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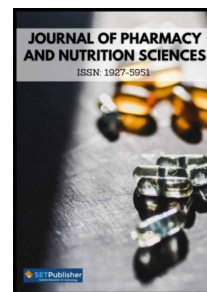

A Generalized Review on Extraction of Biomolecules

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Abstract:

Nature has given us a wide range of biological compounds that can be utilized to help combat health problems, but sometimes with over-processing, these advantages are diminished or lost. Food and pharmaceutical companies have developed a range of new approaches to harness and retain the naturally occurring diversity and quality of bioactive compounds efficiently and effectively. Pharmaceutically important plant products have been known for millennia; they have been used in crude and unrefined forms. One of the best ways to pick the best plant bioactive is through genetic engineering, omics, and plant tissue culture. Many laboratories routinely screen plant species for bioactive compounds to discover new ones. All extraction methods depend on the researcher's preference and what exactly the research entails. Successful extraction begins with the careful selection and preparation of plant samples and thorough knowledge and review of the appropriate literature. Here we have attempted to describe the different stages and methods of extraction from the medicinal plants. From the review, it can be concluded that no universal extraction method is ideal and that each extraction procedure is unique.

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INTRODUCTION

Photochemistry is the study of chemical compounds derived from plants. The word "vital" in Greek means "plant" and chemistry is the science of chemical elements and compounds. The word "menstruum" is a substance that dissolves a solid or in simple terms, it means "solvent" [1]. It has been observed that the quality of active components in two species of the same plant can differ slightly. Some of the advanced research highlights how one can target and predict some of the biomolecules characteristics based on the attributes of the plant, climates, soil and geography. This ecological difference can play a major role in the biosynthesis pathways and various other factors within the plant that will affect the possible final products. Since biotechnology is more concerned with genotype than phenotype, researchers need to keep a closer watch on plant genetics and epigenetics. The first step is to use genomics, proteomics, and metabolics to identify the plant of interest. The second step would be gene transfer from the plant of interest to the target plant using transformation, vectors, expression, and selection. Then the transformed cell or explant can be modified into callus formation, suspension, or protoplast culture, followed by plant regeneration through organogenesis, somatic embryogenesis, and synthetic seed production [2] The developed plants are screened through high-throughput screening, looking for plants that could have new bioactive compounds. It is often observed that a lot of the active constituents in plants are of small quantity, and most of the components are water and cellulose; this is the reason why we need to concentrate the sample of interest [3]. In this review, we describe an overview of the preparation of extracts from plants using organic solvents, with an emphasis on common problems encountered and methods of reducing unwanted side reactions.

Phytochemical or biological studies on plant secondary metabolites pose unique challenges that must be addressed throughout the extraction process. Separation of individual plant parts is followed by drying in the shade or in the sun, pulverization, an artificial dryer, and a hot oven with carefully controlled extraction. Cold extraction is usually slow, but with low denaturation. In hot extractions, active components can be degraded, but it is fast. This is followed by separation, purification using column chromatography (TLC, GC, HPLC, etc.), and electrophoresis for purification. Pure compound identification is accomplished by comparing physical or spectral data to standards.IR, UV, and mass spectra can be used to perform elemental analysis on unknown samples.

In addition to low extraction efficiency and poor selectivity, conventional extraction separation procedures have a number of drawbacks. Traditional techniques could potentially raise the price of the finished product due to complex and time-consuming separation processes. Additionally, hazardous organic solvents are frequently used that are toxic. Researchers have been working on creating substitute extraction and purification methods with "greener" and more sustainable techniques.

Solvent Extraction

Successful determination of a biologically active moiety from plant material is largely dependent on the type of solvent used in the extraction procedure. Based on selectivity, polarity, boiling point, chemical and thermal stability, safety, flammability, and cost, solvents are categorized into 6 groups [4].

1. The solvent power should be used selectively to extract only the active constituents.
2. The boiling point of the solvent should be as low as possible to easily remove it from the product.
3. a low-viscosity solvent with good heat and mass transfer.
4. The solvent should not react chemically with the extract. If the constituents are thermolabile, cold maceration, percolation, are preferred extraction methods. Soxhlet extraction (if non aqueous solvents are used) and decoction (if water is the solvent) are useful for thermostable constituents.
5. The solvent used should be Generally Regarded as Safe (GRAS).
6. The solvent should be inexpensive and easily accessible, and its recovery should be good enough to allow it to be easily separated from the extract.

The solvents used in most of the plant extract are polar (*e.g.,* water, methanol), intermediate polar (*e.g.,* acetone, dichloromethane), and nonpolar (e.g., n-hexane, ether, chloroform). Organic solvents have been found to produce a more consistent amount of active moiety compared to water extract. The more

nonpolar or polar the solvent is, the more refined and specific the extraction can be. Acetone dissolves many hydrophilic and lipophilic components from plants; it is miscible with water and can easily remove the active constituents, particularly flavanols and phenolic compounds, from the extract [5].

Methanol and ethanol can easily penetrate the plant's cellular membrane to extract the intracellular ingredients. Methanol is used for the extraction of anthocyanins, terpenoids, saponins, tannins, xanthoxylene, totarol, quassinoids, lactones, flavones, phenones, and polyphenols. Though methanol is more polar than ethanol, the toxic nature of methanol makes it unsuitable for extraction [6]. Because of its higher polarity and low evaporation, 70 to 80% ethanol is considered superior to pure ethanol. Ethanol is good for the extraction of tannins, polyphenols, polyacetylenes, flavonols, terpenoids, sterols, and alkaloids. Ether is selectively used for the extraction of alkaloids, terpenoids, coumarins, and fatty acids. Dichloromethane is especially used for the extraction of terpenoids [7]. Chloroform is a nonpolar solvent that has the ability to extract several groups of bioactive compounds, particularly terpenoids, and it has the lowest threshold limit value (TLV). Water is very polar and can draw out relatively nonpolar compounds, but it will also cause diffusion of the extract. Water is good for the extraction of anthocyanins, starches, tannins, saponins, terpenoids, polypeptides, and lectins [8].

Best Practices for Biomolecule Extraction

i) Plant material should be authenticated before extraction is carried out. Any foreign substance must be entirely removed.

ii) An appropriate plant portion should be selected for quality assurance purposes; the plant's age as well as the time, season, and location of collection should be noted.

iii) The plant material's drying conditions are considerably determined by the content of its chemical components. For drying, hot or cold airflow or dryers are recommended. If a material with high moisture content is to be utilized for extraction, appropriate weight corrections should be made and reported based on a dry or wet weight basis.

iv) Specific grinding procedures should be used, wherever possible.

v) To obtain the desired particles of uniform size, powdered plant material should be run through appropriate sieves.

vi) When dealing with constituents that degrade when kept in organic solvents, such as flavonoids and phenylpropanoids, appropriate precautions should be taken.

vii) It is critical to standardize the extraction time, i.e., whether it should be short or long. Both the length of each extraction and the total number of extractions required to finish the extraction matter.

viii) The stability and safety of the active ingredients should be guaranteed during the concentration and drying processes.

ix) The extractor's design and material of construction must also be taken into account.

x) To track the quality of various extract batches, the analytical parameters of the final extract should be recorded.

The Extraction of Natural Products Involves Following Steps

1) size reduction, 2) extraction which includes a cold aqueous percolation or hot aqueous extraction, 3) filtration, 4) purification, 5) Concentration, 6) drying. Some of the extraction procedure are provided in Figure **1** and the diagrammatic representation of the extraction apparatus are provided in Figure **2**.

Size Reduction

A hammer mill or disc pulverizer with built-in sieves disintegrates plant raw material. Size reduction increases the surface area, which in turn enhances the mass transfer of active principles from plant material to the solvent. The mesh size of 30–40 is considered optimal because smaller particles may become slimy during extraction and pose problems during filtration [9]. The principle of extraction occurs when a solid material from the plant comes in contact with a solvent. This is carried out when the powdered plant material is fed into the percolator along with a suitable solvent. The material is left in contact with the solvent until equilibrium between the active constituents and the solvent is achieved. An extract is taken from the bottom discharge wall of a percolator. The plant material is washed four to five times, pooled, and then

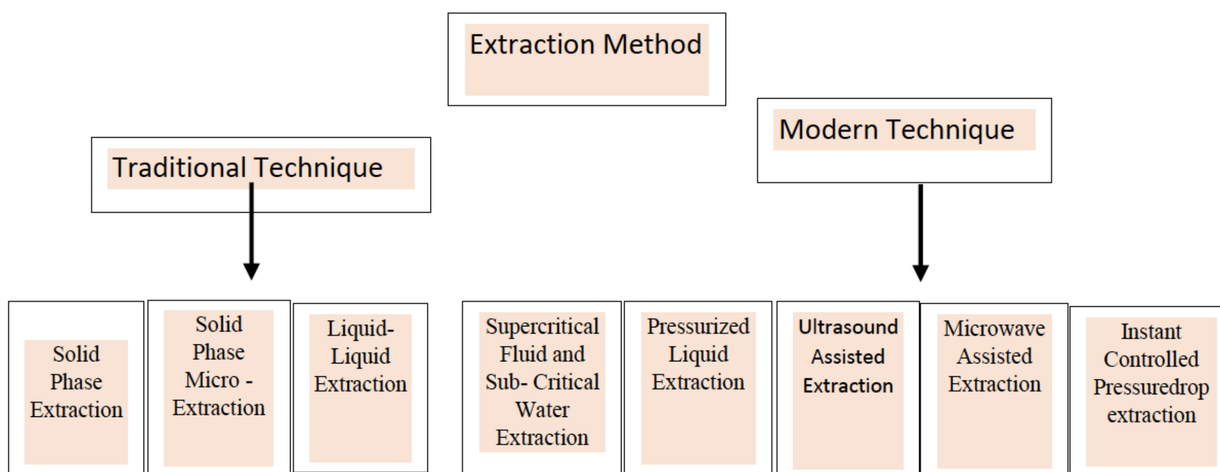

Figure 1: Extraction technique of biomolecules.

concentrated [10]. This is followed by filtration, the extracted plant material that is retained at the flask bottom is decanted into the holding tank and pumped into a filter to remove fine or colloidal particles from the extract. The fourth step is spray drying, which involves spraying the filtered extract with a high-pressure pump at a controlled feed rate and temperature. To get dry powder, the desired particle size of the product is obtained by controlling the inside temperature and pressure of the chamber. Fifth step are concentration and drying procedures [11]. The enriched extract from the percolators or extractors is fed into a fine film evaporator, where it is concentrated under vacuum to produce a thick concentrated extract, which is further fed into a vacuum chamber dryer to produce a solid mass free from solvent. Lyophilization can be performed based on the extract, and although it is expensive, it is increasingly employed [12].

Solvent Extraction

The component of interest needs to be chemically separated from cellulose. This is done several ways, but the simplest choice is solvents. Methanol, ethanol, and solvents like acetone can draw out components based on two very simple differences in the plant matter. Water is very polar and will draw out relatively polar and nonpolar compounds. The more nonpolar or polar the solvent, the more refined and specific the extraction can be.

In this method, it is mostly the crude extract that one can expect to get in response to the polarity of the solvent. This can leave behind everything that is not attracted by the solvent or anything that is not close enough to the polarity of the solvent, which is excellent for extracting target specific compounds. Depending on how much extract one has in the solvent, it may also be saturated; this means that we cannot extract any more of our bioactive compounds, even if there are more in the plant. With crude extract, preliminary assays can be performed that help to narrow down function *in vitro, in vivo* or animal studies tend to be expensive, time-consuming, and often face ethical issues [13].

Maceration

In maceration, the whole or coarsely powdered plant is kept in contact with the solvent in a mixer at room temperature for a defined period with frequent agitation. Crude or fresh infusions are prepared by macerating the solids for a brief period of time with either cold or boiling water, or gentle heat is applied when substances are liable to temperature. The method is best suited for use with thermal herbal drugs [14].

Infusion

This could be either a cold or hot extraction method. In cold extraction, plant material is blended with the solvent in a ratio of 4:1 or 16:1 (e.g., dichloromethane, methanol), and then it is set aside for seven days to let the extraction penetrate between the plant and the solvent. After seven days, the extraction is filtered through filter paper, and the liquid in the plant materials is dried [15]. Cold extraction can achieve a more concentrated and robust plant profile. Hot extraction is typically performed using a Soxhlet apparatus and polar solvents. As the soxhlet apparatus is heated, solvents move from a nonpolar to a polar phase. Hot extraction can be run continuously, and one plant material can be used with many solvents at one time. The hot extraction has a higher range of extracting

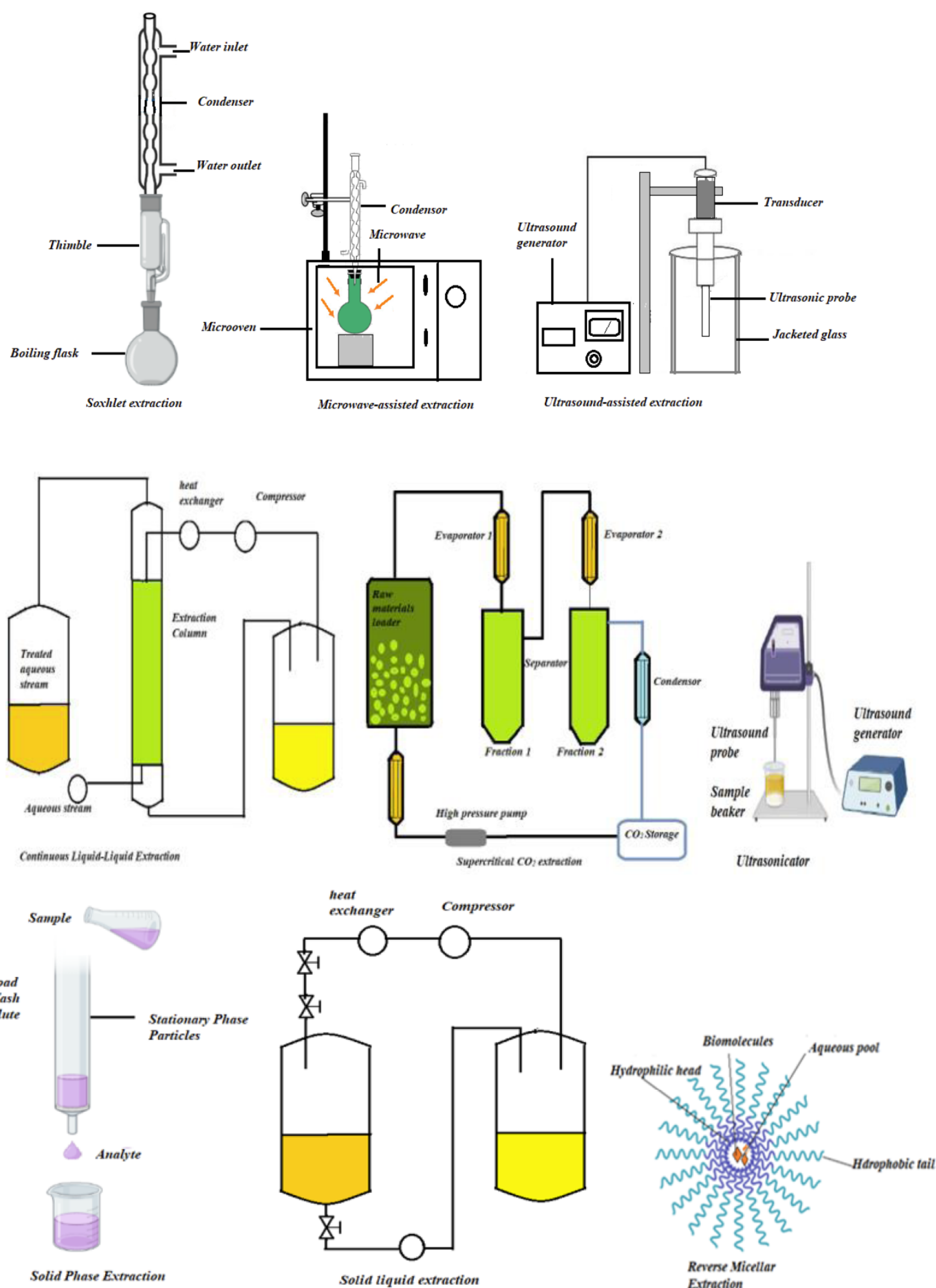

Figure 2: Biomolecules extraction apparatus.

compounds from the plant but can change the chemical structures of the plant [16].

Digestion

In digestion, a moderate amount of heat is applied throughout the extraction process. Powdered plant material is added to the extraction solvent. The mixture is placed over a water bath at a temperature of about 50 °C. The heat used reduces the solvent's viscosity and improves the removal of bioactive components [14].

Decoction

This technique involves continuous hot extraction using a specified volume of water as a solvent. A dried, ground, and powdered plant material is boiled in a

specified volume of water. The content is stirred with heating, cooled, and then strained. The procedure just takes a few minutes, typically around 15 minutes. It is used to extract plant material that is both heat- and water-soluble. Decoction gives more oil-soluble phytochemicals [17].

Percolation

This digestion system is equipped with a Teflon vessel and uses automated temperature and pressure programmes for digestion. It is used for total digestion and extraction of tissues. A percolator is a narrow cone-shaped vessel that is open at both ends and is typically used for solid ingredients. The ingredients are moistened with a specified amount of solvent and allowed to stand for at least 4 hours in a closed container. Percolators come in three types: conical, cylindrical, and stream jacketed percolators. In hot percolation, the high temperature of the solvent increases the solubility of the active moieties, which increases the concentration gradient and therefore improves active principle mass transfer from plant material to the solvent. The temperature of the extract in the percolator is regulated by a steam solenoid wall through a temperature indicator controller [14,17].

Soxhlet Extraction

This extraction technique is used when the desired compound has limited solubility in a solvent and the impurity is insoluble. The finely ground crude compound is packed in a thimble placed in the chamber of the soft slit apparatus. The technique uses a flask and a condenser. The refluxing solvent repeatedly washes the solid, extracting the desired compound into the flask. The condensed extracts drip into the thimble containing the crude extracts. This process is carried out until a drop of solvent is removed from the siphon tube's residue [6].

Microwave Assisted Extraction (MAE)

MAE is a tool used to extract active components by utilizing microwave radiation so that the extraction process can be faster and more efficient. The oven is heated with polar and nonpolar solvents, which causes energy to be lost from the dipole due to molecular friction, collision, and interface sustenance times, resulting in superheating (microwave radiation interacts with the separated positive and negative charge of the dipole). Extractions are performed at elevated pressure and temperature, and because the sample is continuously mixed with the solvent by stirring, extractions are extremely fast, taking just minutes [18]. Now MAE is automated with easy-to-control software that features multi-level users. Factors that affect the extraction include the selection of solvents, solvent volume, time per extraction, extraction temperature, amount of the extraction process, and the particle size of the material. One disadvantage of the method is the increased risk of explosion. Long working times in closed vessels cannot process samples continuously, and the number of samples that can be processed is limited to approximately four hours in a well-sealed container [19]. Due to its many benefits for the extraction process, including its increased extraction rate, reduced solvent consumption, shorter processing times, and improved product output at lower costs, microwave-assisted solvent extraction has become widely used.

Counter-Current Extraction (CCE)

In counter-current extraction, damp raw material is broken down into a fine slurry using a toothed disc disintegrator. The material is fed into a cylindrical extractor and moved in one direction, where it comes into contact with the extraction solvent. The extract becomes more concentrated as it moves further. The technique is quite effective, takes minimal time, and does not involve any risks related to high temperatures. Complete extraction is possible by maximising solvent and flow rates. Unlike the majority of other methods, CCE is carried out at room temperature, protecting the thermolabile ingredients from heat exposure, and less solvent is used. As the extraction is pulverised in moist conditions, water balances the heat produced during comminution [20]. Stilbene-based polyphenolic compounds, alkaloids, terpenoids, and phytocannabinoids are extracted using CCE. A new technology, microdroplet-based CCE extraction, has provided better extraction. The device has a milli-scale column and a cross-flow T-junction microdisperser with a high phase ratio (60–110). The authors discovered that the theoretical stage could reach a column height of up to 25 cm [21]. An online extraction and enrichment–high-speed counter-current chromatography (HSCCC) strategy has been developed to easily and effectively isolate antitumor compounds from solid *C. obtusifolia*. This technology also effectively removed any solvent interference [22].

Supercritical Fluid Extraction (SFE)

An alternate sample preparation technique that uses more sample and fewer organic solvents is supercritical

fluid extraction. The superior characteristic of supercritical carbon dioxide is its fluid nature. The capacity to vary solvent density depends on the pressure and temperature of supercritical carbon dioxide. The critical temperature and pressure for carbon dioxide are, respectively, 31.2^{o}C and 7.38 MPa. Because it is usually regarded as safe, CO_2 is the solvent of choice for SFE [23]. The SFE has an increased capacity for solubilizing non-polar compounds. To reduce solvent losses, supercritical CO_2 flows through the extractor and dissolves plant extracts. After this stage, the CO_2 is recovered and recycled for the process. However, it has a number of polarity constraints. When extracting polar solutes or when there are significant analyte-matrix interactions, solvent polarity is crucial. To overcome the polarity restrictions, organic solvents are added to the carbon dioxide extraction solution [24]. Lately, argon has been used instead of carbon dioxide because it is more inert and less expensive [25]. It has been demonstrated that supercritical fluid chromatography (SFE-chromatography) creates a modifier sphere around the molecules at low modifier percentages in carbon dioxide. As a result, the concentration of modifier is higher around the analyte than it is across the majority of the fluid. A variety of columns, especially those with various polarities, can be utilised in supercritical phase chromatography. SFE-chromatography thus performs both the extraction and purification of the compounds of interest. It allows for the use of different types of columns, especially those with various polarities and good correlation in liquid chromatography [26].

Phytonic Extraction Process

Advanced Phytonics Limited (Manchester, UK) has developed a patented technology termed "phytonics process." Plant materials have been extracted using the new generation of fluorocarbon solvents. 1,1,2,2-tetrafluoroethane, also known as hydrofluorocarbon-134a, serves as the solvent's primary component (HFC-134a). Its remarkable properties in the extraction of plant materials offer significant environmental advantages and safety benefits over conventional processes. The majority of the products produced by this procedure are biological or phyto pharmacological extracts that can be used directly without further physical or chemical processing, as well as the aromatic components of essential and fragrant oils, flavours, and biological extracts [27]. The solvent is not flammable or ozone-depleting, and less threatening to the environment. Vacuum stripping, which is required in other procedures and results in the loss of valuable volatiles, is avoided here. Because there is no oxygen present during the whole process, the products never experience acid hydrolysis damage or oxidation. The method allows for a wide range of working circumstances and, end products. The solvents are completely recycled within the system, which consumes less energy [28].

Liquid-Liquid Extraction (LLE)

This technique requires two layers, an organic layer and an aqueous layer. The organic layer is in the upper phase because it is less dense. There is an exception where we have halogenated organic solvents, like dichloromethane, which has a higher density. The reaction mixture is quenched by adding water. Then the two layers are mixed, first gently and then vigorously, to achieve equilibrium. By doing so, some of the components that were dissolved in an aqueous layer move to the organic layer. This process is known as "partitioning." This is followed by draining the aqueous layer and then the organic layer. The compound of interest is isolated by evaporating the solvent in a rotovapour. Based on how a substance is distributed between the two liquid phases, the extraction efficiency will vary. Its lower viscosity, lower costs, shorter phase separation times, and efficiency in detecting trace substances make it an ideal technique. But the partition behaviour involved is complex and difficult to predict [29].

One of the potentially attractive alternatives is the liquid-liquid fractionation technique known as the aqueous two-phase system (ATPS) or aqueous biphasic systems. It is well known for the extraction, separation, purification, and enrichment of biological molecules. In this system, two water-soluble solutes separate into two immiscible phases based on polymer-polymer, salt-salt, or polymer-salt combinations. Aqueous biphasic systems are key to the extraction of biomolecules and are well known for their enhanced biocompatibility and selectivity in downstream processes. ABS forms two immiscible aqueous phases when water-soluble compounds such as polymers, salts, ionic liquids (ILs), alcohols, and polysaccharides are combined [30].

Protein extraction cannot be done with organic solvents because they lose their biological activity. Proteins are removed by reverse micelles or an aqueous two-phase system. Polymers such as polyethylene glycol (PEG), polyacrylates, dextran, ethylene oxide propylene oxide

co-polymer (EOPO), or a combination of polymers, low molecular weight alcohol, and kosmotropic salts (phosphate, citrate, and sulfate) are used. Ionic or non-ionic surfactants can be used for the formation of micellar and reverse micellar ATPS [31]. Here the partition depends between the phase components, bonds such as electrostatic interactions, steric effects, van der Waals' forces, hydrogen bonds, hydrophobicity, and biospecific affinity. A potent bioseparation process can be achieved by influencing the polymer molecular weights and composition of phase components, temperature, ions, hydrophobicity (neutral salts like NaCl), tie line length (TLL), and pH [32].

Reverse Micellar Extraction (RM)

Reverse micelle extraction is a versatile liquid-liquid extraction for the recovery of by-products. Here the surfactant is dissolved in organic solvents, which form stable nanosized aggregates containing an inner aqueous core where the solute of interest remains. The proteins or enzymes can be extracted from the aqueous medium by forming reverse micelles without loss of biological activity. Surfactants are amphiphilic molecules composed of a hydrophilic head, or polar moiety, and a hydrophobic tail, or nonpolar moiety. The polar head dissolves in water and dissolves the bioactive substances, such as enzymes, proteins, and nucleic acids. This prevents direct contact of active substances with organic solvents outside the micelles [33].

Solid Phase Extraction (SPE)

SPE extraction is used for sample concentration and is considered a cost-effective alternative to liquid-liquid extraction (productivity, solvent, waste), with principles similar to those of high-performance liquid chromatography (HPLC). The chemical constituent is retained in the solid sorbent, and the desired constituent is recovered by elution. The advantages of SPE are better throughput, decreased organic solvent usage and waste generation, higher reproducible recoveries, no formation of emulsions, tuneable selectivity, and automatic workflow. There are several typical applications of SPE, such as cleaning samples, enriching traces, desalting, solvent exchange, and preserving samples. In order to obtain consistent sample processing, SPE materials are available in a variety of polarities. High-throughput operations can be solved affordably with SPE devices, which have the ability to process samples batch-wise [34]. Common sorbents are silica-based, carbon-based, and polymer-based sorbents; magnesium silicate or alumina; Amberlite XAD type, Dowex 50WX8 or Bond Elut C18 cartridges.

Solid Phase Micro Extraction (SPME)

With efficient sample preparation, SPME is used to analyse bioactive compounds that occur at extremely low concentrations. The fibers are coated on the outside for heating the sample, and the volatile sample can be analysed by Gas chromatography. The fibers can also be immersed directly in liquid analytes to be examined by HPLC. The SPME technique can be consistently used in combination with Mass spectrophotometer for versatile detection [35].

Supported Liquid Extraction (SLE)

Inert supports are used in SLE, such as synthetic and diatomaceous earth in the form of cartridges or 96-well microtitre plates. The samples are absorbed onto the inert support and eluted using an organic solvent. The extracted materials are dried for further analysis. After being dried, the resulting extract can be used for additional analysis. This method enables rapid and reliable sample preparation without the formation of emulsions. It is ideal for biological, food, and environmental samples [36].

Pulsed Electric Field (PEF) Extraction

The PEF device consists of electrodes, a treatment chamber, and an electrical pulse generator; the electrical pulse is passed between or through the electrodes. The product between two electrodes is subjected to brief electrical pulses from a few nanoseconds to milliseconds at high voltage (between 100 and 300 V/cm and 80 kV/cm). Due to the high-intensity electric field pulse discharges caused by PEF treatment, cell membrane characteristics can change, increasing cell membrane permeability and accelerating cytoplasmic disintegration [37].

Ultrasonic Extraction (USE)

The technique utilizes ultrasonic waves, which are found between audible waves and microwaves, with frequencies between 20 kHz and 10 MHz. When the plant extract is exposed to ultrasound, microbubbles form in the liquid phase and enlarge; this is known as acoustic cavitation. Mechanical stirring agitates the batch volume and moves the material into the cavitation zone. This accelerates the mass transfer of

the extract, increasing the permeability of cell walls and producing cavitation [38]. Large-scale applications are limited due to the higher cost and undesirable changes in active constituents, particularly at energies higher than 20 kHz. Additionally, the bioactive compounds produced by UAE from plants exhibit superior quality, a higher yield, and less degradation.

Enzymatic-Assisted Extraction

Enzymatic-assisted extraction utilizes water as a solvent rather than organic chemicals. In this technique, the cytoplasm bound bioactive are made accessible to the solvent, thus increasing the yield of the extract. Enzymatic extraction uses a variety of enzymes, including cellulose, pectinase, and amylase, to break down plant cell walls and polysaccharides. In enzyme-assisted cold pressing, the enzyme used hydrolyzes the cell wall without forming a colloidal polysaccharide-protein complex. Enzyme-assisted aqueous extraction entails the development of a colloidal polysaccharide-protein complex [39].

Membrane Filter Processes

In filtration, membranes with specific pore sizes are used to separate molecules that are thermolabile according to differences in size. However, the clogging of filters is a major limitation. There are two types of membrane filtration: static filtration and cross-flow filtration. In cross-flow filtration, the culture broth is injected transversally across the membrane, the membrane, compared to static filtration, this reduces clogging. Polyphenolic adherence is dependent on membrane polarity. Periodic back-flushing for a short time increases the functional life of filters [40]. Microfiltration, ultrafiltration, nanofiltration, and reverse osmosis are four major types of filtration.

In microfiltration, low-molecular-mass solutes and/or solvent particles with diameters in the range of 0.1–10 μm are separated. The osmotic pressure of the sample is negligible, and low hydrostatic pressures are used. Symmetrical, microporous membranes are used for microfiltration. When separating low-molecular-mass (2000 Da) solutes, osmotic pressure is preferred. The membranes used in reverse osmosis generally have asymmetrical structures, and the chemical nature of the membrane has a major role to play in reverse osmosis [41]. Under high hydraulic pressure (2-10 atmospheres), a very fine pore membrane with pore sizes ranging from 0.001 to 0.1 m is forced through a solvent containing low molecular weight solutes in order to retain high molecular weight solutes. The separation of macromolecules is made feasible by a range of membranes with varying molecular weight cutoffs (500–500,000). An asymmetric membrane consists of a membrane that is supported by a mesh that is roughly 0.3 μm thick because the flux through such a membrane is inversely proportional to its thickness. On the membrane surface, a protein slurry may build up and produce a gel layer that is difficult to remove by agitation. Careful selection of variables, such as pH, may help to partially regulate the formation of the gel layer [42].

Drying

Drying is a crucial step in the creation of products. In essence, it entails applying heat to a wet product to remove moisture. Since the majority of the biological by-products of fermentation are heat-sensitive, moderate drying techniques are needed. Drying equipment can be characterized as contact, convection, or radiation dryers, depending on how heat is transferred.

For drying huge quantities of liquids, spray drying is employed. Small liquid droplets comprising the product are directed over a stream of hot gas by a nozzle during spray drying. The solid particles are left behind as the water evaporates. Dryers specifically used for medical, pharmaceutical, or food processing applications [43].

Freeze-Drying/ Lyophilization

Here, the product is dried after it has been frozen under a vacuum, causing the ice to melt directly from solid to vapor without going through a liquid phase. Freeze-drying, or lyophilization is the most commonly suggested method for manufacturing and drying a variety of medications, foods, and microorganisms. Freeze-drying does not cause the targeted product to lose any biological activity [44].

Purification Techniques

Chromatography is one of the easiest methods for dividing a substance into more focused groups. It is a method of separating the components based on the extracts' size or polarity.

Adsorption Chromatography

It involves adsorption of solute on stationary phase. Separation of the sample mixture occurs due to

differences in the adsorption affinities of the sample mixture components. The mixture of components having greater adsorption towards the stationary phase will be adsorbed in the upper part of the column. The component that has less adsorption towards the stationary phase will be adsorbed in the lower part of the column. The common adsorbents used are alumina (Al_2O_3) and silica gel (SiO_2). The silica gel has silanol groups (Si–O–H) at the surface and extended out from the surface in the internal channels of the pore structure, which interact with polar groups [45].

Gel-Filtration Chromatography

In this technique, molecular separation is based on size, shape, and molecular weight. Beads with different-sized pores act as molecular sieves, separating small and large ones. Smaller molecules penetrate through the pores of the gel beads. Larger molecules cannot pass through the pores, so they are ejected first with the mobile liquid.

Ion-Exchange Chromatography

It entails dividing molecules according to how charged their surfaces are. Cation-exchangers, which contain negatively charged groups like carboxymethyl and sulfonate, and anion-exchangers, which have positively charged groups like diethylaminoethyl, are the two types of ion-exchangers (DEAE). Dowex HCR and Amberlite IR are the two most widely used cation exchangers, whereas Dowex SAR and Amberlite IRA are the two most widely used anion exchangers. The pH of the medium is highly important in ion-exchange chromatography because the net charge changes with pH [46]. In other words, both the effective charge on the target molecule and the ion-exchanger depend on the pH. Increasing the concentration of a salt solution or altering the pH of the elutant are two ways to elute the ionic-bound molecules from the matrix.

Affinity Chromatography

A protein and an immobilized ligand interact to form the basis of affinity chromatography. A particular antibody, substrate, substrate analog, or inhibitor can serve as the ligand. Utilizing the immobilized ligand on a solid matrix to fish out complementary structures is useful. By lessening their interaction, the protein that is bound to the ligand can be released. This can be accomplished by adjusting the buffer's pH, and ionic strength or by using a different free ligand molecule. In the following steps, the newly employed ligand needs to be eliminated [46].

Infrared Spectroscopy (IR)

In IR, we can know the functional group of the molecule. The IR spectrum knocks out the electron in the atom, creating molecular vibrations through irradiation with infrared light. The stretching and bending of molecules give lead to the functional group.

High Performance Liquid Chromatography (HPLC)

HPLC is considered for the purification of peptides, proteins, phenolics, and other small to medium-sized organic molecules. HPLC is used in analytical chemistry to identify and determine the concentration of a sample. The column packing is done with significantly smaller particles, giving the stationary phase and molecules much more surface area to interact [47]. In preparative HPLC, the columns are large and are preferably used to collect sample peaks in pure form. There are many types of HPLC columns developed for specific applications, such as normal-phase HPLC (NP-HPLC) and reversed-phase HPLC (RP-HPLC). In RP-HPLC reverse phase, the stationary phase is non-polar and the mobile phase is polar. Analytical chromatography is a small-scale chromatographic method used to analyze the qualitative and quantitative contents of a mixture, while preparative chromatography is used to purify a specific component from a mixture of molecules.

Gas Chromatography (GC)

Gas chromatography is the technique used for the separation of volatile compounds The sample that is to be separated is mixed with appropriate volatile solvents such as heptane, acetone, or methanol Just before the column, there is a septum that allows injection of the sample; the temperature of the injection region is kept 20 to 50 ^{o}C higher than that of the column, which allows rapid volatilization of the sample. Once the sample is volatilized the sample moves down the column where the separation occur where the separation occurs. During analysis the temperature of the column is kept between 150 to 300 ^{o}C. The interaction of molecules between the mobile phase and the stationary phase causes separation [48].

Mass Spectrometry (MS)

Molecular fragments are detected after bombarding the sample with electrons. The mass and connectivity of atoms within a molecule are provided by this function. Here, the organic molecule is hit with electrons from

lasers that allow each of the atomic elements within it to be converted into a charged ion. They are then thrown off at different rates. The software plots this as a relative abundance of the ion against the charge ratio of the ion. The machinery provides a high level of accuracy [49]. Mass spectrometry measures how ions accelerate in an electric field and deflect in a curve in a second electric field. An electric field further deflects them after they bend through a magnetic field, the ions then enter a detector. Electric and magnetic fields can be varied to allow ions of different masses into the detector.

Nuclear Magnetic Resonance (NMR)

The NMR spectroscopy technique is based on the interaction of externally applied radiofrequency radiation, which causes the atomic nuclei to be excited, resulting in nuclear spin. This provides extensive information about molecular structure and atom connectivity and uses the magnetic properties of certain atomic nuclei, such as ^{l}H and ^{13}C. In NMR, the frequency of the excited nucleus spins can be measured from the electric current. The NMR spectrum contains a series of peaks of different intensities known as the chemical shift and provides the structure, dynamics, and molecular interactions of biomolecules [50]. The molecular standard for both ^{1}H and ^{13}C NMR is tetramethylsilane (TMS). Bruno *et al*. [51] have used NMR spectroscopy for the first time to build a molecular fingerprint of the two species, *Posidonia oceanica* and *Ascophyllumn odosum*, for identification and comparison. By using solid-state nuclear magnetic resonance (ssNMR), samples without a crystalline lattice that exhibit both static and dynamic disorder can be studied. ssNMR applications include protein misfolding and aggregation disorders, lipid bilayer membranes, membrane proteins in a lipid bilayer environment, protein-protein interfaces, substrate-enzyme interactions, and crucial structural information on oligomers and amyloid fibril aggregates. ssNMR has grown to encompass a wide variety of biological assemblies, including viral capsids, protein-protein interactions, non-amyloid protein aggregates, revealing three-dimensional structures, and molecular biology [52].

X-Ray Crystallography

The final purification of a wide variety of chemicals uses the well-established technique of X-ray crystallography. Crystallization is a two-stage process, forming nuclei in a supersaturated solution and crystal growth, which proceed simultaneously and can be controlled independently. Supersaturation is achieved by cooling or by the removal of solvent. Proteins form crystals when they are purified and concentrated. Within the crystal, many copies of the protein are arranged in symmetrical arrays [53]. Each atom can subsequently be distinguished because the strength of the scattering is related to the amount of electrons in the atom. Crystallization of proteins is tricky because of their irregular shape. It may take several days to produce a large enough crystal.

CONCLUSION

The use of diverse bioactive chemicals has increased recently, which has encouraged the adoption of an appropriate extraction method in order to deliver the required health advantages. Innovative greener technology would be safer because of safety, scalability, consumer acceptance, feasibility. and has considerable potential in the functional food industry.

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