Chapter 3

Phenolics in Foods: Extraction, Analysis and Measurements

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Additional information is available at the end of the chapter

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Abstract

The increasing consumers demands to acquire healthier fruits and vegetables as well as the urgency in looking to natural compounds with antioxidant activity and enhanced antimicrobial activity against antibiotic-resistant pathogenic bacterial strains have encouraged a quick expansion of research studies about enhanced phenolic extraction and identification methods. Considering the importance of phenolics as natural compounds with antioxidant and antimicrobial activity, this chapter aims to present the most updated information about extraction methods, ranging from the traditional to the most advanced processes, as well as the access to the modern methods used in the identification and quantification of phenolics. The main goal of this chapter is to provide the reader with a broad view on the different protocols used to extract, identify and quantify phenolic compounds from different kinds of foods, including fruits and vegetables.

Keywords: phytochemicals, extraction, determination, colorimetric methods, HPLC, HPLC-MS

1. Introduction

The growing resistance of pathogenic bacterial isolates against the traditional chemical antibiotics as well as the resurgent of old disappeared diseases associated with the constant consumers' demanding of healthier, nutritious and safe food has led the researchers to focus on searching for new, safe and effective molecules. One class of such molecules is the class of polyphenols. Polyphenols are a ubiquitous class of compounds largely present in plants as their secondary metabolites, which are synthesized during their normal development [1] in response to several stressful biotic and abiotic factors [2, 3]. This class of compounds are a much diversified group derived from the amino acids phenylalanine and tyrosine and

comprise simple phenols, hydroxybenzoic acids and cinnamic acid derivatives, flavonoids, coumarines, stilbenes and tannins, among others [4–6].

The results from the last decade's research have shown that polyphenols have important beneficial properties for human health, including antioxidative, antiaging, antibacterial and antimutagenic [7–11]. Moreover, the recent evidence of their interaction with proteins, DNA and other biological molecules has enhanced their exploitation for the production of new natural product-derived therapeutic agents. Despite these advantages, several limitations still persist, particularly those related with their extraction efficiency, which affects the large-scale use of some of these substances. The difficulties in screening, extracting, separation and purifying these compounds have increased the development of new and modern methods to address these limitations. In this context, the aim of this chapter is to present an updated review about sources, technologies and methods that have been developed until now to improve the extraction, detection, separation and full characterization of such beneficial compounds, with special emphasis to their possible application in the design of nutraceuticals and functional food products.

2. Foods as natural resources of phenolics

Polyphenols have been exhaustively studied in their different natural matrices such as fruits, vegetables, teas, algae and microalgae and more recently agro-food wastes (peels, seeds, pulps, stems and roots) [12–15]. In the three last decades, there has been a prolific publication of scientific studies showing that plant-derived foods and agro-food wastes from industrial transformation have huge quantities of polyphenols. In **Table 1** are summarized some recent studies, and as result from these and other studies, there is a diverse source of polyphenols in plant materials, but both type and amount seem to be highly influenced by their chemical nature, extraction methods, sample particle size, storage time and conditions, as well as by the presence other of interfering substances [25]. Also, their chemical structure and nature vary from simple to highly polymerized substances that include varying proportions of phenolic acids, phenylpropanoids, anthocyanins and tannins, among others [26–28]. Moreover, they might also exist in complex mixtures with carbohydrates, proteins and some quite insoluble high-molecular-weight phenolics [28]. Therefore, the phenolic extraction from plant materials is always a mixture of different steps, and many modifications of a particular method are often needed for the removal of unwanted non-phenolic substances such as waxes, fats, terpenes, pigments (chlorophylls and carotenoids). Solid-phase extraction (SPE) techniques, purification and fractionation based on acidity, are commonly used to remove unwanted non-phenolic substances or even other unwanted phenolics [29].

Although the recent advances in the technology had providing innovative approaches to obtain enriched polyphenol natural extracts, we must ware that their extraction efficiency will always be dependent of several factors in which the nature of samples and solvent, pH, temperature, light, length of extraction period, particle size, solvent/sample ratio and liquid-liquid or solid-liquid extraction process [25], among others, are the most critical.

Polyphenols	Source (some examples)
Phenolic acids	
Hydroxycinnamic acids	Cereals, coffee, cherries, citrus fruits and juices, peaches, plums, spinach, tomatoes, wheat flour, corn flour, rice flour, potato, olive mill wastewaters, winery sludge from red grapes, artichoke wastewaters, almonds
Hydroxybenzoic acids	Oilseeds, cereals, coffee, cowpeas, wheat flour, black currant, blackberry, raspberry, squash seeds and shell
Flavonoids	
Anthocyanins	Grapes, red wine, grape seeds, grape skins, winery by-products, fermented grape pomace, strawberries, back and red currants, raspberries, plums, red cabbage
Chalcones	Apples and apple juices,
Flavanols	Apples, grapes, leeks, tomatoes, curly kale, onions, lettuces, berries, beans, red grapes, black and green tea, red wine and red winery by-products, cider
Flavanones	Citrus fruits, citrus juices, orange peels and seeds wastes
Flavonols	Apples, apple peels, beans, leeks, lettuce, onions, tomatoes, olive leafs, broccoli inflorescences, chestnut, olives and olive fermented pomaces
Flavones	Spinach, citrus fruits, celery, pepper, capsicum pepper,
Isoflavones	Soybeans, soy flour, soy milk, soy processing waste
Stilbenes	Red grapes, grapes skins, grape seeds, red grape fermented pomaces
Xanthones	Mango fruits and mango peels fermented pomaces
Tannins	
Condensed tannins	Apples, grapes, peaches, pears, chestnut, hazelnuts, nuts
Hydrolyzable tannins	Pomegranate, raspberries

Table 1. Most common types of polyphenols found in foods and plant-derived products [14–24].

3. Methods used in extraction of polyphenols

It is widely accepted that the extraction step is one of the most important stage in isolation of polyphenols, but based in literature, there is no consensus about one single and effective standard extraction method. On contrary, there are several reported methods with very accurate results, and according to the literature in some cases, the solid-liquid extraction with different types of solvents is more adequate [30], and in others, the ultrasound-assisted extraction method (UAE) increases the extraction efficiency [31], while in others, this increment is higher when a microwave-assisted extraction (MAE) is used [32], and advanced methods such as pressurized fluid extraction (PFE), supercritical CO_2 extraction (SC-CO_2)

and enzyme-assisted extraction (EAE) are even better to enhance the content of polyphenols in the extracts [33–39]. Despite this diversity, all have the common fact that the extraction must be conducted carefully but exhaustively with simple, rapid and feasible procedures, and if possible open to automation [40]. In the next paragraphs, we present a summarized information of the most commonly used methods for the extraction of polyphenols in several pant and food matrices.

3.1. Classical solvent extraction

The classical solvent extraction of polyphenols usually includes extraction by maceration and percolation and by successive Soxhlet extraction [41–45].

The maceration, widely used in the past, is nowadays in underuse since other methods are more feasible. It is a simple procedure in which the powdered sample is soaked in an appropriate solvent in a closed container, normally under room temperature with constant or sporadic agitation [41, 46]. In the end of the extraction, the solid parts need to be separated from the solvent, which can be done by filtration, clarification and/or decantation [47]. This method is quite simple to hand but has the main disadvantage of time-consuming, requires a large volume of solvent [41, 42, 48, 49].

Similar to the maceration, the percolation method is characterized by placing the powdered sample in a closed container (normally cylindrical) in which the solvent is discharged from the top towards the bottom in a slow movement (drop wise). [41, 42, 50]. In this case, the filtration is not necessary because the percolator device has itself a filter which placed at the bottom, and we can only collect the final liquid. This method faces the same issues of maceration, which are time-consuming, large volumes of solvent, solubility of polyphenols, particle size of sample and contact time between solvent and sample.

In Soxhlet extraction [41, 42], the powdered samples are sealed in cellulose bags and placed in an extraction chamber located on top of a collecting flask beneath a reflux condenser, and after the addition of the solvent, the system is heated and the solvent condenses after reaching certain level of temperature [51–53]. A reflux occurs continuously. At the end, the liquid extract is collected to the flask positioned beneath the system [51–53]. The Soxhlet extraction is a continuous process with the advantage of being less time and less solvent consuming than the maceration or percolation methods [54]. However, some authors have stated that Soxhlet extraction must be handled carefully because the excess of temperature, always near to the boiling point, can destroy or modify some thermolabile polyphenols [44]. Others reported that Soxhlet extraction is used widely because of its convenience [41, 42, 44, 54]. Although these have variations, all these three methods have the common usage of organic solvents in a solid/liquid ratio. Solvents such as water, methanol, ethanol, acetone, n-hexane, chloroform, propanol and ethyl acetate have been most commonly used for the extraction of polyphenols (**Table 2**). The difference between solvents resides in their polarity (**Figure 1**) which affects their capacity in extract phytochemicals. The miscibility of organic solvents (**Figure 2**) with each other's or even other types of solvents is another fact to be considered in order to improve the polyphenol extraction yield as shown by several studies [59–63].

Solvent (alphabetical order)	Boiling point (°C)	Density (g/mL)	Solubility in H_2O (g/100g)	Polarity Index[1]	Eluent strength[2–4]	Dielectric constant at 20°C[1, 2, 4]
Acetone	56.2	0.786	Miscible	5.10	0.56	21.1
Acetonitrile	81.6	0.786	Miscible	6.20	0.65	36.64
Chloroform	61.2	1.498	0.8	4.40	0.40	4.81
Ethanol	78.5	0.789	Miscible	5.20	0.88	24.6
Ethyl acetate	77	0.894	8.7	4.30	0.58	6
Hexane	69	0.655	0.0014	0.06	0.01	1.89
Methanol	64.6	0.791	Miscible	6.60	0.95	32.6
2-Propanol	82.4	0.785	Miscible	4.30	0.82	18.3
Water	100	0.998	Miscible	10.20	>>1	78.54

[1]Data collected from http://macro.lsu.edu/HowTo/solvents.htm [55].
[2]Data collected from Speight (2005) [57].
[3]Data collected from Singh et al. (2014) [57].
[4]Data collected from Hakansson et al. (2016) [58].

Table 2. Important properties of some solvents commonly used in the extraction of polyphenols[1–4].

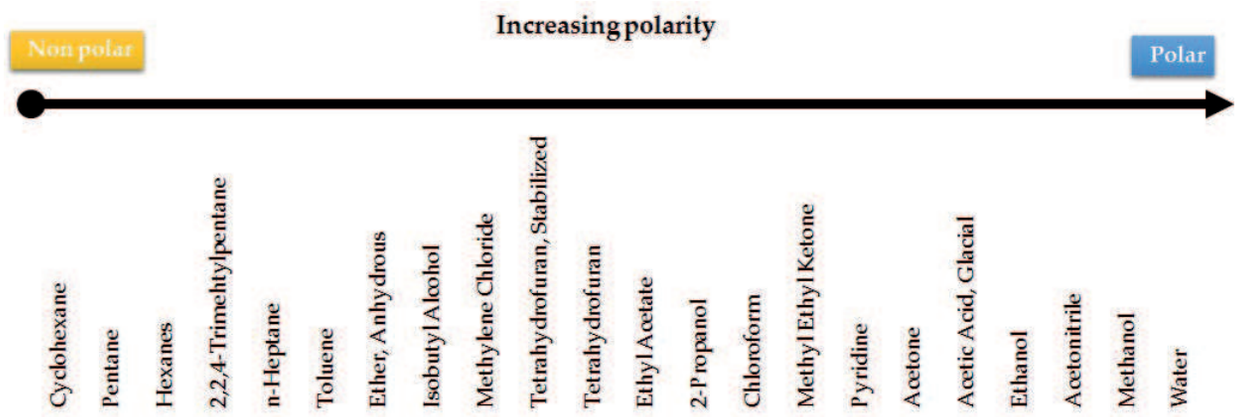

Figure 1. Polarity of the organic solvents most commonly used in phytochemicals extraction from natural sources. Adapted with permission from Refs. [55–56].

In general, organic solvents and their aqueous formulations are mostly used in the extraction of phytochemicals, but it is still no clear which solvent is most adequate for the extraction of polyphenols. For example, acetone showed to be very efficient in the extraction of polyphenols [59] from lychee (*Litchi chinenesis* Sonn.) flowers in comparison with methanol, ethanol or water. While in walnut (*Juglans regia* L.) green husks, the highest extraction yield of polyphenols (44.1%) was obtained when water was used as extraction solvent [60]. By other hand, in a recent study [61], it was found that aqueous and organic solvent have a higher extraction efficiency than absolute organic solvents. Similar situation was observed in *Phoradendron californicum* oak extracts [62], in which aqueous methanol was the solvent most efficient for the extraction of polyphenols.

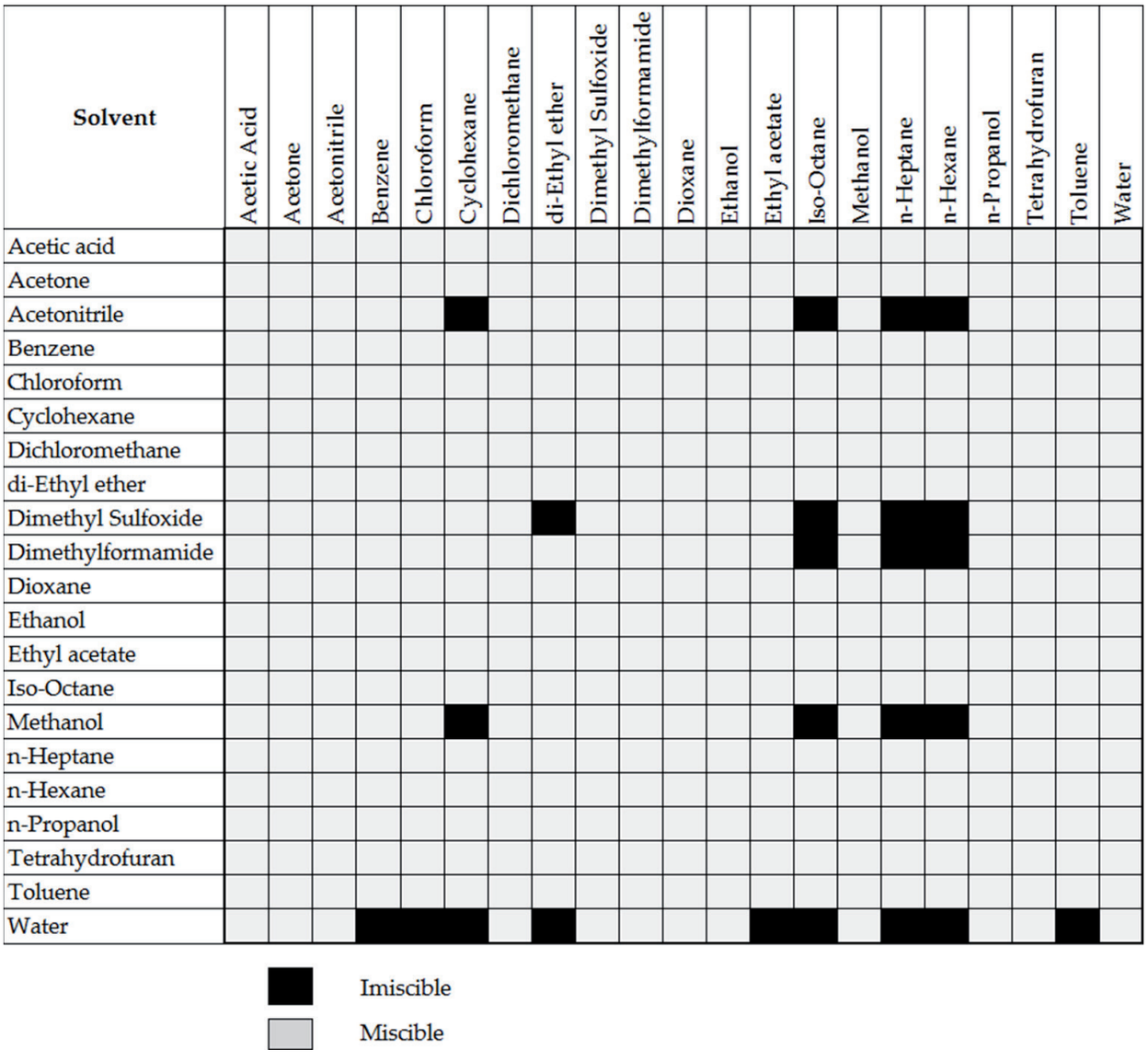

Figure 2. Miscibility of organic solvents used in the extraction of phytochemicals from natural sources. Adapted with permission from Refs. [56–58].

Based on the literature, there is no consensus about the best solvent to extract polyphenols. However, it has been widely accepted that higher polarity usually means better solubility of polyphenols into extraction solvents; however, differences in the structure of phenolic compounds may be critical for their solubility. Thus, the extraction of polyphenols and other phytochemicals must be prior tested and adapted to the solvent, because the diverse structures of polyphenols, such as multiple hydroxyl groups, conjugated or not with sugars, acids or alkyl groups, interfere in the extraction process. Therefore, it is very difficult to say what type of solvent is better to develop a standard method for all type of polyphenols, but the majority of the authors seem to agree that a good solvent system is the one that allows the maximization of polyphenols extracted without any modifi-

cations of their chemical nature. In this context, several factors must be considered when a specific solvent is selected, including (i) solvent power (selectivity); (ii) polarity; (iii) boiling temperature (should be low in order to facilitate removal of the solvent from the product); (iv) reactivity (the solvent should not react chemically with the extract neither should be decomposed quickly); (v) viscosity (low); (vi) stability (should be stable to heat, oxygen and light); (vii) safe in use (should be nonflammable and nontoxic for consumers and environment); (viii) if possible, suitability for reuse; and (ix) compatible with legislation for food applications.

3.2. Advanced methods of extraction

Classical extraction methods are dominated in many laboratory facilities mainly due to its simplicity and low economic cost. Nonetheless, many scientific reports have shown that maceration, percolation and Soxhlet extraction have low efficiency and several environmental issues due to the pollution caused in the environment when large volumes of organic solvents are used. Moreover, the classical extraction often requires a recovery step followed by evaporation to concentrate the extract, which makes it a high time-consuming process. To overcome these constraints, a number of methods have been developed in the last years such as microwave-assisted extraction (MAE); ultrasound-assisted extraction (UAE); supercritical CO_2 extraction (SC-CO_2); pressurized fluid extraction (PFE); enzyme-assisted extraction (EAE) or even combined approaches. From the hundreds of papers published until now, it seems that these novel extraction techniques can be an interesting choice for classical extraction methods, offering several advantages such as less extraction time length, less volume of solvents, less final toxic residues, higher extraction yields and better reproducibility.

Hundreds of works have been published (some of them listed in **Table 3**) [63–73] about the use of such methods to improve the extraction yield of polyphenols in different matrices. In the next paragraphs is presented a detailed description of each method.

3.2.1. The microwave-assisted extraction (MAE)

The MAE is a method that uses energy of microwave radiation to heat solvent in contact with the sample [74–77]. The heat produced increases the diffusivity of the solvent towards the powdered sample, extracting and diffusing the phytochemicals out of the matrix [77]. The disruption of hydrogen bonds, as a result of microwave-induced dipole rotation of molecules, enhances the penetration of the solvent into the matrix, allowing the dissolution of the components into the liquid matrices [78]. This method has the advantage of less consuming time and solvent volumes than the classical approach [77–79]. This method has been largely used for the extraction of monomeric polyphenols (short chains) such as phenolic acids and flavonoids [78–80], but it has been less used to extract polymeric polyphenols such tannins and anthocyanins, because polyphenols with a higher number of hydroxyl-type substituents (long-chains) and those sensitive to higher temperatures (e.g.,

Method	Botanic matrix	Results reported by the authors	References
MAE	Blueberries (*Vaccinium corymbosum* L.)	The usage of **MAE increased the yield of anthocyanins extracted**.	[63]
MAE	Grape seeds (*Vitis vinifera*)	The **MAE** was able **to extract maximum antioxidant phenolics** with lower solvent volume in a shorter time.	[64]
UAE	*Cassia auriculata* leaves	The **UAE** process **enhanced the phenolics extraction in less time**.	[65]
UAE	Mulberry pulp (*Morus nigra*)	The **UAE can be a reliable and economic** tool to extract both **anthocyanins** and **total polyphenols**	[66]
PLE	Mango (*Mangifera indica* L.)	**High yields** of **polyphenols** were obtained at flow rate.	[67]
PLE	Asparagus (*Asparagus officinalis* L)	**PLE revealed cheaper, faster and environmental friendly** and a **good alternative** for the extraction of natural compounds.	[68]
SC-CO_2	Pitanga leaves (*Eugenia uniflora* L.)	**SC-CO_2** allowed to obtain **extracts more concentrated in polyphenols**.	[69]
EAE	Pomegranate peels (*Punica granatum* L.**)**	The incorporation of **enzymes** in the maceration **improved the release of bound phenolics**.	[70]
EAE	Grape residues (*Vitis vinifera*)	**Celluclast®**, **Pectinex® Ultra®** and **Novoferm®** were used to release phenolic compounds from grape wastes. The pretreatment with **enzymes increased the yield of polyphenols extracted**.	[71]
Combined approaches	Broccoli inflorescences (*Brassica oleracea* L. var. italica)	A combined method of **EAE + UAE** was used to extract polyphenols. The combined methods enhanced the content of polyphenol and antioxidant activity	[72]
Combined approaches	Lemon balm (*Melissa officinalis L.*)	**EAE + PLE** were applied for the extraction of phytochemicals. The results showed that **EAE + PLE enhanced the total phenolic content and the antioxidant capacity**.	[73]

Table 3. Application of advanced methods in extraction of polyphenols in different foods and agro-waste matrices [63–73].

anthocyanins) may be degraded under MAE extraction conditions [79, 80]. The temperature used for extraction is proportional to the power (watts) and time and inversely proportional to the heat capacity of the solvent and the mass of sample [80]. Higher temperatures and small amounts of sample increase the rate of solvent diffusion and promote faster extraction kinetics [80].

Numerous phytochemical compounds, including polyphenols, have been extracted MAE system as shown in **Table 4**. It seems that MAE system provides higher polyphenols yield in less consuming time and solvents. However, there are some concerns when the MAE is used to extract polyphenols. Factors such as type of matrix, type and purity of solvent, the microwave application time, power, contact sample surface area and temperature can affect their efficiency. One of the most critical factors is the nature of solvent, which affects not only the solubility of the target components but also the efficiency of all physical process. The choice of solvent must take into account not only the affinity to the target phytochemicals but also the ability to absorb microwave energy [81]. For example, solvents like hexane or dichloromethane, which are transparent to microwaves, do not heat up under microwave [82, 83]; thus, they should not be used in this system. Others, such as ethanol, methanol, or even water, have good microwave absorbing capacity [83], and they get heated up faster; thus, the length of the time and microwave power must be adapted to the solvent to enhance the extraction process without any deleterious effect on thermolabile components.

3.2.2. The ultrasound-assisted extraction (UAE) method

The UAE is a very simple method that relies on the mechanical effect caused by the implosion of micro-sized bubbles, which cause a rapid tissue disruption allowing the release of compounds into the solvent [84]. This is a very simple method with relatively low cost, and it can be used on both small laboratory and large industrial scale [84, 85]. The use of UAE has been widely used in the last years in the extraction of polyphenols from different parts of plants such as leaves, stems, stalks, fruits, seeds [85–93]. In general, the experimental procedure involves the use of ultrasounds with frequencies ranging from 20 to 2000 kHz, which increases the permeability of cell walls and produces cavitation.

Several studies have reported that UAE allows a better and faster extraction of polyphenols with less degradation when compared with other extraction methods. For example, UAE shown to be highly efficient in the extraction of carnosic acid and rosmarinic compared to classical methods of extraction [94]. In a recent study [95], the maximum extraction yield of total polyphenols (13.2 mg/g dry weight) from spruce wood bark was obtained when UAE system was used. Also, an increment in anthocyanin content in purple sweet potato was observed when UAE was used [96]. All these studies have in common the same trend: under UAE, the rate speed dissolution of compounds into extraction solvent was always higher, and thus, the solvent volume used and need to extract phytochemicals was lower compared to the classical extraction methods. Based on these studies and others, it seems that UAE has the advantage of being less expensive due to lower solvent volume used, higher amount of samples tested and lower time needed to perform the extraction process. Also, they agree that the lower temperatures and shorter sonication periods (time) are better to enhance the extraction of polyphenols contributing also to the preservation of the thermolabile and unstable compounds. However, some studies [97, 98] reported that sonication for long periods (>40 min) with higher energy levels (above >20 kHz) could have a deleterious effect on phytochemicals due to the decrease of diffusion area and diffusion rate and increased diffusion distance, leading to a

global decreased yield of total phenolic and flavonoid content. Moreover, under these conditions might occur the formation of free radicals and consequently undesirable changes in the drug molecules [97].

3.2.3. Pressurized liquid extraction (PLE)

The PLE method, also known as "accelerated solvent extraction (ASE)," is a very recent new technology for phytochemicals extraction including polyphenols, which associates high temperature and pressure [99]. In this method, high level of pressure (normally between 3.3 and 20.3 MPa) is combined with high level of temperatures (between 40 and 200°C) to improve the solubility and desorption of molecules, increasing their movement from matrix into solvents, and thus increasing the yield of polyphenols extracted [54]. According to Nieto et al. [99], the PLE method is an advanced technique that provides a faster extraction processes and requires a small amount of solvents when compared with the classical extraction approach. Moreover, it allows better the usage of water as extraction solvent, which is limited in the other previous methods. The use of water as an extraction solvent in PLE, as so-called subcritical water extraction (SWE), is always possible, particularly when elevated temperatures are used [100]. When temperatures around 200°C are used, a change in the dielectric water properties occurs, and then, the water behaves like a normal organic solvent, increasing their extraction efficiency [101]. The main advantages of PLE often reported by several researchers are cleanness of the extracts that PLE provides in comparison with classical maceration, Soxhlet, MAE and UAE, which results in reduced background noise during the subsequent analytical quantification, is especially important when the LC-MS analysis due to ion-suppression effects [102]. By opposition, the main limitations often reported are the low selectivity towards the analytes during extraction, and many interferents may be extracted during the extraction process, an exaggerated dilution of the analytes, especially when a large number of cycles are used, and the high requirements in instrumentation, which increases their costs [103–105]. However, these limitations in PLE are a well-known extraction technique and have been used for the extraction of polyphenols from several different matrices [106–111].

3.2.4. The supercritical CO_2 extraction (SC-CO_2)

The SC-CO_2 extraction is a process in which the CO_2 is used as supercritical fluid and probably is one of the most widely used fluid because it is nontoxic, nonflammable, inert cheap and easily available in high quantity with high grade of purity [112]. SC-CO_2 extraction is possible to use different combinations of temperature and pressure [112], making this method one of the most versatile for creating a multitude of end products. Due to the multitude of combinations, low temperatures (31.6°C, the critical point of carbon dioxide) and pressure (7.386 MPa) are needed, and the SC-CO_2 has been considered very popular in a lab-scale laboratorial facilities. Moreover, since low temperatures and pressure are used, there is a good preventing of thermal degradation of phytochemicals. The main advantage s of SC-CO_2 are [112–116] as follows: (i) more extraction capacity due to their higher diffusion coefficient and lower viscosity than the liquids, which increases a higher mass transfer from solid matrix towards solvents; (ii) it allows higher penetration of solvents into the matrices which increase

the effectiveness and polyphenols extraction yield; (iii) it allows different combinations of pressure and temperature and thus allows a better adaptation of the extraction conditions to the different types of food and plant matrices, increasing the solubility of their different components in the supercritical fluids; (iv) it allows the CO_2 recycling at the end of the process, without any disgrace of chemical residue to environment at the end of the extraction and separation process.

3.2.5. Enzyme-assisted extraction (EAE)

The EAE is a recent method and is based on the capacity of the enzymes to degrade cell wall components into solvents, in general water, with high stability and high bioactivity [117]. In EAE, the enzymes added to food, plant matrices or agro-food wastes are capable to break and weaken the cell walls, increasing the exposure of their cellular components to extraction [71, 118], and thus increasing the capacity to extract polyphenols from the matrices. In fact, some phytochemicals are dispersed in plant cell cytoplasm, and even, some compounds are bound with the polysaccharide-lignin by hydrogen or hydrophobic chain, which are not accessible with a routine organic solvents [119, 120]. Thus, a previous treatment with enzymes can be the only choice, and an enzymatic pretreatment might be the unique and effective way to release bounded compounds from cells [121].

Cellulases, hemicelullases, pectinases and other enzymes may be used to hydrolyze efficiently the cell wall components, enabling the efficiency of extraction of phenolic compounds. Several papers have been published about the positive effect of EAE on increment of polyphenol extraction yield. In 2012, in a study with grape wastes [71], it was found a strong increment in the release of polyphenols when celluclast®, pectinex® and novoferm® enzymes were used. Similar trends were noted in other works [122, 123] which concluded that EAE should be regarded as an alternative method for improved extraction of insoluble-bound phenolics (linked to carbohydrates and proteins of cell wall matrices) from winemaking by-products. These and many other authors observed that the ability of enzymes to degrade cell walls and membranes enables the extraction efficiency of bioactive compounds, and in several situations, the EAE technology might be the unique way to extract effectively bioactive compounds from foods and agro-industrial by-products. In addition to these advantages, the EAE method has been recognized as one of the most eco-friendly methods, because it uses water as solvent instead of organic chemicals, often toxics [119], and is one of the modern extraction methods that are gaining more attention because of the need for eco-friendly extraction technologies.

3.2.6. Combined approaches

In some circumstances, it is possible to find different studies in which the extraction of phytochemical is done throughout combined methods. This occurs, particularly in situations in which a single extraction method is not as efficient as we would expect, and thus, a combination of extraction processes could be the unique effective method to obtain extracts with different polyphenols.

4. The identification and measurements of phenolics

There is a great diversity of studies about the development of new methods for polyphenol quantification. The high-performance liquid chromatography (HPLC) with or without mass spectrometry (MS) is one of the most commonly applied method to identify and quantify polyphenols. However, the classical spectrophotometric assay is still used, even if their results are limited.

4.1. The classical colorimetric methods

The classical spectrophotometry UV/Vis method [124], even with modifications, is still widely used to measure total phenolic content in plant materials. This method is based on the chemical reduction of polyphenols in an alkaline medium to form a blue chromophore complex (phosphomolybdic/phosphotungstic acid) that can be quantified by visible-light spectrophotometry (at 760 765 nm). Many studies have discussed the advantages and disadvantage of using routinely this method to quantify the level of polyphenols, and most of them seems to agree that although they are easy to perform, low cost, rapid and applicable routinely in the most laboratories, they are not accurate. In addition, the reagents used in the method do not react specifically with only polyphenols, and they react with any reducing substance like ascorbic acid, pigments, aromatic amines and sugars [125], and thus, these methods measure the total reducing capacity and not just the polyphenols compounds. Also, their reagents react with some nitrogen-containing compounds such as hydroxylamine and guanidine [126], thiols, many vitamins and some inorganic ions [127] Therefore, many researchers have chosen to use this method only as an indicative tool of total reduction capacity and not for a specific quantification of polyphenol compounds. However, these methods are still considered useful for a quick and prior screening of numerous samples, and for many applications, a simple measure of total amount of polyphenols is enough.

Similar to total polyphenols, total flavonoids can be measured by spectrophotometry methods, and the $AlCl_3$ method [128, 129] is the most vulgarized method used to determine the total flavonoid content. Vanillin and 4-(dimethylamino)-cinnamaldehyde (DMCA) assays are often used to determine the level of proanthocyanidins, in which the flavonoid catechin is used as standard [130, 131]. Like in total polyphenols, the vanillin or DMCA method can overestimate the amount of total flavonoids present in samples. The proanthocyanidins can also be determined by butanol-HCl [132] and bovine serum albumin (BSA) [133] methods. The butanol-HCl method is based on the cleavage of the flavonoid bonds by hot acid, followed by an auto-oxidation reaction which converts flavan-3-ols into anthocyanidins. The red extract formed has a maximum absorbance at around 550 nm. In the BSA method, the flavonoids complex is dissolved in an alkaline solution (sodium decyl sulphate-triethanolamine) followed by a reaction with ferric chloride solution to form a violet complex with a maximum absorbance at 510 nm.

Another spectrophotometric method widely used in the quantification of polyphenols is the UV/Vis spectrophotometry method to determine the anthocyanin content. The anthocyanins constitute one of the main class of polyphenols largely present in plant samples, particularly

in red, blue and black color fruits such as grapes, blueberries, raspberries, redcurrants, blackcurrants, pomegranates and strawberries, among others. The quantification of anthocyanins is in general performed by the differential pH method [134] based on the property of the anthocyanin pigments to change the color with pH, in the wavelength ranging from 490 to 550 nm [134]. The anthocyanins suffer reversible structural modification with a change of pH, and this change allows to estimate spectrophotometrically the total amount of anthocyanins, even in the presence of degraded pigments and other interfering compounds.

All these spectrophotometric methods are considered simple and cheap, but only gives a general estimation about the content of each class of polyphenols but do not allow the quantification of polyphenols individually.

4.2. Chromatography

In the course of the last four decades, several chromatography methods were developed to overcome the main constraints of the classical spectrophotometry methods. The development of new technologies and software led to the appearance of improved methods capable of separation, identification and quantification of phytochemicals individually. These methods are generally based on the principle that a sample is composed of a mixture of components which are separated when the mixture passes through two phases: a mobile (liquid or gaseous) and a stationary (solid, liquid or gel). It is used for the qualitative and quantitative analysis, and the components are separated and analyzed according the properties of a given solution. The great diversity of combinations between the two phases makes possible an existence of several differentiated techniques.

4.2.1. Principles

The basic principles of chromatography are universal [135] and thus widely accepted by all researchers. The main principles are more or less the following ones: (i) chromatography is a physical and chemical method of separating, identification and quantification of different components of samples; (ii) the separation always dependent of the interaction between the components of the mixture with the mobile and stationary phases, and, thus a large combinations of the three are possible; (iii) the interaction of the matrices components with the mix of both phases is influenced by different intermolecular forces including ionic, dipole, nonpolar and effects of specific affinity or solubility; (iv) the mobile phase is generally named as eluent, and the absorbent material, named as stationary phase; (v) the analyte is the compound to be separated; (vi) a chromatogram is the visual output of the chromatograph; (vii) the instrument used for qualitative and quantitative detection of analytes after separation is named as detector; (viii) the separation of components present in the mixture occurs according to the different chemical affinity for the stationary phase, and it happens as the eluent advances on the stationary phase; (ix) the separation of compounds is slower when compounds have strong interaction with the stationary phase and faster when the components have weaker interaction with the stationary phase, and by this, the compounds will be separated from each other as they move over the support material; (x) the component to be analyzed must have solubility with the mobile phase, and different compounds have different retention time

values; (xi) the identification and quantification of components in the mixtures are done by comparison with pure commercial standards of known commercial concentration, through analytical curves.

The chromatography can be classified according to several criteria [135, 136], but in general, the chromatography applied in separation, identification and quantification of phytochemicals is classified as:
(i) Gas chromatography (GC), when gas chromatography makes use of a pressurized gas cylinder and a carrier gas (e.g., helium), to carry the solute through the column. The most common detectors used in this type of chromatography are of thermal conductivity and flame ionization detectors. There are three types of GC as follows: (1) gas adsorption, (2) gas-liquid, and (3) capillary gas chromatography.
(ii) Liquid chromatography (LC), when a liquid adsorbent is used. This method is used in large-scale applications since adsorbents are relatively inexpensive. There is a liquid-liquid chromatography which is analogous to gas-liquid chromatography. The three types of modern LC are as follows: (1) reverse phase, (2) high performance and (3) size exclusion liquid chromatography, along with supercritical fluid chromatography.
(iii) Ion exchange chromatography (IEC), when charged molecules mobile phase passes through the column, until a binding site in the stationary phase appears and retains the molecules. There are two types of ion exchange chromatography: (1) cation exchange in which the stationary phase carries a negative charge, and (2) anion exchange in which the stationary phase carries a positive charge. The method is mainly used in the purification of biological materials.
(iv) Affinity chromatography (AC), which is a technique that involves the chemical modification of a given compound by attaching another compound with a specific affinity for the desired molecules. This method requires that the compounds to be analyzed must be inert and easily to modify, and otherwise, it can be very difficult to perform, and a large number of impurities can appear. Therefore, this type of technique is only used in advanced processes of purification.

4.2.2. The use of HPLC-DAD/UV-VIS and HPLC-MS

The high-performance liquid chromatography (HPLC), referred in the past as high-pressure liquid chromatography, like other chromatography methods is a technique used to separate, identify and quantify phytochemicals from plant mixtures and relies on pumps to pass a pressurized solvent containing the plant samples, foods or other matrices through a column filled with a solid adsorbent material (e.g., silica) [137]. The HPLC methods, however, differ from other liquid methods, particularly from "low pressures," because it uses high pressures (ranging from 50 to 350 bar), while the others normally use the force of gravity to pass the mobile phase through the column [137, 138]. Each component of the samples interacts differently with the adsorbent material of the column, causing a different flow rate for the different components in the mixture, thus leading to the separation of the components as they flow out the column. The columns used in the HPLC methods are made with smaller adsorbent particles size ranging from 2 to 50 μm [137, 138].

Although there is many variations in the HPLC equipment available in the market, the basic HPLC equipment includes a sampler (to carry the sample mixture into the mobile phase), pumps (to deliver mobile phase through the column, with a specific flow) and a detector (such as UV/Vis or photodiode array (PDA), which generates signal proportional to the amount of compound present in the sample mixture [138]. The signal detected allows the identification and quantification of sample components. Each compound detected has a specific retention time; however, due to the interaction strength of interactions between the analytes and stationary phases, the retention time can vary. Nowadays, modern HPLC equipment has a digital processor, which uses a software interface to control the instruments and provides data analysis. Other modern models are equipped with several pumps, which allow different combinations of various solvents at different ratios changing in time, creating a gradient in the mobile phase.

Nowadays, the classic HPLC evolved to HPLC coupled with a mass spectrometry detector (MS), called as LC-MS or HPLC-MS [138]. This new technique allows a more accurate identification which is based on the specific fragmentation of each separated molecule. This enhances the sensitivity and is oriented for the separation of chemicals with specific masses in a complex mixture. The separation of molecules or fragments occurs according to their mass-to-charge ratio in an analyzer by electromagnetic fields. The ions are detected by a qualitative and quantitative analysis, and the signal is processed into mass spectra. The HPLC-MS equipment is in general composed of three modules: (1) an ion source, which converts gas phase sample molecules into ions; (2) a mass analyzer, which sorts the ions by their masses by applying electromagnetic fields; and (3) a detector, which measures the value of the signal detected and provides data for the quantification of each ion present. In the last years, this new method has been strongly

Method	Purposes	Reference
HPLC-ESI-MSn,	Separation and identification of the main polyphenols in black currant (*Ribes nigrum* L.)	[139]
HPLC-MSn	Identification and quantification of phenolic compounds in hazelnut (*Corylus avellana* L.) kernels, oil and bagasse pellets	[140]
HPLC-DAD-ESI-MS/MS	Comparative fingerprint and quantification of polyphenol of aqueous extracts of *Cornus mas* and *Crataegus monogyna*.	[141]
HPLC-PDA–ESI-MS/MS	Identification of phenolic acids, flavonol glycosides in blueberry (*Vaccinium corymbosum* L.), blackberry (*Rubus fructicosus* L.), raspberry (*Rubus idaeus* L.) and cranberry (*Vaccinum vitis-idaea* L.)	[142]
HPLC-DAD-ESI-MS/MS	Characterization of chemical profile of mango (*Mangifera indica* L.)	[143]
HPLC-PDA–ESI-MS/MS	Determination of anthocyanins in cherry (*Prunus avium* L.) and cranberry (*Vaccinium oxycoccos* L.)	[144]
HPLC-DAD–ESI-MS/MS	Characterization of phenolic compounds from Turkish black tea (*Camellia sinensis* L. Kuntze)	[145]

Table 4. Some recent examples of the usage of HPLC-MS system for separation and quantification of polyphenols in different matrices.

implemented in academies in basic research, pharmaceutical and agro-chemical industries to study physical, chemical and biological properties of a great diversity of compounds, as well, and quality control of drugs, foods and natural products. In **Table 4** are presented some recent works [139–145] in which HPLC-MS was effectively used for polyphenol characterization of plants and food with very accurate results.

5. Conclusions

This chapter discusses the importance of polyphenols as well as the availability of different methods to extract them from its natural sources. The most widely used methods in the extraction of polyphenols are the classical ones which usually includes maceration, percolation and successive Soxhlet extractions. Although these methods are still in use, they involve long extraction times, huge quantities of solvent, higher accumulation of residues and very limited results. Therefore, new methods such as UAE, MA, PLE, S-CO_2 and EAE have been developed in the recent years with very feasible results. Also, the evolution of separation and identification techniques of polyphenols has evolved from a simple colorimetry method to the most advanced chromatography techniques. However, the growing demand for new bioactive molecules from natural sources enhances the continuous search for new and innovative methods to extract and separate new molecules, which never ends.

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