using FT-IR [81]. By using cutting-edge HPLC and GC techniques, Pellati F et al. reported multi component study of bioactive chemicals found in *C. sativa* (hemp) of several varieties [82]. Recent research has employed 2D-CMC-HPLC methods to screen and assess bioactive chemicals. These techniques use CMC-UV to retain possible active components in one dimension, and examined using HPLC or GC-MS in the second dimension, either offline or online. This is successfully utilized to screen and examine bioactive molecules, but it has two significant drawbacks: the molecules with little or no absorption of UV may go unnoticed by UV detectors and second dimension takes a lot of time to analyse. Chen et al. created a 2D HepG2/CMC/monolithic column/TOF/MS system to screen and characterize putative bioactive components faster to get over these constraints [83]. Moaddel et al. presented several significant studies on CMAC to screen bioactive chemicals from natural product extracts in recent years [84-86]. A recent study used HPTLC-UV/Vis/FLD-bioassay to build a simplified quantitative bio profiling investigation of 17 widely available ginger varieties. HPTLC-ESI-HRMS was also used to find and characterise multipotent chemicals. Such chromatographic techniques were used to analyse chemical marker components along with the activity of ginger extracts [87]. Recent studies used reversed-phase HPLC in conjunction with ESI-TOF-MS to analyse the bioactive molecules present in citrus by-products. Six new metabolites are reported for the first time in citrus by-products out of the forty or more that could be determined using the method [88]. For the purpose of screening chemical components in natural products, a number of online SEC-HPLC-DAD and SEC-HPLC-MS systems are recently developed [89-91]. Using UPLC-ESI-q-TOF-MS/MS technique a thorough characterization of *P. peruviana* extract has been developed and a total of 59 phytochemicals were provisionally discovered. Metabolite profiling of brown macro-algae has recently established through the application of UPLC-MS [92-94].

III. DIFFERENT SPECTROSCOPIC METHODS

Although chromatographic techniques are widely considered the benchmark for analyzing bioactive molecules, spectroscopic methods hold equal significance since they can effectively offer insights into all species within a sample in a single experiment. Electromagnetic radiation used in spectroscopy travels via organic molecules which partially absorb the incident light and partially transmit the remainder. The measurement involves assessing the electromagnetic energy that has been either absorbed or transmitted, and the subsequent spectrum is utilized for analysis. Particular bonds within the molecule are responsible for absorbing specific electromagnetic radiation. Consequently, spectra are generated from various electromagnetic regions, including UV-Visible, infrared, radiofrequency *etc*. Several different spectroscopic methods, such as UV-Vis., FTIR, NMR and mass are employed to ascertain the structural composition of bioactive molecules that have been isolated from plants. Atomic adsorption spectroscopy (AAS) and inductively coupled plasma mass spectrometry (ICP-MS) or inductively coupled plasma optical emission spectroscopy (ICP-OES) are used to analyse or determine the heavy metals and other elements present. In the following section the spectroscopic techniques like UV-Vis., FTIR, NMR and mass that are used in the structure determination of the bioactive molecules will be discussed with specific emphasis on the recent advancements in instrumentation and methodologies.

1. UV-Visible Spectroscopy

UV-visible spectroscopy is versatile in its ability to perform both qualitative and quantitative analysis and to identify specific classes of substances. This technique is especially advantageous for quantitative studies due to the strong chromophoric nature of aromatic compounds in the UV range. Additionally, UV-visible spectroscopy proves valuable in identifying natural substances, as it can detect saturation in molecules containing aromatic moieties and other chromophores that absorb in the UV region. The UV-Visible spectrum allows for the identification of phenolic substances such as sugars, pigments, flavonoids, coumarins, anthocyanins, tannins *etc.* [95]. This approach is not only cost-effective but also less time-consuming compared to alternative methods [96].

2. Fourier-Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopy utilizing the Fourier transform is a significant technique in locating functional group of bioactive molecules. It provides high-resolution analytical data to identify the complicated compounds. It contributes to the recognition of molecular properties and assists in determining the structure of molecules. The type of vibrational shifts that a polar molecule experiences in response to IR radiation determines how well it absorbs that energy. Therefore, it is also named as vibrational spectroscopy. When IR radiation passes via a sample, certain frequencies get absorbed, and others will pass via sample.

The molecule being studied should exhibit polarity, characterized by a dipole moment to receive a response from IR spectroscopy. The distinctive absorption bands at specific frequencies in the infrared spectrum can be utilized to ascertain the presence of these types of bonds in an organic molecule [97]. FTIR is an efficient, cost effective, and non-destructive investigative technology which can provide fingerprints for the structural information of

isolated bioactive compounds. There are several techniques available for sample preparation in FTIR analysis. The easiest approach for liquid samples involves placing a drop of the sample between two sodium chloride plates, creating a thin layer between them. Solid materials are often milled with potassium bromide (KBr), producing a thin pellet that can be analyzed. In the case of solid samples without a solvent, they can be dissolved in a suitable solvent. A few drops of the resulting solution are then applied to an attenuated total reflectance (ATR) plate, and spectra are recorded. To determine the signatures of main functional groups and bondings, the peaks are examined in relation to the spectral band locations. The acquired spectra were analyzed, and the principal bands were assigned by comparing them to the available literature. Ismail et al. recently investigated and measured bioactive molecules present within grapes seeds using FTIR. This group discovered carboxylate group from proanthocyanidingallate and gallic acid within the water extract of the seeds [98]. The functional moieties observed in extract were qualitatively analyzed using the ATR-FTIR technique, and the samples were authenticated and distinguished by employing a multivariate statistical technique known as Principal Component Analysis (PCA) in which multi-dimensionality of a data set are reduced to 2-D or 3-D data/graph by computing the eigenvalue of the covariance matrix [99, 100].

3. NMR Investigation of Bioactive Molecules

Nuclear Magnetic Resonance spectroscopy, a uniquely capable non-destructive technique provides comprehensive analysis of structure, conformational analysis dynamics, molecular interaction and three-dimensional structure of simple to complex molecular system in both solution and solid state. Moreover, continuous developments in methodology, introduction of high field super conducting magnets, FT and PFG techniques have has established it as one of the most preferred methods for structural investigation of bioactive molecules at atomic resolution [101, 102]. Due to this, NMR spectrometers are now widely employed as a primary tool in laboratories that focuses on bioactive compounds. The complex structural details of the molecules can be obtained using 2D NMR methods instead of the frequently utilized one dimensional methodology. The common NMR methods used in the characterization of bioactive molecules are shown in Table 2 [103-105].

Table 2: Common NMR methods with potential use in the characterization of bioactive molecules

S.N.	NMR Experiment	Application
Solution state NMR		
1.	Chemical shifts	Chemical structure and stoichiometry.
2.	COSY	Correlates protons that are either geminal or vicinal.
3.	Magnetization transfer (NOESY/ROESY/EXSY)	Spatial interactions, structural/ conformational determination, internuclear distance measurement and chemical exchange.
4.	TOCSY	Identifies proton signals within the same spin system.
5.	HSQC	Determines the multiplicity of carbon nuclei and establishes correlations between carbon signals

		and directly bound protons.
6.	HMBC	Enables the identification of long range correlations.
7.	Relaxation times T1/T2	Molecular mobility and spatial interactions.
8.	Magnetization transfer (NOESY/ROESY/EXSY)	Spatial interactions, structural/ conformational determination, internuclear distance measurement and chemical exchange.
9.	DOSY	NMR signals are separated according to the diffusion coefficient of molecular system.
Solid state NMR		
10.	CP-MAS	Spectra of hetero-nuclei X that are directly bound to hydrogen atoms.
11.	HETCOR	Correlate heteronuclear resonance frequencies via their one bond couplings.
12.	CPXT1	Dynamic character of the molecule.

Compounds must first be chromatographically purified before they may be detected by NMR spectroscopy. NMR is often incorporated into the separation, isolation, and purification procedures utilizing the preparative, semi-preparative, thinlayer, and HPLC to elucidate the structural details of the compounds. By connecting the hydrogen signals in aromatic compounds with their corresponding carbon signals via ^{13}C NMR spectroscopy, HMBC is used to provide more light on these structures. These methodologies are very crucial because the biological activity of bioactive molecules may be greatly influenced by their structural integrity. In order to explore the metabolomics profile of citrus juices, Villa-Ruano and co-workers have used the ^{1}H NMR methods to collect spectra for 35 metabolites [106]. Cérantola and co-workers utilized HSQC and HMBC 2D NMR experiments to determine structures of several bioactive compounds in Fucus spiralis [107]. Several bioactive molecules have been quantitatively measured in brown algae *Cystoseira tamariscifolia* using HRMAS SS- NMR [108]. The application of SS- NMR in the investigation of brown algae is extremely beneficial as it can be analysed without the requirement of solvent extraction and eliminates the associated matrix effects. The same scientists also examined a brown macroalgae *Cystoseria* of the Fucaceae family, and reported the comparision between LC/ESIMS and ^{1}H HRMAS experiments [109]. The concentration of phloroglucinol was qualitatively measured using ^{1}H HRMAS in conjunction with ^{1}H qNMR. Cystoseria fingerprints were discovered to be helpful in differentiating between the studied species, which were never able to be separated because of identical metabolic profiling. Cicero et al. used HRMAS NMR for quantitative investigation of main metabolites found in two hybrid varieties of citrus fruits *i.e.*, Messina and Turkish lemon [110]. In recent years, NMR based metabolomics for the application of functional genomics and plant differentiation have also gained considerable attention. This protocol has the sample preparation, extraction, NMR analysis, and chemometric procedures steps used in plant metabolomics employing NMR spectroscopy [111-113]. Dinesh Kumar and co-workers performed thorough metabolite profiling of numerous tissues from the *Crataegus rhipidophylla* Gand plant, using 1D and 2D NMR methods and identified 58 compounds belonging to various classes [114]. The ability to identify metabolites via comparison of NMR spectra with standards or via employing 2D NMR to elucidate structure is main benefit

of NMR metabolomic studies. This procedure is best suited for examination of primary and secondary metabolites as well as phenolic compounds, which are often plentiful in plants.

4. Mass Spectrometry (MS)

MS is a potent method of identifying the unknown molecules, quantifying known molecules, and determining the structural and chemical characteristics of molecules. This method uses electrons or lasers to bombard organic molecules and turning them into charged ions with high energy. The internal electric and magnetic fields of the instrument segregate different-sized fragments based on their mass to charge (m/z) ratio. Energetic species pass towards detector with varying kinetic energy according to their mass. To create a mass spectrum, the relative abundance of fragmented ion is plotted against the ion's m/z ratio. Mass spectrometry allows for the precise calculation of relative molecular mass and the determination of an accurate molecular formula by analyzing molecular fragmentation patterns. This technique is primarily utilized for the structural analysis of organic compounds and for monitoring the presence of known compounds in complex mixtures with a high level of specificity by simultaneously defining the molecular weight and diagnostic fragments of the molecules. Assignment of bioactive molecules is typically based on parent ion's size. Mass spectrometry provides a wealth of data for the structural determination when applied as tandem mass spectrometry, or MS/MS. General setup of a typical tandem mass spectrometer is depicted in Figure 3. It has frequently been employed in conjunction with different LC techniques to track the pattern of compound fragmentation as it elutes on the column [115-117].

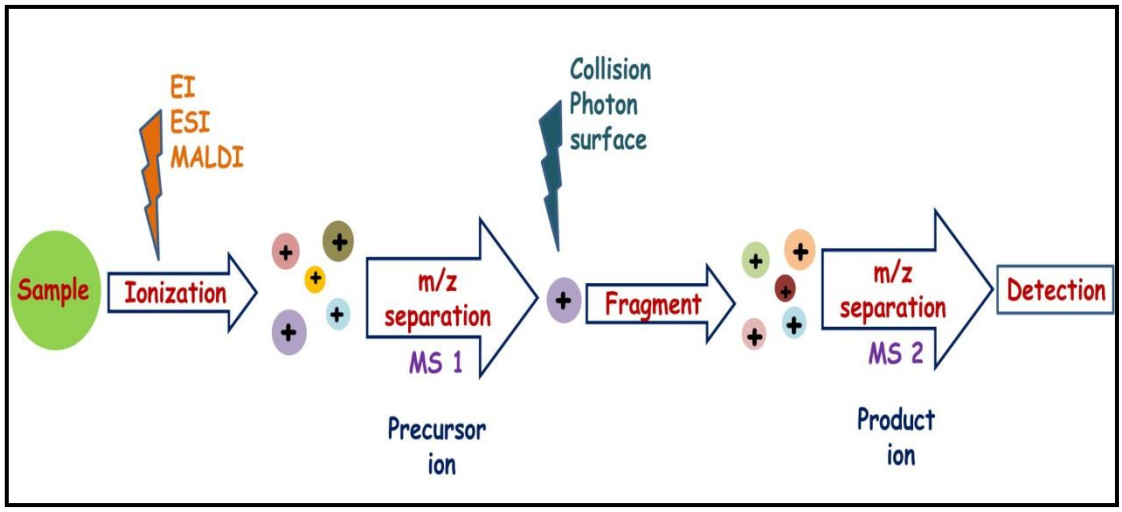

Figure 3: General setup of a typical tandem mass spectrometer

The HPLC and MS combination makes it easier to identify chemical components quickly and accurately even in the absence of purified standards. This method has recently been used for elucidating the structure of bioactive compounds recovered from citrus waste [59]. The type of the ionisation source is also crucial for the investigation. Enhancing the analysis's efficiency, including improved sensitivity and the ability to conduct high-throughput analysis of phytochemical compounds, depends on the selection of various ionization sources. These sources serve as interfaces between LC MS. Some of the

commonly employed ionization sources for the fragmentation of complex botanicals include electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and, more recently, atmospheric pressure photoionization, as well as atmospheric pressure solids analysis probe (ASAP [118]. LC-MS is widely employed to analyse phenolic compounds using ESI as a recommended source because of its higher ionization efficiency. For assessing bioflavonoids, ESI/MS and APCI/MS offer significant advantages in terms of mass scale and sensitiveness [119]. Charge transfer through dopant compound to analyte occurs during LC/MS-APCI method, and analyte is then ionized utilizing photons through a vacuum UV lamp. Analysing non-volatile molecules with MALDI is helpful [120]. For bioactive molecules with higher degrees of polymerization including bioflavonoids, peptides, sugars, carbohydrates, glycoconjugates and proteins, it is recommended to adopt MALDITOF, an ionization technology which utilize laser light to create ions from big compounds without fragmentation [121, 122]. It has also been demonstrated that MALDI has the potential to analyse saponins that have been isolated from alfalfa [123]. This useful method can offer a direct examination of plant extracts without requiring any separation or derivatization steps [124]. Additionally, it was shown that MALDI can differentiate between different oligosaccharide isomers [125, 126]. Because of the appearance of the peaks as numerous charged species, HRMS can also quantify larger bioactive molecules with higher degrees of polymerization. The determination of phenolic compounds using HPLC coupled to UV-PDA and ESI/MS has been proven to be effective [127]. Their potential molecular structural details are further validated by using NMR spectroscopy. The primary applications of GC/MS are for volatile compounds such essential oils. Due to less volatile nature of flavonoid glycosides, this is seldom employed in flavonoid investigation. APCI has been proven to be quite helpful in the analysis of carotenoids since it offers increased sensitivity towards the charged ions. APCI's great sensitivity has led to it becoming the most popular ionization method for carotenoids.

IV. SUMMARY

There are numerous methods for identifying, isolating, and extracting bioactive substances from plants. There is a pressing demand for innovative, cutting-edge approaches for extraction, identification and isolation of bioactive chemicals in amounts suitable for their significant uses in a variety of fields, yet these methods are typically time-consuming and extremely expensive. A wide variety of bioactive chemical substances, either in its purest form or as homogeneous extracts, have been produced from medicinal plants. These compounds provide pharmaceutical companies with several prospects for the creation of new therapeutic leads due to their wide structural and functional diversity. The evolution of novel and more efficient investigation and separation methods is becoming extremely important as the food, pharmaceutical, and cosmetics sectors become more and more interested in naturally occurring bioactive molecules and secondary plant metabolites. The goal when searching for bioactive molecules is to identify a suitable method that can rapidly screen the source material for various bioactivities like antioxidant, antibacterial, or cytotoxic properties, while ensuring simplicity, specificity, and speed in the process. The first stage in isolating all biologically active compounds is selecting the optimum extraction technique to maximise yield while utilising the least expensive solvent volumes, time, and energy requirements. Different techniques function differently as per the nature of the target components. An overview of the characterization methods available for biologically active

molecule is presented in this chapter, with a focus on contemporary chromatographic and spectroscopic methods. Each technique's advantages and disadvantages are explored. The methodologies that have been outlined here can provide valuable insight for a comprehensive investigation of plant raw components as well as the bio-actives. Natural product researchers may find the information in this chapter to be a useful resource for producing better pharmaceutical products using the most potent natural bioactive constituents.

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