

## CHAPTER 02

# Extraction, Isolation and Characterization of Bioactive Natural Compounds



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**ABSTRACT:** Natural products have traditionally played a pivotal role in drug discovery and development, providing an immensely large library of therapeutically potent bioactive compounds. This chapter lays down complete information on the procedures involved in the extraction of bioactive natural compounds of a variety of biological substances, their subsequent purification and analysis processes. Professional extraction techniques, maceration, Soxhlet extraction as well as ultrasonication, and supercritical fluid extraction, are chosen depending on the type of chemical and thermal stability of the targeted molecules. The chapter describes the purification methods of bioactive constituents following the extraction using methods such as high-performance liquid chromatography (HPLC), column chromatography and thin-layer chromatography (TLC). Moreover, the chapter provides an explanation on how to find chemical structures by using Nuclear magnetic resonance, mass spectrometry, and HPLC-coupled detectors that are superior spectroscopic techniques. It is a combination of these analytical processes that results in valid identification and description of natural substances that improve their analysis in therapy evaluation as future pharmacological agents.

Naturally occurring substances from prebiotic, microbial, plant, and animal sources have always piqued human curiosity. Traditional remedies, fragrances, flavorings and preservatives plant extracts of different parts have been widely utilized in traditional remedies, fragrances, food flavorings, and preservatives. They are also more widely used in both chronic and acute diseases. The active substances in plants include alkaloids, glycosides, steroids, tannins, volatile and fixed resins, oils, phenols and flavonoids, and are accumulated in different parts of the plant. It is the combination of these active chemicals that makes the plant have therapeutic and medicinal properties (Tonhubthimthong et al., 2001). Many plant extracts have been put to large extent as fragrances,

folk medicines, food preservatives and food additives. In anti-inflammatory, analgesic and antipyretic therapy, hormone replacement therapy substitutes and treatment of infectious and chronic diseases like cancer, diabetes and asthma, bioactive natural ingredients are used more often. Additionally, it is used as a treatment for bronchitis, arthritis, edema, cough of pneumonia and fever, hepatitis, nephritis, gastropathy & bronchitis (Ahmed et al., 2025). In addition to meeting the bulk of people's basic healthcare needs in underdeveloped nations, natural medicines are also becoming more and more popular in wealthy nations because of their low or nonexistent side effects and lower cost. About 49% of people in the USA have experimented with natural remedies for

disease prevention and treatment. The global statistics show that traditional medicine, which mainly uses plant extracts as its primary medical care, serves over 80% of the total world population (Sandhya et al., 2006).

Medicinal plants are the richest source of bioresources of pharmaceutical intermediates, nutraceuticals, nutritional supplements, and chemical substances in synthetic and conventional drugs (Pandey & Tripathi, 2014). The natural compounds, which are of importance to the bioactive compounds, are not enough even though they are highly utilized. These days, it is crucial to create efficient and focused techniques and promote research on natural products. The current chapter discusses the basic principles and importance of extracting bioactive compounds from natural sources. Moreover, various techniques used to isolate pure natural compounds from complex mixtures, and their characterization is also discussed.

## EXTRACTION METHODS

Extraction is the process of employing standard and selective methods to separate the parts of a plant that have medicinal activity. Because the required chemical components must be extracted from the plant materials for additional separation and characterization, it is the most important initial stage in the investigation of medicinal plants. There are various extraction techniques of natural products from existing plants. These methods fall into two categories: traditional (long-standing) and modern (more recent). Modern methods use pressure and/or higher temperatures, whereas conventional methods use organic solvents or water and are typically conducted at atmospheric pressure.

As per the principle of extraction, the extraction techniques are of the following types, namely, pressing, sublimation, solvent extraction, and distillation. Solvent extraction is the most commonly used method. Extraction of natural products follows the following steps: The solute is diffused out of the solid matrix, the solvent is diffused in it, the solute is dissolved by the solvents and the extracted solutes are collected (Zhang et al., 2018). The prevalent solvents used to perform the extraction procedures are water, hexane, ethanol, dichloromethane, chloroform, ethyl acetate and

methanol. Conventional means of extraction have been reported to incorporate the use of organic solvents and take too long before the extraction to be made and a lot of solvents are used. Modern methods have also been used to extract natural compounds and these have many advantages which include increased yield of the extraction, it also uses less organic solvents and also consumes the shortest time of extraction (Brusotti et al., 2014).

## ISOLATION AND PURIFICATION

The extracts are a complex combination of a large number of natural substances of different polarity. Further To get a pure bioactive molecule, it must be separated and purified. The purification of natural products having pure bioactive components is very difficult in the identity and purification of pure bioactive compounds. This has been of titanic magnitude in the the last few years witnessed purification and separation of natural products (Sasidharan et al., 2011). Thin-layer chromatography and column chromatography (TLC) is the most popular and it is used to determine the location of the substance. It is easy to use, cheap and they come in all types of stationary stages. In addition, non-chromatographic methods are utilized such as phytochemical screening tests and monoclonal antibody-based immunoassays (MAbs). The biological and structural purification of the pure chemicals is then determined. (Zhang et al., 2018).

The summary of the characterization process is given below:

### Evaluation of Physicochemical Properties

- **Extraction yield:** The extraction process yielded the percentage of plant material content.
- **Moisture content:** The extract's moisture content.
- **Ash content:** The extract's ash percentage.

### Evaluation of Phytochemical Properties

The analysis of alkaloids, glycosides and flavonoids, phenolics and other phytoconstituents through qualitative methods makes up preliminary phytochemical screening. Several phytoconstituents are specifically analysed using HPLC (Li, 2019). The analysis of volatile and semi-volatile substances is completed through GC-MS technological identification (Kotowska et al., 2012).

## Evaluation of Spectroscopic Properties

Using Fourier Transform Infrared Spectroscopy (FTIR), the extract's functional groups can be identified. Nuclear Magnetic Resonance (NMR) Spectroscopy is for phytoconstituent structural clarification (Breitmaier, 2006)

## Evaluation of Biological Properties

- Assessment of the extract's capacity to prevent the growth of microorganisms i.e., antimicrobial activity (Humphries et al., 2018).
- The extract's capacity to scavenge free radicals is assessed in Antioxidant activity (Brand-Williams, 1995).
- Assessment of the extract's capacity to stop the growth of cancer cells i.e., cytotoxicity (Mosmann, 1983)

## TECHNIQUES FOR THE EXTRACTION AND PURIFICATION OF BIOACTIVE NATURAL COMPOUNDS

Bioactive Natural compounds isolation and purification are fundamental processes of natural product studies, especially in pharmacology, nutraceuticals, and biotechnology. The choice of extraction and purification procedures is determined by the physicochemical characteristics of the bioactive constituents, such as the solubility, the stability, as well as the intended use.

### Extraction Techniques

The extraction method is used on large scale to determine the yield and purity of the desired bioactive molecule. An appropriate choice of extraction solvent also plays a decisive role because it directly influences the quality of the end bioactive product (Tiwari, 2015). Besides, it is crucial to optimize extraction conditions in order to make the process efficient. After optimization, use of a combination of extraction methods can be used in an effort to increase the yield and functionality of the bioactive compound (Pinelo et al., 2005)

## CONVENTIONAL EXTRACTION METHODS

The extraction methods commonly used in laboratories depend on organic solvents, which need long extraction times along with high solvent usage.

**Maceration:** Plant extraction can be done by placing plant material in its coarse or powdered form in a solvent and letting it stand between two to three days at room temperature with frequent agitation. The extraction vessel is carefully covered to reduce solvent evaporation at the ambient pressure. The process assists in disruption and degradation of the plant cell walls that increases the release of soluble phytoconstituents. The mixture is then left after a specified extraction time, and decantation or filtration is then applied (Adegbola et al., 2017). The simplest extraction technique used and the most common is called Maceration; the process is based on the mechanism of molecular diffusion, which is also naturally time-consuming. The process is used to ensure that the fresh solvent continues to view the surface of the plant particles and further extraction occurs, as well as dispersing the concentrated extract that forms on the surface of the plant particles (Patel et al., 2011).

**Percolation:** The procedure implicates a stable flow of a solvent through a compressed film of plants, driven by gravity or a minute amount of pressure. Then, the plant is moisturized by the solvent and let the period of maceration begins, it stimulates swelling and rises the permeability of the material.

Fresh solvent is then added, penetrating the plant matrix and dissolving the bioactive components, which are subsequently collected as an extract (Handa et al., 2008). Because percolation produces a higher yield and requires less time to extract than traditional maceration, technique has gained popularity in studies on natural products and herbal medicine.

**Soxhlet Extraction:** It is a widely used method that is often used in the recapture of bioactive substances, mostly lipophilic substances for example oils and fats in vegetable material. It is a liquid extraction method, according to which the target compounds are selectively extracted with the help of solvents at the boiling point under ambient pressure. Due to its effectiveness and dependability, Soxhlet extraction is usually used when the effectiveness of other extraction methods is to be tested (Macías-Sánchez et al., 2010).

The advantages of conventional Soxhlet extraction include its simplicity and cost-effectiveness. However, this technique is associated

with significant drawbacks, including prolonged extraction times and the use of large volumes of solvent, which may contribute to environmental concerns (Wang & Weller, 2006). Additionally, the extensive use of solvents not only increases the environmental impact but also raises the overall cost of the extraction process.

**Hydrodistillation:** Hydrodistillation is the conventional extraction technique used by plants to get bioactive chemicals. Plant samples are placed in a still by researchers, who then add adequate water and boil the mixture. Steam can be introduced directly into the plant material, or it can be introduced indirectly by boiling water. As the vapour combination proceeds through indirect water-cooled condensation, water and essential oils make up the condensate. After the mixture condenses, it passes through a separator that separates the water from the oil and bioactive substances. Although the hydrodistillation process has several advantages, its application for heat-sensitive phenolic compounds during extraction is limited by temperature constraints (Azmir et al., 2013).

## ADVANCED EXTRACTION TECHNIQUES

The industrial sector's need for bioactive compounds necessitates improving extraction methods because current methods are insufficient. By combining several procedures, scientists have improved extraction technologies and increased their efficiency. Because it generates higher yields in fewer cycles, this method is highly regarded as a solution for the growing market (Boyadzhiev et al., 2006).

**Ultrasound-Assisted Extraction (UAE):** UAE is a quick extraction method that operates by using high-power, low-frequency sonication. Power ultrasound, one of the two main types of ultrasonic therapies, has a long history in the medical field due to its vital role in sterilisation and defoaming for degassing and extraction of bioactive compounds. This approach offers a quicker and more affordable substitute for traditional methods while greatly increasing extraction efficiency (Keglevich, 2014).

Cavitation, or the creation of cavities, is how UAE functions. Sonoporation is the practice of applying ultrasonic waves to liquid cells to produce

holes that allow extraction solvents to enter plant materials deeply while increasing transfer efficiency (Tiwari, 2015). More intensity and amplitude are needed to achieve the necessary degree of cavitation, particularly for viscous materials. Because viscous materials absorb vibrational energy, the vibrational effects of UAE devices deteriorate when employed with them (Roselló-Soto et al., 2015).

**Microwave-Assisted Extraction (MAE):** By activating polar molecules and dipole movement, MAE uses microwave irradiation to heat solvents. The target chemicals are transferred to solvents when they are subjected to microwave irradiation conditions because of their unique characteristics (Ying et al., 2011). The reason why MAE has high extraction efficiency is the rapid heating of electromagnetic waves. Ekezie et al. (2017) provided a proposal of the use of the microwave irradiation as a potent technique of extracting bioactive molecules in the food sources in plants. Their study revealed that excessive application of heat to plant cells causes evaporation thus straining the walls of the cell. Eventually, this pressure leads to the breakdown of the cell walls and as a result, the escape of metabolites into the external environment is made possible. Also, the treatment of enzymes combined with the extraction using a microwave is even more advantageous because more extractants that are desired will be released and this will maximize the yield. (Jha & Sit, 2022).

**Supercritical Fluid Extraction (SFE):** Once a compound attains its critical temperature ( $T_c$ ) and pressure ( $P_c$ ) greater than the critical points of temperature and pressure, then it is considered a supercritical fluid. This is the case in which the fluid is both a gas and a liquid. Carbon dioxide ( $CO_2$ ) is the most popular supercritical fluid as it is economical, accessible, exhibits good heat transfer conditions, is non-toxic, and has the characteristic of passivity when it comes to most chemicals (Budisa & Schulze-Makuch, 2014). Extraction and purification are the two key processes in supercritical fluid extraction (Raventós et al., 2002). The leveraging of the extraction is typically increased in the solid forms as opposed to the liquid ones and this is the reason why the solid ones are mostly preferred. The dried and milled samples are then put in columns where the supercritical solvents

are run through to dissolve the extractable components. After dissolving, these materials are dispersed into a separator. This kind of separator separates the solvent-extract mixture by lowering pressure, raising temperature, and sometimes doing both (Schuster et al., 2018).

**Pressurized Liquid Extraction (PLE):** Pressurized Fluid Extraction (PFE) or Pressurized Liquid Extraction (PLE) is based on the idea of high pressure (Nieto et al., 2010). Even when a liquid grasps its boiling point, it can remain liquid because of high pressure. This method prominently quickens the extraction process at high temperatures. The viscosity and surface tension of the solvents have an influence on the rate of extraction. The rate of mass transfer and solubility rates increased by decreasing these features and resulting in increased extraction effectiveness. The main assistances of PFE are its high solubility, minimal solvent usage and fast extraction time. Due to potential for high pressure and temperature to mark their structural integrity and activity, bioactive chemicals under the category of heat-sensitive compounds are not suitable in this manner.

**Enzyme-Assisted Extraction (EAE):** Researchers explored the enzyme technology added to both traditional methods of extraction (maceration and distillation) and the contemporary one. Scientists have used Enzyme-Assisted Extraction (EAE) to extract phenolic products and polyphenols and oils, extracting fatty acids and essential oils, sugars and di-and-triterpenes and vitamins and polysaccharides and proteins (Bhatkar et al., 2021). Enzyme pre-treatment is done on a wide range of plant and fungal species belonging to different families to improve the yield of bioactive metabolites. Different hydrolytic enzymes, together with enzymatic mixtures and commercial enzyme solutions, are researched for their extraction effectiveness by scientists who use cellulases, hemicellulases, pectinases, and proteases. Scientific studies primarily measured the total yield between metabolite groups that included polyphenols, flavonoids, lipids, carbohydrates, and proteins (Vardakas et al., 2021). The method of enzymolysis is commonly used to increase the recovery of polyphenols and plant phenolic compounds. Almost every study demonstrated that enzymatic treatments produced substantial improvements in the release of

both total phenolics and targeted compounds as measured against untreated samples. These enhancements totaled hundreds of percentage points. Experimental findings showed that enzymes, including cellulase, together with pectinases and different enzyme blends, particularly Viscozyme and Kemzyme, demonstrated excellent results (Krakowska-Sieprawska et al., 2021).

**Purification of bioactive natural compounds:** Numerous bioactive substances and phytochemicals with varying polarity can be found in extracts from plants, animals, and marine life. Following extraction, the desired chemical is separated by bioactive molecule purification. The goal of the purifying process is to increase the amount of the bioactive component chosen for extraction. In addition to stability concerns pertaining to the intended active components, the extraction process must separate the target bioactive molecules from other components in the mixture, which presents a significant difficulty. Different purification techniques that satisfy these criteria are required in order to pursue the stable and pure bioactive molecule.

**Chromatographic Techniques:** Polysaccharides can be purified using ultrafiltration, preparation ultracentrifugation, salting out, metal coordination, zone electrophoresis, and quaternary ammonium salt coordination.

Major techniques of chromatographic include flash chromatography, TLC, Sephadex chromatography, HPLC, and column chromatography. Bioactive compounds and polysaccharides are commonly purified using gel, affinity, and anion exchange column chromatography (Table 1).

**Thin Layer Chromatography (TLC):** The method of Thin Layer Chromatography (TLC) has gained popularity because it offers a straightforward setup and basic but successful purification combined with separation features. TLC runs its operations using silica-coated plates that have uniform silica layers spread across polymer/plastic sheets or glass plates. The advantage of the Thin Layer Chromatography (TLC) method is in its simplicity of configuration, as well as simplistic yet effective purification as well as separation characteristics. TLC operates its business with silica-coated plates that are coated with uniform layers of silica on polymer/ plastic sheets or glass plates (Garcia et al., 1993). This

**Table 1.** Techniques for Polysaccharide Purification

Category	Methods
General Purification	Preparation zone electrophoresis, ultrafiltration, ultracentrifugation, salting out, metal coordination, quaternary ammonium salt coordination
Chromatography	Column Chromatography, Flash Chromatography, Sephadex Chromatography, Thin Layer Chromatography, High-Performance Liquid Chromatography,
Column Chromatography Types	Affinity Column Chromatography, Gel Column Chromatography, Anion Exchange Chromatography

process gives effective isolation of compounds due to their attraction to the stationary phase and the mobile phase.

The separated substances that have been obtained using Thin Layer Chromatography (TLC) analysis can be subjected to additional tests, which confirm and identify them. These tests include screening reagent usage that determines the presence of phytochemicals and the analysis of the samples under UV light. The process of identifying will provide the separating compounds in addition to the knowledge of the purity levels of their extraction. The confirmatory methods are procedures that are necessary to confirm isolated compounds in TLC procedures.

**High-Performance Liquid Chromatography (HPLC):** HPLC functions as a prevalent analytical method to obtain purified components from solution combinations with several related substances as well as unwanted contaminants (Sarker & Nahar, 2012). HPLC features ideal resolving power for processing and separating unwanted components extracted from samples. The separation technique HPLC operates in two categorizations for different functions: Analytical HPLC assesses compound quantity and quality, then Preparative HPLC (prep-HPLC) isolates or extracts pure compounds from sample mixtures. Modern automated prep-HPLC systems provide facilities for large-scale purification operations that shorten the overall processing time (Mahato et al., 2019).

UV detectors stand as the most commonly used detection method in HPLC systems because they demonstrate high sensitivity and benefit the analysis of rare compounds in samples. The instrument detects trace amounts of substances because of its high sensitivity levels. DAD and MS serve as detection methods in HPLC systems to enhance compound validation alongside quantification (Tsao & Deng, 2004).

**Medium-pressure liquid chromatography:** The purification process of bioactive compounds through Flash chromatography relies on medium-pressure applications on columns to separate components from extensive sample mixtures in efficient and quick operations. Automated robotic systems that combine with online detectors operate to make target compound purification and isolation methods more proficient and automated.

**High-speed counter current chromatography (HSCC):** HSCC is predicated on solvent-based systems via two phases that flow in opposite directions simultaneously. This liquid-liquid separation method is mainly applicable in the purification of flavonoids without the presence of a solid support media. The important attribute of HSCC is that it can enable the purification of compounds in a single run, without any pretreatment of the sample (Duo-Long et al., 2011). Wang et al. (2005) were the first to report the application of this technique, and they were able to purify polymethoxy flavones (PMFs) in tangerine peel extract.

**Molecular sieve chromatography:** Using biological size differences, the sample mixture of organic molecules is separated using the molecular sieve chromatography method. Among other materials of the same or even larger size, the method works well with proteins and pectin. This method uses porous dextran polymer beads arranged in a column as the filtration medium. When the sample molecules pass through the column, separation occurs; larger molecules are pushed out because the pore previously excluded them, while smaller molecules take longer to exit the column. The process operates to isolate small and large pectin fragments comprising galactans and arabinans that have smaller molecular dimensions (Zhang et al., 2007).

### Non-Chromatographic Purification Methods:

Tracing technologies utilized for purification involve three different assays:

1. Phytochemical screening assays,
2. Fourier-transform infrared spectroscopy
3. Monoclonal antibody-based immunoassays (Sasidharan et al., 2011).

**Liquid-liquid partitioning:** Liquid-liquid (partition) chromatography is a simple, cost-effective, and straightforward technique for purifying and isolating compounds. The target's differential distribution compound between two liquid phases with differing split coefficients is the basis for this technique. Decantation is used to separate the phases of immiscible liquids, whereas a rotary evaporator is used to separate solvents based on their different boiling points in miscible liquids. Before using other purification techniques, this approach is frequently employed as a first step. Researchers employed liquid-liquid partitioning with butanol, hexane, and ethyl acetate fractions from clove (*Syzygium aromaticum*) to isolate four novel flavonol glucuronides: rhamnetin-3-O- $\beta$ -d-glucuronide, rhamnazin-3-O- $\beta$ -d-glucuronide, rhamnazin-3-O- $\beta$ -d-glucuronide-6"-methyl ester, and rhamnocitrin-3-O- $\beta$ -d-glucuronide-6"-methyl ester from the ethyl acetate extract (Ryu & Oh, 2016).

**Recrystallization:** One purification technique for eliminating contaminants from a solution is recrystallisation. This method's basic idea is based on temperature effects, which increase the solubility rates of most compounds. The crystals exhibit a variety of characteristics that are influenced by cooling pace, solvent composition, practitioner experience, and environmental factors. In general, the temperature at which a compound recrystallises should be between one-third and half of its melting point (Ryu & Oh, 2016). There are two main steps in the recrystallisation process. First, solute particles are deposited from the saturated solution according to their morphologies; in the second step, called crystal growth, crystal lattices are formed. The phase separation or "birth" of new crystals occurs during the first stage, known as nucleation, and these crystals enlarge during the second stage. Isolating a chemical with this approach can be time-consuming, which is a significant drawback. Ryu & Oh (2016) applied this technique to purify a

flavonoid compound from clove (*Syzygium aromaticum*) using methanol as a solvent, in addition to a tannin compound and a triterpenoid from the same source.

**Membrane filtration:** Membrane filtration is the process of separating oligomers according to their molecular size using a selectively permeable membrane. The preservation of the structural integrity of polyphenolic acids (PAs) at room temperature, ease of use, and less production of laboratory waste are only a few benefits of this technique. However, it also has certain drawbacks, such as the high cost of membranes and the slow filtration rate. The filtration membranes are divided according to the pore size or molecular weight cutoff, which include microfiltration, ultrafiltration, nanofiltration, and reverse osmosis membranes. In industrial applications, membrane filtration technology can be employed to separate and purify PAs with varying degrees of polymerization.

**Precipitation:** A mix of solutes, starting with a natural extract, goes into solution in an organic solvent before subjecting it to compressed CO<sub>2</sub> or a similar anti-solvent substance. The selected organic solvent needs to dissolve all components in solution while mixing well with the anti-solvent. The homogeneous mixture created by antisolvent contact with liquid solution results in solvent solvation power reduction because of volumetric expansion. Some solutes in the mixture become insoluble to the new medium because of dissimilarities in polarity, along with differences in CO<sub>2</sub> affinity and molecular weight, and thus they precipitate. Compounds that maintain high interactions with organic solvents remain in solution due to the presence of strong intermolecular forces. When CO<sub>2</sub> precipitates occur, the organic solvent functions as a complementary solvent by dissolving the precipitate (Shimizu & Abbott, 2016).

## STRUCTURAL ELUCIDATION OF BIOACTIVE NATURAL COMPOUNDS USING SPECTROSCOPIC METHODS

**Structure elucidation** typically refers to the thorough determination of a molecule's structure from scratch, resulting in an explicit molecular connectivity map that includes accurate stereochemistry. Nuclear magnetic resonance spectroscopy is most frequently linked to this

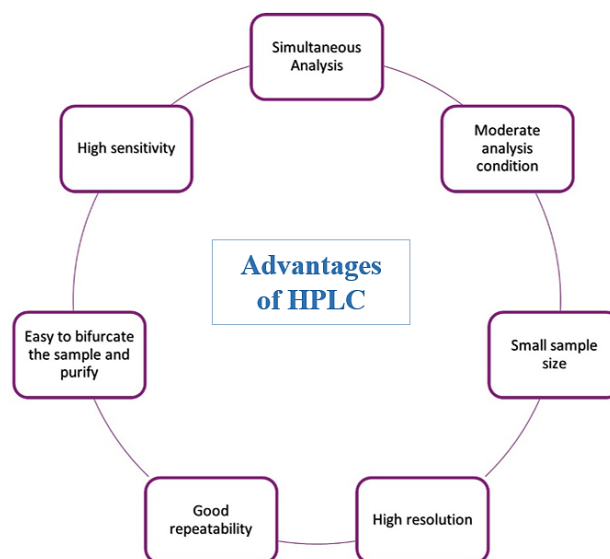
procedure, which is done without prior information or beliefs. Conversely, dereliction often describes how to identify known natural substances, frequently using mass spectral library matching or by looking for well-known fragmentation patterns in mass spectrometry (Gaudêncio & Pereira, 2015).

**High Performance Liquid Chromatography:** In analytical chemistry, High Performance Liquid Chromatography (HPLC) is an essential technique that can separate, identify, and quantify molecules in any liquid-soluble material. It is thought to be the most accurate technique for analysing bioactive natural substances both qualitatively and quantitatively (Rao et al., 2015). A solution of the bioactive natural ingredient is inoculated into a column containing a porous material (the stationary phase) as part of the HPLC process. The mobile phase, a liquid, is then delivered at high pressure into the column. Segregation of the sample occurs due to differences in how briskly the compounds migrate through the column, which is determined by their varying affinities for the stationary and mobile phases. Consequently, different compounds elute at different times (Rajan, 2015). Compounds with a stronger affinity for the stationary phase move more slowly and proceed shorter distances, while those with a weaker affinity travel faster and move farther (Kumar et al., 2015).

HPLC offers significant versatility as compared to gas chromatography because (a) it is not confined to volatile or heat-stable samples, (b) there is an extensive selection of mobile and stationary phases to select from (Gupta et al., 2012). The advantages of using HPLC has been shown in Fig. 1.

HPLC can be classified in several ways;

1. **By scale of function:** analytical HPLC or preparative HPLC
2. **By separation theory:** adsorption chromatography, affinity chromatography, chiral phase chromatography, ion exchange chromatography, and size exclusion chromatography
3. **By elution approach:** isocratic separation and gradient separation
4. **By means of operation:** normal phase chromatography and reverse phase chromatography (Vidushi et al., 2017).



**Fig 1.** The advantages of HPLC modified from Sonia & Nappinnai (2016)

## Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy is a fundamental technique in analytical chemistry used for determining the molecular structure of bioactive natural compounds (Elyashberg et al., 2008). The first phase in determining the structure of an unspecified compound is by using accessible MS and NMR records. If the range of the unspecified matches a reference spectrum exactly, it indicates that the structure of the unspecified is the same as the reference. The process is known as structure identification. If spectra do not match, the challenge of elucidation of structure emerges. Once the constitution is determined, it is crucial to check if the composite is novel. This process, involving a structural search in relevant databases, is called dereplication. The recognition of a known compound based on earlier spectroscopic and analytical data forms the foundation of the NMR method.

Elucidation of structure is undoubtedly a novel task, as it falls under the genre of inverse problems (Liu et al., 2014), which are identified by solution ambiguity. In general, the objective of determining a structure from 2D-NMR particulars can be regarded as a blend of two inverse issues that must be resolved sequentially (Halabalaki et al., 2014). The foremost objective is identifying all the possible sets of atoms in a molecule that indicates

correlations in 1D and available 2D-NMR spectrums. This phase involves interpreting the spectra that has other infusions due to resonance overlap and many other factors. The secondary objective is to pin down all the possible structures that fit the coupled nuclei and choose the most likely structure by applying accessory limitations, such as characteristic features of the spectrum, knowledge about chemicals, and predictions of NMR chemical shifts. Understandably, solving the second problem is based on the first one. If errors in spin couplings happen during the first problem, an error-free structure becomes difficult to determine.

By analyzing the reasoning of spectroscopists during elucidation of structures, it was found that the initial NMR data can be regarded as a set of “axioms,” forming a partial axiomatic theory (Elyashberg et al., 2008; Halabalaki et al., 2014). Therefore, the task is reduced to inferring all possible structures based on these axioms. These axioms can be formalized with no trouble, providing a theoretical foundation to develop codes for computer-assisted structure elucidation (CASE).

Prediction of NMR chemical shift is of importance in estimating proposed structures (Elyashberg et al., 2008; Molinski & Morinaka, 2012). Both empirical and quantum-mechanical (QM) methods can be used to determine chemical shifts of nuclei  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{31}\text{P}$ ,  $^{15}\text{N}$ , and  $^{19}\text{F}$ . The most common prediction models to use NMR spectrum are HOSE codes, incremental and neural networks (Elyashberg et al., 2008; Molinski & Morinaka, 2012). The expert systems can incorporate these methods.

The verification of structure based on the  $^{13}\text{C}$  chemical-shift prediction method is useful in the selection of a specific structure out of a large number of possible structures that are produced by the use of expert systems (Elyashberg et al., 2008). In order to determine the structures of large compounds, the  $^1\text{H}$  chemical-shift prediction method is usually most desired due to its rapid method of obtaining a spectrum with minimal amounts of sample. Nevertheless, this technique has a drawback of low efficiency due to solvent, pH, and temperature, which may influence proton chemical shifts.

## Mass Spectrometry

Mass spectrometry (MS) has exhibited its exceptional quality to analyze complex mixtures with sensitivity and remarkable accuracy. Mass spectrometry is a resourceful method that controls ions (either positive or negative) in the gas phase using magnetic fields or external electric. With the proper ionization methods, mass spectrometry can analyze many compounds. The ionization process can occur either under high vacuum or ambient settings (Feider et al., 2019).

Once ionized, the ions are hastened, fixed, and directed to the mass analyzer in sequence. Ions having variable mass-to-charge ratios are distinguished in a mass analyzer depending upon the variations in their flight times, kinetic energy, or the frequencies of specific motions regulated by magnetic or electric fields. A major advantage of mass spectrometry is its ability to provide detailed structural data about the analyte ions and determine the molecular weight from molecular ions or their (de)protonated forms. Intact ions can also undergo fragmentation processes. This allows for the production and detection of key fragment ions, which can then be used to reconstruct the structure of an unknown molecule (Feider et al., 2019).

The precision of mass and structural analysis has increased with the use of hybrid instruments, which incorporate many mass analysers (Glish & Burinsky, 2008). Also, there are numerous Mass spectrometers set up to enable a reaction in the gas phase to obtain additional structural information when fragment ions do not suffice to establish the structure of a precursor in the absence of a reaction, for example, in the form of reaction products (Liu et al., 2023; Warneke et al., 2020; Ma et al., 2022). Additionally, easily affected molecules that are difficult to capture in the condensed phase on MS can be studied, yielding valuable insights into their behaviour and role in important chemical and biological processes (Lubes & Goodarzi, 2018; Asteggiano et al., 2021).

## APPLICATIONS IN BIOACTIVE NATURAL PRODUCTS STRUCTURAL ANALYSIS

Mass spectrometry has proven to be a helpful technique for determining the structure of bioactive

natural substances throughout time (Xu et al., 2021). It will be able to determine the sequence of polymers, including proteins and peptides, as well as the percentage of monomers in units. Additionally, a wide range of additional substances are fully identified using mass spectrometry and other comparable techniques. Such molecules can be analysed using a number of MS programs.

## CONCLUSION

Because of their varied structural and therapeutic capabilities, natural products are essential to the drug discovery process. To accurately identify bioactive chemicals, cutting-edge extraction, purification, and analytical methods such as TLC, HPLC, and complex spectroscopic techniques must be used in tandem. The process of transforming natural materials into possible pharmaceutical products is accelerated by using these combination techniques.

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