



Chromatographic Techniques Used in the Bioguided Isolation of Bioactive Compounds from Natural Products

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Abstract

Bioactive compounds are natural substances found in various plant matrices, known for their antioxidant, antitumor, anti-inflammatory, and antimicrobial properties. The growing demand for natural sources of these compounds has driven research into isolating these substances from plants. This process usually involves several stages: extraction, fractionation, purification, and isolation, using chromatographic techniques such as High-Performance Liquid Chromatography (HPLC), open-column chromatography, and Thin-Layer Chromatography (TLC). These techniques allow for separating and purifying bioactive substances such as flavonoids, alkaloids, and polyphenols. Notable examples of plant-derived bioactives include acetylsalicylic acid from the bark of the willow tree of the *Salix* genus and morphine from *Papaver somniferum*. This review explores the methods and steps involved in isolating bioactive compounds from plant matrices, emphasizing the application of chromatography for separating and purifying these substances.

Keywords: Bioguided; Chromatography; Isolation; Natural Products.

Abbreviations: HPLC: High-Performance Liquid Chromatography; TLC: Thin-Layer Chromatography; RF: Retardation Factor about the solvent front; NMR: Nuclear Magnetic Resonance Spectroscopy

INTRODUCTION

Bioactive compounds are natural substances found in various plant matrices. This class of compounds is linked to beneficial properties for humans, such as antioxidant, antitumor, anti-inflammatory, and antimicrobial activities, among others [1,2]. There has, therefore, been a constant increase in the search for natural sources rich in bioactive compounds, with a view to their application in product development and the synthesis of new substances. The process of harnessing these benefits begins with isolating bioactive compounds from plants, which are a rich source of substances such as flavonoids, alkaloids, and polyphenols [3,4]. This isolation involves several stages: extraction, fractionation and purification, and finally, the isolation of the compounds of interest. Once isolated, these substances can be identified and evaluated for various biological activities, enabling them to be used in medicine, pharmacy, cosmetology, etc. The notable examples of compounds widely used today obtained from plant matrices highlight the relevance of studies in this area. Aspirin, one of the most widely used painkillers in the world, contains acetylsalicylic acid, a substance synthesized from salicin, a compound isolated from the bark of willow trees of the genus *Salix* [5]. Another example is morphine, a drug with high analgesic power that consists of an alkaloid isolated from the opium capsule of *Papaver somniferum* [6].

However, isolating bioactive compounds from plants is a complex process involving various steps and techniques, mainly chromatography. Different chromatographic methods, such as High-Performance Liquid

Chromatography (HPLC) in analytical and preparative modes, open column chromatography, and thin layer chromatography, are used to separate and purify substances, allowing pure compounds to be obtained and enable the biological activity of these substances to be assessed. In this review article will present the steps involved in isolating bioactive compounds from plant matrices and address how the different chromatographic techniques are employed in this process.

PLANT MATRIX

The first step in the bioguided isolation of bioactive compounds consists of choosing the plant to be studied. Ethnopharmacology, a field that investigates biologically active agents and the therapeutic use of plants based on traditional knowledge and human observations, plays a key role in this process. By starting with a plant that has established roots in folk medicine, researchers can scientifically validate its properties, bridging the gap between traditional uses and modern scientific evidence [7]. Once the plant is chosen, the next step is to determine which part of the plant will be used, which could be leaves, flowers, stems, fruit, etc. The leaves are very rich in bioactive compounds since several studies have shown the presence of phenolic substances, rich in antioxidant, anti-inflammatory and antimicrobial activities [3,8,9]. The material is collected after selecting the plant part, and the preparation stage begins. Leaves must be cleaned, dried, ground, and stored under refrigeration to preserve their bioactive properties for further analysis.

SOLID-LIQUID EXTRACTION (OBTAINING THE CRUDE EXTRACT)

The second part of the process consists of extraction, where the compounds present in the plant leaves are extracted and migrate to the liquid medium. Various extraction methods can be used, varying parameters such as temperature, solvent, time, and the use of unconventional techniques such as ultrasound-assisted extraction, which facilitates the process through mechanical sound waves [10]. Among the various extraction methods, cold maceration is widely used to extract compounds. This technique avoids high temperatures, thereby preventing the degradation or loss of compounds and preserving their functional properties. In this process, the previously dried and ground plant material is placed in a flask in contact with a solvent. The solvent is chosen according to the class of compounds to be extracted. For bioactive compound extraction, solvents with medium to high polarity are typically

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chosen, as many bioactive substances, such as phenolic compounds, also exhibit polar characteristics. Ethanol solutions are commonly employed due to their highly polarity and effectiveness in extracting a wide range of bioactive compounds [11].

Jacobsen et al. [12] extracted bioactive compounds from the *Pereskia aculeata* leaves using cold maceration in exhaustive mode, using a mixture of ethanol:water (80:20 v/v) as the solvent. Their studies produced extracts rich in phenolic compounds such as rutin and isoquercetin [12]. In the exhaustive cold maceration process, the plant in contact with the solvent is stirred continuously on a shaker table, with the solvent being renewed every day until complete extraction is verified, evidenced by the discoloration of the solvent. The solvent is then filtered to remove any remaining particles from the plant and eliminated them. In contrast, in an ethanolic mixture the ethanol is evaporated in a rotary evaporator and the water is freeze-dried. The product remaining after this process is the crude extract.

SOLID-LIQUID EXTRACTION (FRACTIONATION)

In the fractionation stage, the crude extract is subjected to solid-liquid extraction using different solvents with different polarities. The aim is to produce fractions rich in certain classes of compounds. For example, the hexane solvent is apolar and will, therefore, extract compounds with a similar polarity, such as lipids, giving rise to a fraction concentrated in this class of substances. Ethyl acetate, on the other hand, is a solvent with medium to high polarity and, therefore, extracts compounds with similar polarity, producing a fraction with more polar characteristics [8]. Thus, to isolate phenolic compounds, apolar and low polarity solvents are used, which act as “filters,” removing unwanted compounds and making the crude extract more concentrated in the compounds of interest. Then, medium to high polarity solvents produce fractions rich in bioactive substances. After the fractionation process, the solvents are evaporated using a rotary evaporator, and the fractions, together with the crude extract, are tested for biological activity.

The biological activity evaluated is defined according to the research interest. Jacobsen et al. [12] performed the extraction and fractionation process of *Pereskia aculeata* leaves. They monitored the antioxidant activity of all the extracts and fractions produced, concluding which solvents were capable of producing the most bioactive fractions [12]. Oldoni et al. [3] carried out the same process with *Moringa oleifera* leaves, monitoring the antioxidant and anti-glycemic activity [3]. In addition to the biological activities, High-Performance Liquid Chromatography (HPLC) is used to identify and quantify the compounds in the samples. Chromatography is a technique for separating the components present in a complex mixture, which are separated due to the interaction of the sample with two distinct phases, the mobile phase and the stationary phase. In liquid chromatography, the mobile phase is a liquid, while the stationary phase can be a solid or a liquid immobilized in a solid [13,14].

In HPLC, the mobile phase is driven by high-pressure pumps and passes through the chromatographic column, which is filled with the stationary phase. As a result, the mobile phase carries the sample and the components present with it, causing them to pass through the column. During this passage, the compounds will interact chemically in different ways with both phases, generating a “competition” between adsorption on the surface of the solid and desorption by the solvent [13,14]. Reverse phase chromatography separates a highly polar sample containing bioactive compounds, where the stationary phase is composed of an apolar filler, such as modified silica, and the mobile phase consists of a solvent or a mixture of polar solvents. In this technique, highly polar components spend less time in contact with the stationary phase. They are displaced more quickly by the mobile phase due to the difference in polarity and less interaction with the column packing [14]. After the compounds have passed through the chromatographic column, there is the detector, which will record the output of the compound as a signal

consisting of a chromatographic peak. Thus, the result obtained in this analysis is a chromatogram, where each peak has a specific retention time according to the interaction time during the chromatographic run [13].

In addition to separating the components, HPLC can identify and quantify the compounds. This process occurs using standard solutions. By injecting a standard solution of the analyte, it is possible to compare each molecule's retention time and maximum absorption profile characteristics. If both parameters are the same for the standard and the compound analyzed in the sample, it is possible to state that this compound corresponds to the analyte present in the standard solution, and by constructing calibration curves with this solution, it is possible to quantify the content of this compound [13]. By using chromatography to evaluate the crude extract and the fractions produced, it is possible to determine which sample has the largest amount of bioactive compounds, as well as which has the highest levels of certain compounds, making it easier to choose the best fraction to follow in the other stages.

PURIFICATION (OBTAINING SUB-FRACTIONS)

After the fractionation process, by evaluating the biological activity and determining the compounds by chromatography, the most bioactive fraction is selected for the purification stage, where even richer sub-fractions of the substances of interest will be obtained. The open-column chromatography technique is used for this. This technique is carried out on a glass column, where the stationary phase is initially prepared. At this stage, the glass tube is positioned vertically, and an absorbent cotton pad or glass wool is added to retain the stationary phase. The column is then filled with the stationary phase suspended in a solvent, where silica powder is commonly used. This step is carried out very carefully to avoid forming air bubbles, which can impair the separation efficiency due to the difference in homogeneity of the mobile phase flow. It is also important to ensure that the column never dries out, always keeping the solvent level above the top of the stationary phase deposit throughout the chromatographic run [13].

The sample is subsequently introduced into the chromatographic column. To optimize this process, it is advisable to incorporate the sample into the stationary phase matrix before carefully packing it into the column. The mobile phase, consisting of a solvent or solvent mixture, is added. This phase may be adjusted progressively depending on the specific requirements of compound separation. During elution, the mobile phase carries the sample through the column. As with HPLC, the interaction between the sample and both the stationary and mobile phases drives the separation of the compounds, which is visually indicated by distinct colored bands. A flow control valve at the base of the column regulates the elution rate, allowing for the collection of various fractions in separate vials as they emerge [13].

Grouping The Sub-Fractions

Open column fractionation results in many sub-fractions, making the practical stage more difficult due to the large number of samples to be analyzed and reducing the final yield of compounds to be isolated. This way, sub-fractions with a similar chemical composition can be grouped and referred to as a single sub-fraction. The chemical composition of each sub-fraction can be assessed using Thin-Layer Chromatography (TLC).

TLC is used for rapid qualitative analysis and, in some cases, for isolating small quantities of substances. The stationary phase, a chromatographic plate, consists of a thin layer of a finely divided solid (e.g., silica) coated on a rigid, inert support, such as aluminum. A drop of the sample is applied to the base of the plate, and the plate is added to a chromatographic vat containing the mobile phase. The mobile phase consists of a solvent that, by capillarity, migrates through the stationary phase, dragging the sample with it and separating the compounds due to the different interactions between the two phases. Finally, a chromatogram is obtained with several spots arranged in different



positions on the plate since due to the interactions of the sample with both phases, each component travels a certain distance, designated as the Retardation Factor about the solvent front (RF) [13,15].

Using this technique, after the chromatographic run with the sub-fractions, it is possible to check which samples have a similar chemical composition, i.e. which samples have the same spots and the same Rf. Thus, when different sub-fractions have a similar chemical composition, they can be grouped as one. Finally, the biological activity is evaluated and the compounds are quantified by chromatography of the remaining sub-fractions, where the most bioactive are selected for the final stage of isolating the compounds.

ISOLATION OF COMPOUNDS

The sub-fractions evaluated with the best bioactivity and highest concentration of compounds are subjected to high-performance liquid chromatography in preparative mode for the large-scale isolation of compounds. While conventional HPLC works on an analytical scale and aims to separate, identify, and quantify the components, the main objective of the preparative mode is to obtain the purified compounds on a large scale. The operating principle of both techniques is the same, but there are instrumental differences. In analytical HPLC, a chromatographic column with a diameter ranging from 2 to 4.6mm is used. In contrast, in preparative HPLC, the diameter is more significant, ranging from 20 to 50mm, which allows the injection of a larger volume of sample, with a higher flow rate of the mobile phase and, therefore, a higher yield in obtaining the compounds. In addition, the equipment operating in preparative mode has the addition of a fraction collector. As the compounds leave the column and are detected, they are directed and collected in separate vials. This collection process can be manual, developed by the analyst himself, or automated, where the detector signals for the flow to be diverted to the collector [14].

After separating and obtaining the isolated compounds, it is necessary to evaporate the solvent from the mobile phase to obtain the pure compound, usually in powder form. After this process, different techniques are used to elucidate the molecule's chemical structure and identify the isolated compounds. The most used techniques are Nuclear Magnetic Resonance Spectroscopy (NMR), Infrared Spectroscopy, and High-Resolution Mass Spectrometry. NMR provides information on magnetically distinct atoms, commonly hydrogen and carbon, making it possible to determine the environment in which the proton is located and the number of different non-equivalent protons. Infrared spectroscopy involves the absorption of infrared radiation, which promotes vibrational movement in the structural units of the molecule. As a result, the functional groups present in the compound's structure will generate bands that absorb at characteristic frequencies, and, by consulting data from the literature, it is possible to analyze the spectrum generated and suggest which bonds are present in the molecule [16].

High-Resolution Mass Spectrometry complements and provides more information for the molecule identification stage. In this technique, molecules are converted into ions, separated due to their mass/charge ratio, and considered fragments, representing distinct fractions of the molecule. In the high-resolution technique, it is possible to use a mass spectrometer coupled to a liquid chromatograph, allowing analysis of non-volatilizable samples without the need to use standards to identify the compounds, also providing greater accuracy in the mass value of the compound [17]. Finally, after obtaining and identifying the isolated compound, it is interesting to evaluate the biological activity of this substance, allowing us to ascertain whether the property present in the plant comes from this compound or even from a synergism between different components of the plant matrix. This also makes it possible to determine whether this substance is promising for future applications in different fields, such as medicine, cosmetology, and pharmaceuticals (Figures 1,2).

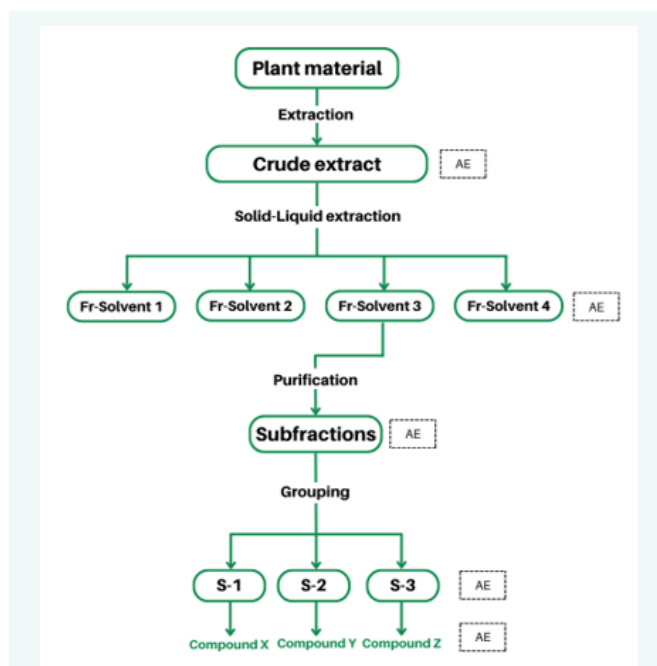


Figure 1: Flowchart of the steps involved in the extraction and isolation of bioactive compounds from plant matrices. AE: Activity Evaluation.

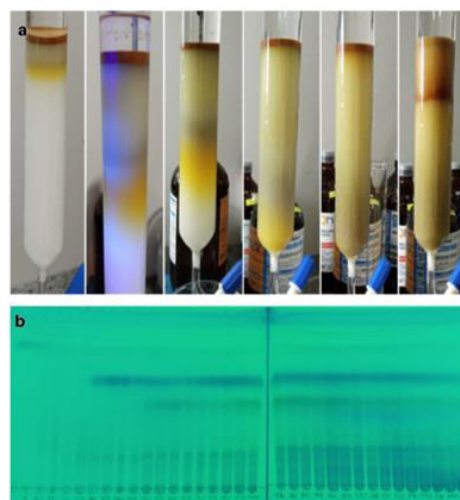


Figure 2: (A) Open column liquid chromatography technique and (B) Thin layer chromatography technique for samples obtained from plant leaves [17].

DISCUSSION AND CONCLUSION

The isolation of bioactive compounds from plants represents a crucial step in discovering and developing novel products and therapeutic agents. Advanced chromatographic techniques enable the purification of these compounds, facilitating detailed evaluation of their biological activities. By harnessing the potential of plant bioactive, this approach not only promotes the sustainable use of natural resources but also drives technological innovation in various fields, including pharmaceuticals, cosmetics, and functional foods.



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