

Review

Advanced Analytical Approaches for the Analysis of Polyphenols in Plants Matrices—A Review

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Abstract: Phenolic compounds are plants’ bioactive metabolites that have been studied for their ability to confer extensive benefits to human health. As currently there is an increased interest in natural compounds identification and characterization, new analytical methods based on advanced technologies have been developed. This paper summarizes current advances in the state of the art for polyphenols identification and quantification. Analytical techniques ranging from high-pressure liquid chromatography to hyphenated spectrometric methods are discussed. The topic of high-resolution mass spectrometry, from targeted quantification to untargeted comprehensive chemical profiling, is particularly addressed. Structure elucidation is one of the important steps for natural products research. Mass spectral data handling approaches, including acquisition mode selection, accurate mass measurements, elemental composition, mass spectral library search algorithms and structure confirmation through mass fragmentation pathways, are discussed.

Keywords: extraction; high-resolution mass spectrometry; fragmentation pathway; non-targeted analysis; chemical profiling



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1. Introduction

Plants have been used for centuries as remedies in several forms: unprocessed, as complex mixtures of different species as in Traditional Chinese Medicine [1], or, more recently, in commercial products as phyto-pharmaceuticals and dietary supplements [2–4]. The bioactivity of those natural products is generally high [4–6], supporting the high use of those products as a primary form of healthcare for a large part of the population [7]. Furthermore, about 80% of all synthetic drugs are directly or indirectly derived from them [8,9]. The pharmaceutical industry is currently showing an increasing interest in the development of new formulations with integrated vegetal extracts as a source of bioactive compounds [9]. Therefore, the assessment of the chemical components of herbs, spices or functional foods has become an essential part of our understanding.

Polyphenols are secondary plant metabolites, and comprise a wide range of compounds that strongly differ in their structure, physicochemical and nutritional properties. Dietary polyphenols are one of the most important groups of natural antioxidants and chemopreventive agents in human diets, playing a vital role in supporting the functioning of biological systems [6,10]. Epidemiological, clinical and nutritional studies strongly support the suggestion that polyphenolic compounds enhance human health by lowering risk and preventing several diseases and disorders [5,10]. It has been reported that polyphenolic

compounds exhibit anticarcinogenic, anti-inflammatory, antimicrobial, antiviral, antidiabetic and hepatoprotective activities, as well as estrogen-like activities [11–13].

In the last few decades, different studies have intensely investigated the antioxidative, antimicrobial, antiproliferative or enzyme-inhibition effects of polyphenols [6,12,14]. Studies using in vitro and in vivo approaches together with LC-MS analytical techniques have led to a better understanding of the bioavailability and bioactivity of polyphenols. However, the theory that the beneficial effects of polyphenols are direct consequences of antioxidant activity in vivo is obsolete. Their protective activity was firstly attributed to their antioxidant, free radical-scavenging, and metal chelator properties, and then to their ability to inhibit different enzymes [12]. New research supports the hypothesis according to which polyphenols' interaction with signal transduction pathways and cell receptors induce adaptive responses that drive antioxidant, antiplatelet, vasodilatory or anti-inflammatory effects [6,11,15]. Thus, the current research places more emphasis on the individual identification/quantification of each compound rather than on the in vitro assay of bioactive properties [16].

The occurrence of polyphenols in herbs or food and their chemistry have been intensively debated, and as such, they are not the subject of this study. This review aims to provide updated information on the advances in the analytical approaches currently used in sample preparation and subsequent analyses for the determination of the polyphenolic profiles in plant samples. Recent literature (2015–2020) was reviewed for a comparative overview of the advanced analytical techniques.

2. Extraction

The extraction of bioactive compounds from plant material is a key step in the development of various analytical methods. The extraction methods should be simple, safe, reproducible, less expensive, and also suitable for industrial applications in phyto-chemistry.

2.1. Conventional Methods

Conventional methods provided by European Pharmacopoeia, [17] such as infusion, decoction, percolation or maceration, as well as extraction under reflux and Soxhlet extraction, are still currently used. Methanol, ethanol, acetonitrile, acetone, or their mixtures with water are the most used solvents.

2.2. Advanced Methods

The current tendency is to develop and apply new fast, efficient and selective techniques, which are able to meet the special extraction requirements of bioactive compounds and are environmentally clean.

2.2.1. Ultrasound-Assisted Extraction (UAE)

Among the new extraction methods, ultrasound-assisted extraction (UAE) is a modern technique that offers a high yield of active compounds with simple manipulation, energy efficiency and high reproducibility [18]. Commonly used for solid/liquid systems, UAE lead to a disruption of the cellular walls of the plant material, and enhances mass transfer across cell membranes, thus increasing the solvent access to the analytes [18,19]. Several factors, such as solvent composition, solvent-to-sample ratio, ultrasound amplitude and cycle, solvent pH, and temperature, can impact the extraction efficiency [19]. The selection of solvents and temperature were shown to be the most important factors influencing the efficiency of UAE [18,19]. It has been observed that, in the case of highly polar phenolic compounds, extraction with pure organic solvents has low efficiency [19,20]. Ethanol/methanol mixtures with water in various proportions (e.g., 80:20, 60:40, 70:30) are widely used as extraction solvents [19], sometimes with the addition of acids for the adjustment of pH, e.g., ethanol 70% and acetic acid 2% at 60 °C was used for flavonoids extraction in *Dendranthema indicum* [21], and 80% aqueous MeOH with 1% formic acid was successfully used for the extraction of flavonoids from culinary herbs and spices [22].

The extraction of polyphenols is also affected by the duration of contact between phases, and the liquid–solid or liquid–liquid ratio. Bajkacz et al. [23] studied the influence of extraction duration (2 and 5 h) on the extraction efficiency from plant material. An increase in polyphenol content with the extraction duration was observed. However, the potential production of free radicals with prolonged sonication (>40 min) in frequencies above 20 kHz was reported as a disadvantage of the method [24]. The possible degradation of some active principles in plant matrices can also occur due to oxidative pyrolysis caused by hydroxyl (OH) radicals during the cavitation phenomenon [25].

2.2.2. Microwave-Assisted Extraction (MAE)

In recent years, due to the tendency to reduce the quantity of organic solvents used in the extractions, microwave-assisted extraction (MAE) has been developed and optimized. With the main benefits of extraction time reduction, low cost and sustainability, this technique was found to be suitable for the extraction of phenolic acids, flavones, flavonols and isoflavones from blackberry (*Rubus Fruticosus* L.) [26], myrtle (*Myrtus communis* L.) leaves [27], blackthorn (*Prunus spinosa* L.) flowers [28], basil (*Oscium basilicum*) [29], Chilean superfruits (*Aristotelia chilensis*) [30] and pistachio green (*Pistacia vera* L.) [31].

Microwaves consist of electric and magnetic fields that oscillate perpendicularly to each other in a high-frequency range from 0.3 to 300 GHz [24,32]. This condition produces localized heating and causes the destruction of the plant matrix leading, to the easier diffusion of the compounds of interest into the solvent [28,32]. A microwave power between 300 and 900 W and extraction temperatures ranging from 50 to 100 °C are generally used [19].

The main challenge of this extraction technique is to obtain the maximum extraction yield via the destruction of the cellular tissue without affecting the chemical structure of the natural compounds. A special piece of equipment was developed in 2017 by a Romanian research team using a slot end coaxial antenna with a microwave applicator, provided with a cooling system [33]. Consequently, a high specific absorption rate (SAR) was achieved at low temperatures. Polyphenols were extracted from sea buckthorn leaves, showing higher polyphenols contents and higher antioxidant capacities than extracts obtained via conventional methods performed with the same temperature profile [33].

2.2.3. Accelerated Solvent Extraction (ASE)

As an alternative to the extraction and fractionation of nutraceutical compounds from different natural products, accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE), allows faster extraction and, by adjusting the process parameters, enhances the extraction selectivity for particular groups of compounds [19]. Due to the high pressure, solvents remain in the liquid state even at high temperatures, allowing high-temperature extraction. These conditions enhance the solubility of target compounds in the solvent and the desorption kinetics of plant matrices. Furthermore, as it is performed in a closed system, the occurrence of oxidation reactions during ASE is limited [19]. Thus, the method has been successfully used to extract thermally sensible phytochemicals from such plants as purple sweet potatoes [34], passion fruit rinds [35], citrus [36], and olive leaves [37].

Regarding the extraction conditions, studies have shown that ASE is more efficient when mixtures of solvents are used, such as methanol or ethanol in water, instead of pure solvents [19], for the reasons of polarity compatibility. Working pressures ranging from 4 to 20 MPa affect the diffusion of the solvent into the pores of the raw material matrix, enhancing the contact of the target compounds with the solvent [32,38]. Temperature is also an important parameter of ASE extraction. Various works have shown that from 40 to 120 °C, there is an increase in the phenolics extraction efficiency [32].

ASE was evaluated by Garcia-Mendoza et al. [38] at different working pressures using several solvents, including ethanol, water, an acidified mixture of ethanol + water and acidified water, for the extraction of polyphenols and anthocyanin from juçara (*Euterpe*

edulis Mart.). ASE was compared to different low-pressure extraction methods (Soxhlet and UAE) and to supercritical fluids extraction (SFE). The antioxidant activity determined through DPPH was significantly higher in the Soxhlet extracts, suggesting differences in the chemical composition of the extracts, probably due to the long interactions between raw material and solvent. The total polyphenols content obtained in SFE was lower than in ASE and low-pressure extractions, while a significant improvement in the extraction of anthocyanins by SFE compared with ASE was observed [38].

Study results showed that high-pressure methods are, in general, more effective and selective for phenolic compound extraction than low-pressure techniques [37,38]. However, lower recovery rates of thermosensitive polyphenols at high temperatures and incomplete extraction due to limited solvent volume were reported as disadvantages [32,39].

2.2.4. Supercritical Fluids Extraction (SFE)

Besides ASE, supercritical fluids extraction (SFE) is a green extraction technology based on the properties of fluids in a supercritical state (their thermodynamic parameters, pressure and temperature being critical values) to extract bioactive components from vegetal materials [32,40]. Supercritical carbon dioxide (SC-CO₂) is currently considered as an ideal solvent for selectively extracting soluble compounds from vegetable materials [40,41]. For both the food and the pharmaceutical industry, the main advantages of extracts obtained by SFE are the absence of residual organic solvent and the controlled selectivity of the extract's composition, SFE being recognized as safe by the European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA) of the United States of America [32,42].

However, this approach has some limitation. SC-CO₂ is a non-polar solvent that has affinity to non-polar or low-polar compounds. On the other hand, polyphenols have a low degree of solubility in SC-CO₂, which leads to low extraction yields [40]. To overcome this limitation, the addition of chemical modifiers or cosolvents, such as water, methanol, ethanol, acetone, acetonitrile or an acidified ethanol + water mixture, to change the non-polar nature of supercritical CO₂, was tested [42].

2.2.5. Enzyme-Assisted Extraction

Enzymatic hydrolysis is an effective and nontoxic extraction procedure, widely used in various food processes. The enzymatic activity of cellulases, pectinases and hemicellulases leads to cell wall disruption and enhances the extraction of valuable compounds from plants [43]. Furthermore, due to the enzymatic activity of lyases and hydrolases on the glycosidic fractions of natural polyphenols, their biological properties are improved in terms of bioactivity and bioavailability [44]. A mixture of hesperidinase and β -galactosidase was used in a very recent study for the extraction of flavonoids from *Matricaria chamomilla* [44]. Flavonoids, as well as non-flavonoid polyphenols, were significantly structurally modified by the enzymatic treatment, which resulted in the increased bioactivity of the metabolites as inhibitors of pancreatic lipase activity [44].

Mixtures of enzymes, including pectinases, endo- and exo-glucanases, β -glucosidases, β -galactosidase and cellobiases, are used to obtain an overall synergistic effect [45,46]. Enzymatic hydrolysis using cellulose and pectinase was employed for achieving the release of polymeric polyphenols, which are theoretically "non-extractable", from the plant matrix [46].

Extraction parameters such as temperature and pH influence the catalytic activity and the rheological properties of the raw material, as well as the solubilization of bioactive compounds. Although temperature increase enhances the mass transfer rates and solubility of the extracted compounds, it may lead to enzymatic denaturation; therefore, temperatures below 60 °C are usually used [47]. Environment pH values ranging between 4.0 and 6.5 are used for the optimal activity of enzymatic system [43,45]. After incubation for 30 to 90 min, the vegetal mass is subjected to extraction and centrifuged [43].

2.2.6. Extraction with Ionic Liquids (ILs)

Recent studies have focused on the extraction of bioactive components from herbal medicines or other vegetal sources using ionic liquids (ILs) and deep eutectic solvents (DESs) as alternative solvents, showing their potential to substitute organic solvents [48]. ILs are a group of organic salts that are presented in a liquid form below 100 °C and consist of an organic cation (e.g., imidazolium, pyrrolidinium, pyridinium tetraalkyl ammonium, tetraalkyl phosphonium) and an inorganic or organic anion (e.g., tetrafluoroborate, hexafluorophosphate and bromide).

The polarity, hydrophobicity, viscosity and miscibility of the solvent can be selected by choosing the cationic or anionic constituent. The wide variety of possible combinations of cations and anions implies a wide variety of physico-chemical properties that depend on both the nature and the size of the cation, but especially the anion. Depending on their nature, the resulting ionic liquids may have a hydrophobic or a hydrophilic character, higher or lower viscosity and miscibility with water or other different organic phases, as well as specific electrochemical properties [49].

Due to these properties, the use of ionic liquids has been considered a success in stabilizing, pre-concentrating or extracting bioactive analytes. Imidazolium-based ionic liquids have been successfully applied for the extraction of isoflavones by UAE from soy (daidzein, genistein, and glycosides) [50] and from *Iris tectorum Maxim* (tectoridin, iristectorin B, and iristectorin A) [51]. Choline-chloride based DESs have been used for the extraction of polyphenols from *Ficus carica* L. leaves [52].

2.3. Modeling the Extraction Process by Response Surface Methodology (SRM)

Extraction optimization can be a time-consuming process, as many combinations of different solvents mixtures, with different pH values and temperatures, can be used. In order to save time, solvents and resources, empirical models were created and applied to test the functional relationship between a response of interest and a number of associated inputs [18,53–55].

Response surface methodology (RSM) is based on mathematical and statistical techniques for the modeling and optimization of complex chemical and physical processes [55,56]. In a recent study, for the optimization of the extraction of bioactive components from *Medicago sativa* L., Fumic et al. [54] used the Box–Behnken design (BBD), a spherical three-level–three-factor design, employed to determine the best combination of independent extraction variables (solvents' concentration in water, temperature and pH) for the selected dependent variables (extraction yield, radical scavenging activity, the content of phenolic compounds). The conclusions of the study showed ethanol concentrations as the key variable for the achievement of high total phenol and flavonoid contents, while temperature was the most important variable for the extraction of phenolic acids and the antioxidant activity of the extracts [54].

Improvements in UAE yield and the content of phenolic compounds in apple pomace extracts were obtained by Skrypnik and Novikova [55] using nonionic emulsifiers (Tween 20, Tween 80), compared to 70% ethanol/water. pH value, extraction time, and emulsifiers concentration and volume were subjected to RSM.

Considering the complexity of the influence of the extraction conditions, a very careful analysis of the extraction settings is required according to the desired results, which demonstrates the usefulness of these modeling techniques.

3. Extract Hydrolysis and Purification

The hydrolysis can be performed before, during or after extraction, using different procedures—acidic, alkaline or enzymatic hydrolysis—in order to release bound polyphenols and increase the extraction yield of their aglycone form [46,56,57]. While acidic hydrolysis breaks glycosidic bonds, alkaline hydrolysis breaks ester bonds and removes acetyl- or malonyl- groups from glucosides, allowing only β -glucosides and native aglycone forms to remain in the extract [56]. Enzymatic hydrolysis produces aglycone forms, similar to acid

hydrolysis [56]. The official AOAC method 2001.10 for isoflavones in soy and in various foods containing soy uses alkaline hydrolysis [57]. For enzymatic hydrolysis, β -glucosidase, β -galactosidase, β -glucuronidase or mixtures of these are usually used [45].

The purification, fractionation and concentration of the extract are of great importance, both for the analysis of polyphenols and for the subsequent use of the extracts in various fields, including pharmaceuticals.

The extract, hydrolyzed or not, may be subjected to clean-up techniques allowing the accurate identification and quantification of the target analytes. The extraction in the solid phase (SPE) is the most-employed technique in clean-up procedures [58]. In SPE, the target compounds are retained in a specific sorbent and then eluted with an adequate solvent, such as methanol, ethanol, and ethyl acetate. The SPE process allows for the purification and concentration of polyphenols at the same time. SPE columns type C18 [23], HLB [58] or Oasis MCX [59] were used as stationary phases. An alternative to traditional SPE is the matrix solid-phase dispersion extraction (MSPD) method, with the advantage of less solvent consumption [60]. Dispersive solid-phase extraction (d-SPE), another alternative to SPE, was recently evaluated for the determination of phenolic compounds in *Myrciaria cauliflora* peel [61], proving its efficiency in removing the interfering compounds without significant retention of polyphenols.

QuEChERS (quick, easy, cheap, effective, rugged, and safe) is an alternative method of polyphenol extraction and purification that reduces solvent amount and procedure duration. Initially developed for the determination of pesticide residues in food matrices, due to its versatility, QuEChERS has been progressively applied for the extraction of other compounds in different matrices, resulting in the good recovery of the target analytes and lower interference [62]. The technique involves liquid–liquid partitioning with organic solvents and purification of the extract using solid-phase dispersive extraction (d-SPE) with sorbents and buffers [62,63].

QuEChERS assisted by ultrasound extraction was recently optimized for the isolation of polyphenols from several fruit and vegetable samples [62]. Acetonitrile, methanol, ethanol, and a combination of them were tested, methanol being selected in the subsequent experiments. An ultra-sonification time of 5 min was also selected as optimum. A mixture of buffered salts, including disodium hydrogen citrate sesquihydrate, trisodium citrate dihydrate sodium chloride, and $MgSO_4$, was used [62].

However, due to the development of advanced, sensitive spectrometric methods, sample preparation has become simplified, often consisting of the filtration and convenient dilution of the extract.

4. Analytical Detection Techniques

Due to polyphenols' structural diversity and low concentrations, and the plant matrix complexity, their analysis remains challenging. Currently, there is a requirement for sensitive and accurate methods for the analysis of polyphenols, as knowledge of their identity and dosage are prerequisites in evaluating health benefits [64]. Novel techniques have been employed in the past few decades, ranging from high-pressure liquid chromatography (HPLC) to mass spectrometry (MS) and spectroscopic methods. Recent (2015–2020) developments, and the application of analytical methods in qualitative and quantitative studies of polyphenols following extraction, were reviewed in the present work. High-resolution mass spectrometry is particularly addressed due to its applicability in the targeted/untargeted metabolomic analysis of polyphenols.

4.1. Liquid Chromatography with Ultraviolet/Visible (UV/Vis)-Based Detection: HPLC Fingerprint with Chemometric Analysis

High-performance liquid chromatography (HPLC) is still one of the most widely used analytical tools for the identification and quantification of polyphenols [65]. The quality consistency of herbal medicines reflects variations in their chemical composition from batch to batch, depending on several factors, such as botanical species, chemotypes, morphological parts of the plant, geographical area, time of harvest, and storage conditions. With the

continuous development of HPLC technology, chromatographic fingerprint analysis has been recognized as an innovative, rapid, and comprehensive method for the identification and qualification of herbal medicines [66]. The fingerprint profiles show variations in a given herb in an integrated manner, and can identify a particular herb, distinguishing it from closely related species [66,67]. The chromatographic fingerprint of the herbal profile can be defined as the characteristic signal of selected plant that allows unambiguous identification via the evaluation of the chemical similarities and differences in the obtained chromatograms of studied samples [66]. Fingerprint analysis has been internationally accepted as a method for the evaluation and quality control of herbal medicines and preparations, and is currently applied in combination with other chemometric modeling methods, namely, similarity analysis (SA), hierarchical clustering analysis (HCA), principal component analysis (PCA), and partial least square regression [68–70].

For a reliable investigation of a matrix as complex as is found vegetal extracts, the fingerprint method should display good precision, repeatability, and stability, evaluated based on the relative standard deviations (RSD) of the relative retention times ($RRT < 3\%$), and the relative peak areas ($RPA < 3\%$) of the characteristic peaks compared with the reference peak. Similarity values above 0.98 are accepted [69].

The chromatographic fingerprint method can also distinguish authentic materials from substitutes and adulterants, suggesting new applications for food products and pharmaceuticals. Recently, several methods were developed for the fingerprint analysis of different species, such as Flos Carthami (*Carthamus tinctorius* L.), [71], Aurantii Fructus (*Citrus aurantium* L.) [66], chamomile (*Matricaria chamomilla* L.) [72], licorice (*Glycyrrhiza glabra* L.) [67], and selected lavender species (*Lavandulae* spp.) [73] (Table 1). An HPLC-UV polyphenolic fingerprint method was applied on pure cranberry extracts and cranberry-based extracts adulterated with grape at different percentages [2].

For assessing the chromatographic condition, the C18 or C8 reversed-phase LC (packed with particles of silica bonded with alkyl chains) columns are preferred for the separation. C12 columns have also been investigated in herbal drug standardization [74].

Table 1. Some examples of the recent applications of HPLC fingerprinting methods in natural polyphenols analysis.

Plant Material	Extraction	HPLC Condition	Characteristic Fingerprint Peaks	Chemometric Analysis Approach	Ref.
Cranberry (<i>Vaccinium macrocarpon</i>)-based products (fresh and dried fruits, juice)	Lyophilized samples were extracted with an acetone:water:hydrochloric acid (70:29.9:0.1 v/v/v) solution by UAE for 10 min	Kinetex C18 (100 4.6 mm i.d., 2.6 µm particle size) column; mobile phase: 0.1% formic acid in water (v/v) and MeOH; flow rate of 1 mL/min; monitoring wavelength range: 190–550 nm	gallic acid, homogentisic acid, protocatechuic acid, protocatechualdehyde, (+) catechin hydrate, gentilic acid, <i>p</i> -salicylic acid, chlorogenic acid, vanillic acid, (–) epicatechin, syringic acid, syringaldehyde, ethyl gallate, <i>p</i> -coumaric acid, ferulic acid, resveratrol and quercitrin	Partial least square regression and PCA	[2]
27 <i>Salvia</i> L. Species, leaf and root	Maceration in MeOH (2 × 10 mL for 24 h) followed by solvents removal on rotary evaporator under vacuum at 40 °C to dryness	RP C18 Eurospher-100 column, (5 µm particle, 125 mm × 4 mm); mobile phase: 0.2% (v/v) glacial acetic acid in water and ACN; flow rate of 1 mL/min; monitoring wavelength: 280 nm	rosmarinic acid, carnosic acid, caffeic acid, salvianolic acids A and B	PCA	[65]
<i>Aurantii Fructus</i> , dried mature and imature fruits of <i>Citrus aurantium</i> L. (medicinal herbs in TCM);	UAE (200 W) with MEOH for 45 min	Symmetry C18 column (250 × 4.6 mm, 5 µm); mobile phase: ACN and 0.1% aqueous phosphoric acid; flow rate of 1 mL/min; monitoring wavelength range: 285–324 nm	eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, meranzin, poncirin, naringenin, nobiletin, tangeretin and auraptene	Quantitative analysis of multiple components by single marker (QAMS); similarity analysis; standard method difference; HCA	[66]
Licorice root (<i>Liquiritiae radix</i>) - <i>Glycyrrhiza glabra</i> L.	UAE with 80% MeOH-water, 120 W, 40 KHZ, 20 min	Cosmosil column (5C18-MS-II, 5 µm, 4.6 × 250 mm), at 35 °C; mobile phase: 5 mmol/L sodium heptane sulfonate solution phosphoric acid (499:1, v/v) and ACN-MeOH (9:1, v/v); flow rate of 1 mL/min; monitoring wavelengths: 203 nm, 220 nm, 250 nm, 280 nm and 344 nm	glycyrrhizic acid, liquiritigenin, isoliquiritigenin, isoliquiritin, liquiritin apioside, isoliquiritin apioside and glabridin	Geometric linear quantified fingerprint method (GLQFM) and PCA	[67]
Flos Carthami, the dried flower of <i>Carthamus tinctorius</i> L.; (medicinal herbs in TCM)	UAE with 50% MeOH, 270 W, 30 min	C18 column (4.6 mm × 250 mm, 5 µm); mobile phase: 0.1% formic acid in water and ACN	guanosine, hydroxysafflor yellow A, anhydrosafflor yellow B, kaempferol 3-O-β-rutinoside, rutin, quercetin, kaempferol	Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A)	[71]
Chamomile— <i>Matricaria chamomilla</i> L., commercial samples	UAE with MeOH:water mixture (80:20, v/v) at 35 °C for 30 min, three times repeated	Hypersil Gold C18 column (250 × 4.6 mm; 5 µm) at 35 °C; mobile phase: 0.05% TFA in ACN and 0.05% TFA in water; flow rate of 1 mL/min; monitoring wavelength: 254 nm	gallic acid, caffeic acid, syringic acid, <i>p</i> -coumaric acid, ferulic acid, rutin, myricetin, quercetin and kaempferol	ANOVA, PCA, hierarchical cluster analysis (HCA)	[72]
Seven species of <i>Lavandulae flos</i>	UAE with xylen (1:30) under reflux for 4 h followed by solvents removal on a rotary evaporator under vacuum to dryness	Kinetex RP18 column (5 µm, 150 × 4.6 mm); mobile phase: MeOH:water–0.1% formic acid (gradient 5–100% (v/v) of MEOH) at 30 °C; flow rate of 1 mL/min; monitoring wavelength: 280 nm	apigenin, myricetin, luteolin, luteolin 7-glucoside, chlorogenic acid, caffeic acid, ferulic acid	Cluster analysis using SpecAlign program (Pearson correlation coefficient, r, and Euclidean) and PCA	[73]
Pomegranate (<i>Punica granatum</i> L.), dried peel	UAE of the dried samples with 60% ethanol, 26 min	Zorbax SB-C18 column (5 µm, 4.6 mm × 250 mm); mobile phase: glacial acetic acid (99:1, v/v; pH 3.0) and MeOH flow rate of 1 mL/min; monitoring wavelength: 280 nm	gallic acid, punicalagin, catechin, chlorogenic acid, caffeic acid, epicatechin, rutin, and ellagic acid	Similarity Evaluation System for Chromatographic; Fingerprint of Traditional Chinese Medicine (Version 2004A) software	[75]

Table 1. Cont.

Plant Material	Extraction	HPLC Condition	Characteristic Fingerprint Peaks	Chemometric Analysis Approach	Ref.
<i>Sedi linearis</i> Herba, dried whole herb	UAE of the dried samples with 70% methanol, 60 min	BDS Hypersil C18 column (4.6 m × 250 mm, 5 μm), 30 °C; Mobile phase: ACN and 0.1% acetic acid solution; flow rate of 1 mL/min; monitoring wavelength: 265 nm	hyperoside, isoquercetin and astragalin	-	[76]
Black tea samples	Enzymatic extraction using immobilized polyphenol oxidase followed by fractionating using a Mitsubishi SP-207 resin chromatography with an elution gradient of 20%, 30%, 40%, 50% and 70% aqueous ethanol	C18 column; mobile phase: water: ACN: glacial acetic acid (73.5/26/0.5, v/v/v); flow rate of 5 mL/min	caffeine, (−) epigallocatechin gallate, (−) epigallocatechin, (−) epicatechin gallate, (−) epicatechin, (+) gallic acid, (+) gallic acid, (+) catechin gallate, (+) catechin, theaflavin, theaflavin 3-monogallate, theaflavin 3'-monogallate and theaflavin 3,3'-digallate	-	[77]
Raw elderberry (<i>Sambucus nigra</i> L.)	UAE of the dried samples with 80% methanol, 45 min	C18 column, 35 °C, mobile phase: MeOH and acetic acid in water (1.0%, v/v); flow rate of 1 mL/min; monitoring wavelength: 285 nm	gallic acid, chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, rutin, myricetin, quercetin kaempferol and quercetin 3-glucoside	HCA and PCA	[78]
<i>Phyllanthus emblica</i> , dried fruits	Fruit powder was extracted in 70% ethanol (1:8) at 50 °C using a magnetic stirrer	DiKMA C18 column (250 mm × 4.6 mm × 5 μm); mobile phase: 0.2% formic acid in water and methanol; flow rate of 1 mL/min; Monitoring wavelength: 273 nm	gallic acid, corilagin, ellagic acid, quercetin	Similarity Evaluation System for Chromatographic Fingerprints of Traditional Chinese Medicine (Version, 2004A) and HCA	[79]
<i>Medicago</i> spp. in different phenologic stages: vegetative elongation, late bud and late flower; dried leaves	MSPD extraction using C18 column. Elution with methanol: H ₂ O (9:1, v/v)	Luna 5 U C18 column (5 μm, 150 × 4.60 mm) at 40 °C; mobile phase: acetonitrile and acetic acid in water (1.0%, v/v); flow rate of 1 mL/min; monitoring wavelength: 254 nm	puerarin, daidzin, genistin, daidzein, glycitein, genistein, pratensein, formononetin, irilone, prunetin and biochanin A	generalized linear model (GLM) and linear discriminant analysis (LDA)	[80]
<i>Tithonia diversifolia</i> , dried leaves	Maceration in 80% acetone for 72 h; evaporate to dryness at 40 °C	C18 column (5 μm, 4.6 mm × 250 mm); mobile phase: water/acetic acid, 98:2% v/v and methanol/water/acetic acid, 70:28:2% v/v; flow rate 0.6 mL/min; monitoring wavelengths: 254, 327, 366	gallic acid, chlorogenic acid, caffeic acid and p-coumaric acid, and apigenin	analysis of variance model and Tukey's test	[81]
lyophilized leaves of ten mango varieties	UAE with 70% ethanol, 320 W for 30 min; evaporate to dryness by vacuum rotary evaporator at 30 °C	C18 column (250 mm × 4.6 mm, 5 μm); mobile phase: 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile; flow rate: 0.8 mL/min; all wavelengths scanning detection from 200 to 600 nm	neomangiferin, galic acid, 5-caffeoylquinic acid, 3-chlorogenic acid, mangiferin, 4-hydroxybenzoic acid, sinpic acid, isoquercitrin, quercetin	similarity analysis, PCA, HCA, discriminant analysis	[82]

A mixture of water with organic solvents (i.e., methanol, acetonitrile) is usually used as the mobile phase. The development of LC methods for polyphenols analysis is almost always based on a slow solvent gradient (duration between 45 and 80 min) with the advantage of sharper peaks because of the compression effects induced by the gradient, as well as minimizing the LC column contamination due to the increased solvent strength. A gradient based on a combination of acetonitrile/water/methanol is often applied on a C18 column for the separation polyphenols [66,74]. Acetic acid, trimethylamine, phosphoric acid or trifluoroacetic acid (TFA) are usually used as mobile phase additives for enhancing the chromatographic separation [66,73–75,78] (Table 1).

However, conventional HPLC suffers the disadvantages of long analysis time, low resolution and large solvent consumption. UPLC and multi-dimensional separation techniques have emerged as alternatives to HPLC, presenting superior separation capabilities and high levels of system stability, providing technical support for further pharmacological and pharmaceutical research [77,83].

4.2. Liquid Chromatography–Mass Spectrometry Analytical Methods

Although many studies had been published concerning the phenolic profiles of various plant species through RP-HPLC, mass spectrometric techniques have recently gained a considerable advancement in the analysis of complex biological matrices, and thus in evaluating the bioactivity and nutraceutical potential of plants [20,23,84,85].

Due to noise reduction, and improved detection sensitivities and method specificities, these techniques require minimal sample preparation by dilution. Thus, the limitations of LC-UV methods, such as the presence of interferences in complex samples and the high levels of detection and quantification limits, have been overcome [23,84]. Mass spectrometry is currently the most sensitive method of structural analysis, consisting in the ionization of the investigated chemical substances followed by the separation of the ions according to the mass to charge ratio. The mass spectrum represents the plot of the ions' relative abundances versus their mass to charge ratio, and is a characteristic of each compound [20].

4.2.1. Liquid Chromatography (LC) Tandem Low-Resolution Mass Spectrometry

Liquid chromatography (LC) coupled with single–quadrupole low-resolution mass spectrometry, or more often, with tandem mass spectrometry (MS-MS) using ion trap spectrometers (IT) [84] or triple quadrupole (QQQ) [23,86–88], is common in targeted screening and quantification methods for polyphenols. The comparative study of the theoretical mass and fragmentation patterns of the reference standards vs. target compounds is used to unequivocally confirm the identity of polyphenolic compounds in “targeted” analysis. However, due to the limited availability of analytical standards, a limited number of compounds can be identified and quantified using this approach.

Four MS scan approaches are possible, and all can generate valuable information: (i) Full scan (FS) and (ii) selective ion monitoring (SIM) represent the most common data acquisition modes in methods without fragmentation. Confirmatory analysis uses the monitoring of the fragment ions through (iii) product reaction monitoring (PRM) or (iv) multiple reactions monitoring (MRM), which monitor all ion fragments resulting from a single selected precursor ion (MS² spectra) [86].

The most common ionization source in the LC-MS analysis of polyphenols is electrospray ionization (ESI) in the negative mode, providing the deprotonated molecule [M-H][−] [86]. ESI in the positive ionization mode has also been proposed in various applications [89].

For the chromatographic separation of polyphenols, prior to spectrometric analysis, reverse phase chromatography (RP-LC) using the stationary phases C18 and C8 is the most commonly used in LC-MS. Short columns (e.g., 150 mm for HPLC and 100 mm for UHPLC) with small particle diameters (e.g., 5 μm for HPLC or between 1.8 and 2.6 μm for UHPLC) are generally preferred [23,87,90]. Mixtures of ultrapure water acidified with formic acid or

acetic acid 0.05–0.5% and methanol or acetonitrile as organic solvents (often acidified with formic acid or acetic acid) are usually used as mobile phases [20,23]. The addition of 0.1% trifluoroacetic acid can result in improved peak separation, tailing reduction and superior peak symmetry [20].

For adequate detection of the analytes, the parameters of the ESI ion source (nebulizing gas flow, their temperature, voltage and source temperature, declustering potential, etc.) as well as the different parameters of the MS instrument (e.g., collision energy) are optimized by injection of analytical standards.

Liquid chromatography (LC) tandem low-resolution mass spectrometry has recently been used in various targeted analyses of polyphenols in alfalfa (*Medicago sativa* L.), lavender (*Lavandula spica* L.), buckwheat (*Fagopyrum esculentum*), phacelia (*Phacelia tanacetifolia* Benth.) and licorice (*Glycyrrhiza glabra*) root extract [23], Chinese rose (*Dalbergia odorifera*) used in traditional Chinese medicine [88], red clover (*Trifolium pratense* L.) extract [87], and green, black and oolong tea (*Camellia sinensis*) [89] (Table 2).

4.2.2. Non-Targeted High-Resolution Mass Spectrometry Use in “Chemical Profiling”

Although low-resolution mass spectrometry is still used for the identification and quantification of polyphenols in different vegetal matrices, particularly food, high-resolution mass spectrometry (HRMS), represented by quadrupole–flight time spectrometry (Q-TOF) and quadrupole–Orbitrap technologies, have recently gained popularity due to their ability to provide complete information on the exact molecular mass, elemental composition and chemical structure of a given compound [91,92].

The exact mass provided by high-resolution mass spectrometry with a mass error ≤ 2 ppm is currently the ideal tool for the structural characterization of the compounds in various applications, including polyphenols identification [91]. In the MS/MS tandem analysis, the quasi-molecular ion $[M-H]^-$ or $[M-H]^+$ fragmentation model can be studied by the retro-Diels–Alder reaction (rDA) or other fragmentation models [91,92].

For polyphenols’ analysis, the negative ionization mode provides more characteristic fragments than positive ionization [93–95]. A fragmentation pattern characterized by loss of the carboxylic fraction (CO_2 , 44 Da) is observed for phenolic acids. The remaining skeleton showed the ion $[MH]^-$ at m/z 137.02 ($C_7H_5O_3$), and in the MS/MS spectra, the fragment $[MH-COOH]^-$ at m/z 93.03 (C_6H_5O) is generated [91]. The loss of a hydroxyl radical leads to the fragment m/z 107.01, visible in several such compounds [91].

For O-glycosylated flavonoids, the cleavage to the neutral glycosidic residue is characteristic in both positive and negative ionization modes, resulting in fragments such as 162 Da (glucose), 132 Da (pentose), 146 Da (deoxyhexose), 146 Da (rhamnose), 180 Da (glucopyranose) and 308 Da (rutinoside) [92]. In aglycones flavonoids, two characteristic fragmentation pathways can be distinguished: ring heterocyclization and gradual degradation of the molecule [92].

Thus, for negatively ionized flavones, isoflavones, flavonols and flavanones, the MS-MS ion spectra show fragmentation following the retro-Diels–Alder reaction path to the C ring, leading to = molecule cleavage at bonds 1 and 3 (Figure 1). Two product ions containing intact A and B rings result [91–93].

On the other hand, small radical losses such as CO and CO_2 , H_2O , CH_2-CO , and CH_4O were observed [91,92,95]. The fragments resulting from the rDA reaction are particularly useful for elucidating the structure, because they allow not only the positioning of the OH-group, but also for the identification of the position of glycosidic bonds. March et al. [94] proposed an intermediate structure of the C ring, which successfully explained the mechanism of CO_2 removal at the C ring in the negative ionization of isoflavone-glycoside. Fabre et al. [95] found a loss in ketene moiety (C_2H_2O) in flavones and flavanones at the C ring following fragmentation due to the low probability of rearrangement, whereas Kang et al. [86] showed that the loss of ketene occurs at ring A for isoflavones.

Table 2. Examples of the target screening methods used for the identification/quantification of the selected polyphenols.

Plant Material	Extraction	Hyphenated Technique Used	Instrumental Methodology (HPLC Condition, Ionization, Acquisition Mode)	Selected Polyphenols	Ref.
Flowers, leaves, and stalks of alfalfa (<i>Medicago sativa</i> L.) and goldenrod (<i>Solidago virgaurea</i> L.); flowers, leaves, stalks, and roots of phacelia (<i>Phacelia tanacetifolia</i> Benth.); buckwheat (<i>Fagopyrum esculentum</i>); licorice root (<i>Glycyrrhiza glabra</i>); and lavender flower (<i>Lavandula spica</i> L.)	solid-liquid extraction (SLE) consecutively using H ₂ O; mixture H ₂ O/EtOH (1:1; v/v), mixture H ₂ O/MeOH (1:1; v/v), and finally NeOH shaking for 5 h; SPE purification using a C18 column	UHPLC-MS/MS	Zorbax Eclipse XDB-C18 column (50 × 2.1 mm, 1.8 μm); mobile phase: 0.1% v/v formic acid in water and ACN; ESI ionization source in negative mode, acquisition in selected reactions monitoring mode, SRM	3-(4-hydroxyphenyl) propionic acid, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid, quercetin, rutin, glabridin, and naringenin	[23]
Edible lotus (<i>Nelumbo nucifera</i>) rhizome knot	Enzymatic hydrolysis with cellulose and pectinase, at 62 °C, pH 4, 90 min followed by ultrafiltration	HPLC-QqQ-MS/MS	ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μm); mobile phase: aqueous 0.4% acetic acid, and acetonitrile; ESI in negative ion mode Acquisition in MRM mode	chlorogenic acid, B-type procyanidin dimer, (+)-catechin, B-type procyanidin dimer, (−)-epicatechin, propyl gallate, caffeic acid, (−)-epicatechin-3-gallate, and rutin	[43]
<i>Trifolium pratense</i> L. (Red Clover), dried leaves	MAE, 300 W, with MeOH at 70 °C	HPLC-ESI-MS/MS	Zorbax Eclipse XDB-C18 50 mm × 4.6 mm × 1.8 μm column; mobile phase: 0.05% aqueous formic acid and MeOH; ESI ionisation source in negative mode, acquisition in SRM	43 phenolic including: genistein, daidzein, p-hydroxy-benzoic acid, caffeic acids, kaempferol 3-O-glucoside, quercetin 3-O-glucoside, hyperoside	[87]
Heartwood samples of <i>Dalbergia odorifera</i> (medicinal herbs in TCM);	UAE with 70% methanol (v/v) for 45 min	UHPLC-QqQ-MS/MS and UHPLC-Q/TOF-MS/MS	Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm); mobile phase: ACN and water containing 0.05% formic acid ESI negative ion mode. Acquisition in MRM mode for QqQ analysis.	17 flavonoids including: daidzein, dalbergin, 30-hydroxydaidein, liquiritigenin, isoliquiritigenin, alpinetin, butein, naringenin, butin, prunetin, eriodictyol, tectorigenin, pinocembrin, formononetin, genistein, sativanone	[88]
Green, black and oolong tea (<i>Camellia sinensis</i>)	Extraction at high temperature (80 °C) for 3 min with mild stirring	HPLC-QqQ-MS/MS	Capcell Pak C18 MGIII (2.0 mm × 100 mm, 3 m) column; ESI in positive and negative ion mode. Acquisition in MRM mode.	gallic acid, epigallocatechin, catechin, epigallocatechin gallate, epicatechin, gallic acid gallate, epicatechin gallate, catechin gallate, theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate; theaflavin-3,3'-digallate.	[89]
Sour Guava (<i>Psidium friedrichsthalianum</i> Nied) lyophilized whole fruits	UAE of lyophilized fruits with acetone:water (7:3). The extract was submitted to successive partitions with ethyl ether, ethyl acetate, and <i>N</i> -butanol.	UPLC-ESI/QqQ-MS/MS	UPLC BEH C18-column (2.1 × 100 mm, 1.7 μm); mobile phase water/formic acid (99.9:0.1, v/v), and ACN/formic acid (99.9:0.1, v/v) ESI ionization source in negative mode. Acquisition in MRM	22 phenolic compounds including several hydroxybenzoic, phenylacetic, and hydroxycinnamic acid derivatives	[90]
Barks of <i>Conarus var. angustifolius</i> , and leaves of <i>Cecropia obtusa</i> , <i>Cecropia palmata</i> and <i>Mansoa alliacea</i> ; dried samples	UAE in 70% hydroethanolic, butanol/ethyl acetate, 4 h Butanol and ethyl acetate were evaporated at 40 °C	HPLC-ESI/QqQ-MS/MS	SB-C18 Rapid Resolution HD column (2.1 × 50 mm, 1.8 μm); mobile phase 0.1% acetic acid in water and ACN; ESI ionization source in negative mode Acquisition in MRM	gallic acid, catechin, caffeic acid, rutin, ferulic acid, quercitrin and resveratrol	[96]

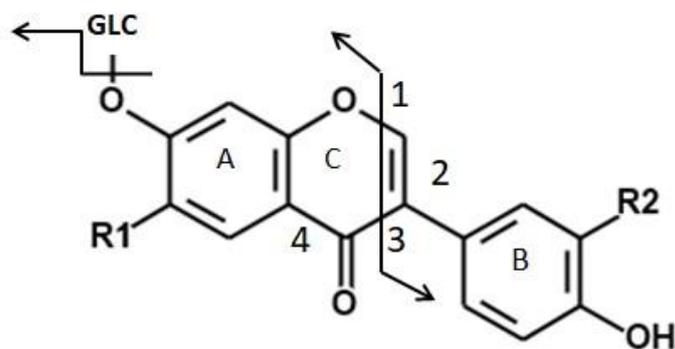


Figure 1. The proposed fragmentation model for flavonoids following the retro-Diels–Alder path to the C ring (after Gao et al. [91]).

In addition, the ion $[MH-C_3O_2]^-$ has shown a relatively high abundance in flavonones fragmentation (luteolin, apigenin, genistein), it being one of the key ions for the differentiation of isobaric compounds apigenin and genistein. The subsequent fragmentation MS^3 of this ion from the apigenin showed the ion $[MH-2C_3O_2]$ resulting from the loss of another C_3O_2 fragment, and in genistein the loss of CO from $[MH-C_3O_2]$ was displayed [97].

For methoxylated flavonoids (biochanin, formononetin, prunetin, calicosin, glycitein), the $[M-H-CH_3]^-$ ion is characteristic of negative ionization [94]. The ionic fragments CH_3 , CHO , and CO_2 , resulting from successive or simultaneous losses, are attributed to the type of isoflavone $[4'-OCH_3]$, [86,97]. In addition, for isoflavones containing a hydroxyl group or a methoxy- group at the B ring, a relatively highly abundant fragment ion at m/z 132 was detected and assigned to $[0.3B-2H]^-$ for prunetin and glycitein, and $[0.3B-CH_3-H]^-$ for formononetin and biochanin A [86,97]. The fragment ion at m/z 117 indicates the presence of a hydroxyl group at ring B or in position 3. On the other hand, the fragments $[MH-CH_3-CO]$ are characteristic of methoxylated isoflavones, CO loss being subsequent to radical loss $[CH_3]$. The fragment $[MH-CH_3-CO-B\text{-ring}]$ was found to be characteristic of the distinction between glycitein and other isomers as collision energy increases [98]. Thus, the mass spectral decomposition of polyphenols under ESI-HRMSⁿ allows a structural characterization of the corresponding compounds by assigning specific key ions.

Analytical approaches based on HRMS have been successfully applied for polyphenols profiling in herbal remedies from traditional Chinese medicine, such as Dingkun Dan [91] or *Aster tartaricus* risoma [99], rare plants such as *Ophryosporus triangularis* (native to the Acatama Desert, Chile) [100], or common plants, e.g., culinary herbs [22], green tea products [101], leaves of green perilla [102], pomegranates [103] and goji [104] (Table 3).

Table 3. Examples of the non-target screening methods used for chemical profiling of the plant extracts.

Plant Material	Extraction	Hyphenated Technique Used	Instrumental Methodology (LC Condition, Ionization, Acquisition Mode)	Data Processing Approach Used for Tentatively Identification	Ref.
Ground dried culinary herbs and spices: dill (<i>Anethum graveolens</i>), marjoram (<i>Origanum majorana</i>), turmeric (<i>Curcuma longa</i>), caraway (<i>Carum carvi</i>), and nutmeg (<i>Myristica fragans</i>)	UAE with 5 mL of 50% ethanol in 0.1% formic acid in ultrapure water; 5 min	UHPLC- LTQ Orbitrap MS	Atlantis T3 C18 (100 × 2.1 mm, 3 μm) column; mobile phase; 1% formic acid in water and 0.1% formic acid in ACN. Full scan MS mode at 60,000 FWHM and MS/MS mode with the Orbitrap at 30,000 FWHM DDA scan.	In-house database Comparison with HR-MS data found in literature PCA, HCA	[22]
Dingkun Dan (traditional Chinese medicine prescription)	Ultrasonic extraction with MeOH, 30 min	UHPLC-Q/Orbitrap –HRMS/MS	Acquity T3 (2.1 × 100 mm, 1.8 μm) column; mobile phase: 0.1% formic acid in water (A) and 0.1% formic acid in MeOH. HESI II ionization source in positive and negative mode; collision energy: 25–60 V. Data-dependent acquisition (DDA).	In-house database Searching in ChempSpider; Pubchem.	[91]
<i>Aster tataricus</i> rhizoma	UAE with MeOH for 30 min	UHPLC-Q-TOF-MS	Poroshell 120 EC-C18 column (100 mm × 2.1 mm, 2.6 μm); mobile phase: water + 0.1% formic acid and ACN + 0.1% formic acid. ESI source in both positive and negative ion mode. DDA and DIA acquisition methods. Multiple mass defect filter (MMDF) and dynamic background subtraction (DBS) by AB Sciex software	Searching for reported metabolites; in-house database. Searching in ChempSpider database using MasterView™1.0. Product ions strategy (KPIs). Clog P (calculated by Chemdraw Ultra 12.0 software)—used for distinguishing isomers.	[99]
<i>Ophryosporus triangularis</i> Meyen, dried aerial parts (leaves and stems) and flowers	UAE with MeOH, 30 min	UHPLC-Q-Orbitrap HRMS/MS	Acclaim UHPLC C18 column, (150 mm × 4.6 mm ID, 2.5 μm); mobile phases: 1% formic aqueous solution and ACN; HESI negative ionization mode; full scan at 70,000 FWHM followed by targeted MS/MS at 17,500 FWHM; collision energy (HCD cell)–30 kv	In-house database comparison with HR-MS data found in literature	[100]
Dried leaves of green perilla (<i>Perilla frutescens</i>)	Extraction with MeOH by shaking for 8 h at ambient temperature	LC- TOF-MS/MS	Poroshell 120 EC-C18, 150 × 2.1 mm, I.D., 4 mm; mobile phase: 0.1% formic acid in water and MeOH; ESI source in negative ion mode; resolution of 2700; collision energy 10 eV.	The analyst TF software (version 1.7); identification of the compounds by comparison with previous studies. Tentatively characterized by fragmentation pathway identification.	[102]
Goji berries (<i>Lycium barbarum</i> L.)	Extraction in 1% formic acid in 80:20 methanol/ water solution by centrifugation at 25,000 rpm/3 min	UHPLC-ESI-QTOF-MS	Zorbax eclipse plus C18 column (50 × 2.1 mm, 1.8 μm); mobile phase: water and MeOH ESI ionisation source in positive; full scan MS ² (30,000 FWHM); mass accuracy ≤ 5	Profinder B.07 software. Phenol-Explorer 3.6 database	[104]
<i>Stellera chamaejasme</i> extracts	reflux with MeOH for 30 min at 50 °C.	UHPLC–LTQ-Orbitrap MS ⁿ	Agilent Zorbax Ecpise Plus C18 column (100 × 3.0 mm, 1.8 μm) at 50 °C; mobile phase water with 0.5% FA and ACN; HESI in positive and negative mode DDA, MS ⁿ scan (four ions for MS ² and one ion for MS ³); resolution of MS ² –60,000 and MS ³ –30,000; collision energy: MS ² –30 V and MS ³ –35 V.	SciFinder database (https://scifinder.cas.org) for chemical formula annotation; comparison with HR-MS data found in literature	[105]

Table 3. Cont.

Plant Material	Extraction	Hyphenated Technique Used	Instrumental Methodology (LC Condition, Ionization, Acquisition Mode)	Data Processing Approach Used for Tentatively Identification	Ref.
The male flowers of date palm (<i>Phoenix dactylifera</i>)	Soxhlet extraction with 80% MeOH for 6 h at 50 °C, fractionation in a C18 column	HPLC-ESI-ITMS	Symmetry C18 column (5 µm, 2.1 mm × 150 mm; mobile phase: ACN containing 0.03% (v/v) formic acid and water containing 0.03% (v/v) formic acid; ESI ion source in negative ion mode full-scan followed by MS-MS	Density functional theory (DFT) study	[106]
<i>Mentha rotundifolia</i> (L.) Huds, aerial parts	maceration in MeOH for 24 h at room temperature	UHPLC-ESI-Q- Orbitrap- HRMS/MS	Kromasil RP-18 column (250 mm 10 mm); mobile phase: water/ACN 75:25, 0.25% FA and ACN, 0.25% FA; HESI ionization source in negative mode; full MS followed by DDA scan	In-house database, confirmation by NMR approaches	[107]
Baoyuan decoction (traditional Chinese medicine formula)	reflux with water for 1.5 h	UHPLC-Q-TOF-MS/MS	Cortecs UPLC C18 column (1.6 µm, 2.1 × 100 mm; mobile phase: 0.05% aqueous formic acid and ACN containing 0.05% formic acid; ESI ionization in negative mode; DIA	UNIFI software. In-house database	[108]
The bark, twigs, leaves, and fruits of <i>Alnus japonica</i> , <i>Alnus hirsuta</i> and <i>Alnus hirsuta var. sibirica</i>	UAE with MeOH (1:10 g DW/g), 60 °C, 60 min, at 60 kHz	UHPLC-Q-TOF-MS/MS	Acquity BEH C18 (100 × 2.1 mm 1.7 µm) column; mobile phase: H ₂ O and MeCN, both of which were acidified with 0.1% formic acid; ESI ionization mode in negative ion; DDA (full MS followed by MS/MS scans for the three most intense ion).	Spectral preprocessing using MZmine; in silico annotation with network; annotation propagation GNPS molecular networking; integration of annotation data using MolNetEnhancer	[109]
<i>Medicago sativa</i> L. and <i>Trifolium pratense</i> L. dried sprouts	UAE with 70% ethanol (1:10 g DW/g), 60 °C, 60 min, at 60kHz	UHPLC-Q- Orbitrap HRMS/MS	Accucore U-HPLC Column C18 (150 × 2.1 mm, 2.6 µm); mobile phase: water containing 500 µL/L ⁻¹ formic acid (pH 2.5) and MeOH; HESI ionization in negative mode; DIA (Full scan 70,000 FWHM, MS/MS 35,000 FWHM). Normalized collision energy: 30, 60 and 80 NCE	Chemspider database; NORMAN MassBank, mzCloud TM Advanced Mass Spectral Database; in silico fragmentation with ACDLabs MS Fragmenter 2019.2.1 software	[110]
Blackcurrant (<i>Ribes nigrum</i> L.) leaves	UAE with ethanol/water (1:1), 20 °C, 60 min	ESI-LTQ-Orbitrap MS	Kinetex Evo C18 5 µm column; mobile phase: water + 0.1% formic acid and ACN + 0.1% formic acid. ESI source in negative ion mode. DDA	MZmine; PCA.	[111]
Persimmon leaves (Chinese traditional medicine)	UAE with methanol/water (80:20, v/v)	LC-ESI-LTQ- Orbitrap-MS	Atlantis T3 column 2.1 × 100 mm, 3 m; mobile phase water/0.1% formic acid and ACN; ESI source in both positive and negative ion mode; DDA approach-full scan at 30,000 FWHM and MS/MS at 15,000 FWHM; Collision energy (HCD cell)–35 kv	Identification by generating the molecular formula using accurate mass (C = 30, H = 100, O = 15), and matching with the isotopic pattern. Searching in polyphenol database: (http://phenol-explorer.eu/). Confirmation by comparison with HR-MS data in literature and databases	[112]
Flowers and leaves of Chilean Mistletoe (Quintral, <i>Tristerix tetrandus</i>)	Lyophilized flowers and leaves were defatted thrice with <i>N</i> -hexane (1:10) and then extracted by UAE with 0.1% HCl in MeOH (1:10) for 60 min. Purification with XAD-7 column.	UHPLC-Q/Orbitrap/HRMS/MS	UHPLC C18 Column, Acclaim, 150 mm × 4.6 mm ID, 5 µm. Mobile phase: 0.1% aqueous formic acid and ACN 0.1% formic acid. HESI II ionization source in positive and negative mode. Full scan MS (70,000 FWHM) acquisition followed by targeted MS/MS analysis (17,500 FWHM). Collision energy: 30 kv. Mass accuracy ≤ 5	Trace Finder 3.2 software	[113]

Hybrid mass spectrometers using linear ion capture technology, such as LTQ-Orbitrap, have also become common in this field. LTQ-Orbitrap provides the possibility of the screening, identification and structural characterization of unknown compounds using MSⁿ fragmentation [111,112]. For example, the UHPLC technique coupled with LTQ-Orbitrap has recently been used for the characterization of components in *Stellera chamaejasme* [105] and in blackcurrant leaves [111].

Regarding the methods' optimization, in the LC-HRMS/MS non-targeted screening analysis, resolution, scan rate and mass acquisition interval are the most critical parameters [99]. In addition to data acquisition modes also common in low-resolution spectrometry (SIM and MRM, PRM), new approaches such as data-dependent acquisition (DDA) and independent data acquisition (DIA) strategies, used so far only in proteomics, have recently been transposed into the analysis of small molecules in HRMS [110,114].

The limitation of the DDA strategy comes from the selection of precursors for MS/MS analysis [99]. Instead, in the recently developed independent data acquisition (DIA) strategy, all molecules within consecutive preselected *m/z* windows are subject to fragmentation, leading to higher specificity compared to the AIF (all ions fragmentation) approach [99,110]. As a disadvantage, the lack of precursor preselection can lead to impure mass spectra and low sensitivity. In this particular situation, the scan speed is the critical parameter [99,114].

In a recent study, Sun et al. [99] compared the DDA and DIA acquisition mode approaches using UHPLC-Q-TOF-MS technology for chemical characterization of the *Aster tataricus* rhizome, a traditional Chinese medicinal remedy. The key product ions strategy (KPIs) was used for the first time for searching for and identifying bioactive compounds. A total of 131 compounds, of which 31 were flavonoids, were identified or provisionally characterized. For the DDA setting, a complete scan and the ten most intense ion fragments from each analyte were applied to perform a TOF scan. For flavonoids, the ionic fragment *m/z* 153.0180 of kaempferol was used as the key ion in positive ionization. Based on the metabolic patterns of apigenin, isorhamnetin and kaempferol, several flavonoids were provisionally characterized: asapigenin-5-ramnoside, isorhamnetin-3-O-neohesperidoside, isorhamnetin-3-O-glucoside and biorobine (kaempferol 3-robin). The DDA strategy allowed the identification of 120 compounds, while 131 was identified by the DIA approach. However, certain glycosides identified in the DDA could not be detected by DIA [99].

The use of the DIA-MS strategy using an HRMS Q-Exactive Orbitrap instrument has recently been reported for the identification of polyphenols in extracts of red clover and alfalfa sprouts [110].

A workflow strategy in chemical profiling and metabolomics using HRMS technologies is shown in Figure 2.

Although HRMS non-target screening offers clear advantages, investigating the enormous amount of data produced by such techniques remains a challenge [110]. The identification of unknown compounds in complex samples always requires MS and MS/MS databases, and the evaluation of the exact mass of the ions obtained by HRMS (molecular ion, ionic fragments, isotopic models) for provisionally assigning the chemical formula based on the rules defined in organic chemistry or using designed software.

Practically, considering polyphenolic compounds, the potential elementary molecular compositions of the compounds are established as C, H and O, then molecular formulas calculated based on mass accuracy are generated with a reasonable degree of measurement error (≤ 2 ppm). Based on molecular fragments and a "match" factor of 70%, presumptive compounds are selected from a database, such as the Chemspider, (<http://www.chemspider.com/>), Pub-chem, (<https://pubchem.ncbi.nlm.nih.gov/>) or SciFinder database (<https://scifinder.cas.org>) or the polyphenol database (<http://phenol-explorer.eu/>) [105,110,111].

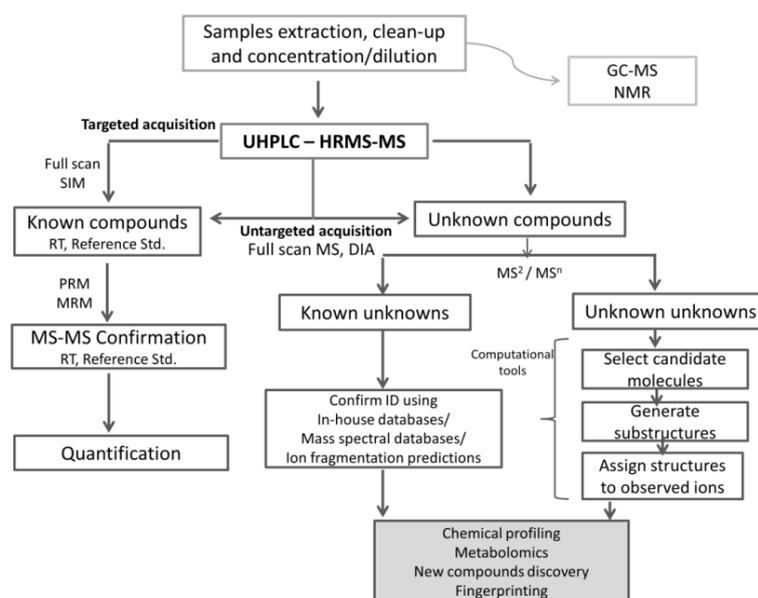


Figure 2. Workflow strategy in chemical profiling and metabolomics using HRMS technologies.

The comparison of the fragmentation patterns in MS-MS databases in the literature led to structure confirmation. There are currently various collections of public and private MS/MS mass spectra, such as NBS/EPA/NIH Mass Spectral Data Base, G.W.A. National Bureau of Standards Washington; “eight peak index” at the Mass Spectrometry Data Center of Royal Society, Cambridge; Norman MassBank (<https://massbank.eu/MassBank/>); network clusters such as as mzCloudTM (<https://www.mycloud.com/#/>); ReSpect, NIST (The National Institute of Standards and Technology; <http://www.nist.gov/srd/nist1a.cfm>) and Global Molecular Social Networking (GNPS). These allow users to compare the obtained MS-MS spectra with reference spectra for chemical structure annotation.

Spectrum processing and comparative analysis can be done manually, requiring extensive work and time, or automatically using software such as Compound DiscovererTM, Trace FinderTM, MassHunter, MasterViewTM, MZmine, MAGMA software, or MetabolitePilotTM.

In addition, predictors of silico fragmentation, such as software tools like ACD Labs MS Fragmenter or Mass FrontierTM, are used to extend the primary annotation of presumptively identified molecules to the confirmation of the chemical structure [110].

Peak selection is one of the key steps in any non-target screening, and it can be done using a software algorithm (e.g., UNIFI platform by Waters Corporation). In the “suspect” compounds screening, the selection is made based on theoretical/predicted reference data. In non-target screening, presumptive peak identification can be performed when the signal strength for both the precursor ion and the fragments is sufficient to elucidate the molecular formula. Recently, Xu et al. [115] applied a UPLCQ-TOF-MSE method using data processing in the UNIFI computer platform for the determination of the chemical composition of Gandou decoction, a traditional Chinese medicine (TCM) formula. In total, 96 compounds, including flavonoids and phenolic acid, were identified or provisionally characterized based on retention time, exact mass (mass separation window of 5 ppm) and MS-MS fragmentation patterns [115].

In addition to MS databases, information on the environmental context (samples source, literature) may also be essential for the compounds’ identification. As well, combined statistical approaches, including chemometrics and bioinformatics, are currently being used to identify unknown or new compounds [116].

Guo et al. [116] performed a chemical profiling of several Keemun black tea classes via LC-Orbitrap-MS/MS. Data were subsequently analyzed using multivariate metabolomics statistics (PCA and HCA), leading to the identification of tea class markers (theasinensin A, afzelechin galate and kaempferol-glucoside).

The reported studies have highlighted that the digitized analysis of MS/MS data significantly improves the phytochemical analysis. Moreover, it demonstrates that molecules with high complexity can be identified and chemically characterized only with specialized software tools and require highly qualified personnel.

4.3. Analysis of Phenolic Compounds in Plants by GC-MS Using Derivatization Techniques

Although the use of GC coupled with MS and tandem MS-MS has been proven a powerful analytical tool for natural products' characterization [117], there is a limited range of polyphenolic compounds that can be analyzed by GC-MS methods. Phenolic acids, phenolic aldehydes, ketones and phenolic alcohols were recently analyzed by GC-MS in wild plant fruits [117], fruit samples [36,118], *Curcuma caesia* Roxb [119], Kodo millet (*Paspalum scrobiculatum*) [120], and *Scambiosa Columbabria* L. [121]. Although newer two-dimensional GC x GC systems improved the separation performance and resolution [122], currently, the GC-MS technique is less commonly employed mainly because complex sample pre-treatments are required to increase the volatility and stability of the phenolic compounds. In addition to sample extraction and clean-up, a previous chemical derivatization step is needed to convert the hydroxyl groups to ethers, or make esters more volatile and thermostable.

Among the variety of derivation reagents, the most used is the trialkylsilyl, substituting the active hydrogen in alcohols and other polar organic compounds by the trimethylsilyl (TMS) group, $-\text{Si}(\text{CH}_3)_3$. N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), N,O-bis-(trimethylsilyl)acetamide (BSA), N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and N-[dimethyl-(2-methyl-2-propyl)silyl]-2,2,2-trifluoro-N-methylacetamide (MTBSTFA) are the most commonly used reagents [118,122]. Regarding the chromatographic conditions, fused silica capillary columns with lengths ranging from 25 to 30 m and inner dimensions from 0.25 to 0.5 mm, coated with 5% phenyl-95% dimethylpolysiloxane, are commonly used. The temperature gradients use initial column temperatures ranging from 40 to 80 °C, and final temperatures between 200 and 370 °C, with increasing rates ranging from 2 to 50 °C/min. High-purity helium is commonly used as a carrier gas at a flow-rate ranging from 0.4 to 3 mL/min, although high-purity hydrogen has also been used. Usually, GC-MS systems use the electron ionization (EI) mode prior to quadrupole MS quadrupole ion trap (QIT) and time-of-flight (TOF) analyzers [118,122].

4.4. Metabolomic Analysis of Polyphenolic Using Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS) are currently the main analytical methods applied in metabolomics studies [123]. Both techniques can enable two approaches for "profiling" studies: a non-targeted approach using chemometric analysis; and a targeted approach for the identification and quantification of known compounds in the plant extract [123,124]. Both techniques have met the requirements of metabolomics by being able to provide information that can lead to molecular structure characterization.

One of the disadvantages of HRMS compared to NMR is the complexity of operations, including elaborate sample preparation, chromatographic separation, molecule fragmentation, and extensive data processing, all of which requiring highly qualified personnel. In addition, because of the complexity and variability of the factors that influence the analysis, analytical protocols are difficult to standardize. Although less sensitive than mass spectrometry, NMR is highly reproducible, allowing the operations' standardization [124]. Due to the distinct advantages of each method, NMR and HRMS are considered complementary techniques for the characterization of plant extracts [123].

Various open databases for the metabolites' identification are currently being developed, such as the Human Metabolism Database (<http://www.hmdb.ca>), and the Biological Magnetic Resonance Datbank (BMB) (<http://www.bmrb.wisc.edu/>). However, most of these databases contain information on human metabolites and very few resources for the identification of secondary metabolites of plants [74].

In NMR spectroscopy, the intensity of the peaks is directly proportional to the number of atomic nuclei, and hence, in combination with chemometric analysis, NMR is currently used to classify plant samples according to species, origin, processing, age or other quality parameters, based on the NMR profiles [74]. The main approach of metabolomics is to compare two data sets (for example, in the case of plant matrices several species of the same genus) and, through data filtering and multivariate analysis (PCA) techniques, a discrimination between these two data sets is performed. This process highlights any differences between the data and aims to identify the compounds responsible for this differentiation [74].

A typical NMR workflow for the identification of unknown compounds in natural extracts involves the collection of fractions containing the metabolites of interest after chromatographic separation followed by off-line NMR analysis. RMB databases and computational methods, such as the prediction and simulation software PERCH, or StrucEluc from ACD Labs, in combination with complete ^1H NMR iterative full-spin analysis (HiFSA approach), provide an accurate distinction between the natural compounds with almost identical NMR spectra. In addition, computer-assisted structural elucidation (CASE) is a methodology that allows users to enter NMR data and identify clusters by comparison algorithms [123–125].

Using an integrated approach (HPLC-DAD-MS/MS) and nuclear magnetic resonance (NMR) techniques, four derivatives of ellagic acid (ellagic hexoside, ellagic pentoside, methyl ellagic deoxioside acid, 4- (alpha-Rhamnopyranosyl) ellagic acid (eschweilenol C)) were characterized for the first time in the biomass of Brazil nuts, *Bertholletia excelsa* Bonpl. (Lecythidaceae) [123]. A triple-quadrupole negative ionization was used for MS analysis, and NMR analysis was performed after an SPE fractionation of the plant extract.

5. Conclusions

Currently, the complete characterization of the chemical profiles of medicinal plants, functional foods and other nutraceuticals, and the identification of their secondary metabolites, is becoming more and more important for better understanding the biochemical and pharmacological actions, as well as the curative/preventive potential.

Recent instrumental developments have resulted in improved chromatographic resolution and MS detection (higher sensitivity, acquisition speed, resolution and mass accuracy). Due to the differences in the fragmentation mechanisms of the $[\text{M} + \text{H}]^+$ and $[\text{M} - \text{H}]^-$ ions, the fragmentation patterns in the MS-MS analysis reflect the structural properties of the polyphenolic compounds and allow their identification in complex plant matrices.

Due to the high mass accuracy, high-resolution MS-MS spectra are particularly useful for identifying polyphenols in plant extracts, establishing new applications in metabolomics and complex phytochemical analysis.

Thus, current technologies enable targeted and non-targeted analysis, and are able to provide the chemical characterization of unknown compounds, chemical profiling and metabolomics in various plant. Chemometric analysis is also a powerful tool that can be used in the preliminary stages of analytical methods optimization, but is also efficient in data processing.

These new approaches may be the key to the analysis of polyphenols, although there are challenges in identifying and annotating those compounds due to the limited availability of analytical standards and structural diversity.

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