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Review

A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables

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ABSTRACT

Phenolic compounds, ubiquitous in plants, are of considerable interest and have received more and more attention in recent years due to their bioactive functions. Polyphenols are amongst the most desirable phytochemicals due to their antioxidant activity. These components are known as secondary plant metabolites and possess also antimicrobial, antiviral and anti-inflammatory properties along with their high antioxidant capacity. Many efforts have been made to provide a highly sensitive and selective analytical method for the determination and characterisation of polyphenols. The aim of this paper is to provide information on the most recent developments in the chemical investigation of polyphenols emphasising the extraction, separation and analysis of these compounds by chromatographic and spectral techniques.

Contents

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1. Introduction

Phenolic compounds are secondary plant metabolites, which are important determinants in the sensory and nutritional quality of fruits, vegetables and other plants. (Tomas-Barberan, Ferreres, & Gil, 2000; Lapornik, Prosek, & Golc, 2005).

These compounds posses an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular mass polymer (Balasundram, Sundram, & Samman, 2006).

These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants. As a large group of bioactive chemicals, they have diverse biological functions. Phenolics may act as phytoalexins (Popa, Dumitru, Volf, & Anghel, 2008), antifeedants, attractants for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light, amongst others (Naczk & Shahidi, 2006). These bioactive properties made these compounds play an important role in plant growth and reproduction, providing an efficient protection against pathogens and predators (Popa, Agache, Beleca, & Popa, 2002; Bravo, 1998), besides contributing to the colour and sensory characteristics of fruits and vegetables (Alasalvar, Grigor, Zhang, Quantick, & Shahidi, 2001).

In particular, natural phenols have been reported to have excellent properties as food preservatives (Valenzuela, Nieto, Cassels, & Speisky, 1992) as well as having an important role in the protection against a number of pathological disturbances, such as atherosclerosis, brain dysfunction and cancer (Gordon, 1996). Moreover, polyphenols have many industrial applications, for example, they may be used as natural colourants and preservatives for foods, or in the production of paints, paper, and cosmetic.

For these reasons, great effort has been made to characterise the phenols occurring in different plant tissues (Pinelo, Del Fabbro, Manzocco, Nunez, & Nicoli, 2005).

The aim of this study is to present some valuables sources of polyphenols, the main classes of phenols found in fruits, vegetables and other herbs along with their bioactive proprieties. Isolation and characterisation techniques of this bioactive compound are the most important steps in the practical application of the polyphenols. The paper presents different separation and characterisation methods, that were used in the last years. The main advantages as well as the limitation of each method were reported in order to establish the most feasible methods for polyphenols analysis.

2. The main classes of polyphenolic compounds

Phenolic compounds comprise a wide variety of molecules that have a polyphenol structure (i.e. several hydroxyl groups on aromatic rings), but also molecules with one phenol ring, such as phenolic acids and phenolic alcohols. Polyphenols are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another. The main groups of polyphenols (Fig. 1) are: flavonoids, phenolic acids, tannins (hydrolysable and condensed), stilbenes and lignans (D'Archivio et al., 2007).

2.1. Flavonoids

More than 8000 polyphenolics, including over 4000 flavonoids have been identified, and the number is still growing (Harborne, Baxter, & Moss, 1999). Flavonoids can be further classified into anthocyanins, flavones, isoflavones, flavanones, flavonols and flavanols (Tsao and Yang, 2003). The chemical structures of the main classes of flavonoids are presented in Fig. 2.

Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C6–C3–C6 configuration. Essentially the structure consists of two aromatic rings, A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C. The aromatic ring A is derived from the acetate/malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway (Merken & Beecher, 2000).

Variations in the substitution patterns of ring C result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins (Hollman & Katan, 1999), of which flavones and flavonols are the most widely occurring and structurally diverse (Harborne, Baxter, and Moss, 1999). Substitutions to rings A and B give rise to different compounds within each class of flavonoids (Pietta, 2000). These substitutions may include oxygenation, alkylation, glycosylation, acylation, and sulphonation (Balasundram, Sundram, & Samman, 2006).

Flavonoids are especially important antioxidants due to their high redox potential, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelating potential (Tsao & Yang, 2003).

Flavonoids are the most commonly found phytochemicals, that typically these chemicals help to protect the plant against UV light, fungal parasites, herbivores, pathogens and oxidative cell injury (Cook & Samman, 1996). When consumed regularly by humans, flavonoids have been associated with a reduction in the incidence of diseases such as cancer and heart disease (Beecher, 2003; Cook & Samman, 1996; Liu, Cai, & Shao, 2008). There is currently great interest in flavonoid research due to the possibility of improved public health through diet, where preventative health care can be promoted through the consumption of fruit and vegetables. Flavonols are a class of flavonoids commonly found in many fruits and vegetables, their content varying widely, depending on environmental factors, such as growing conditions, climate, storage and cooking conditions (Caridi et al., 2007).

Flavanones are characterised by the presence of a saturated three-carbon chain and an oxygen atom in the C4. They are generally glycosylated by a disaccharide in C7. Flavanones are present in high concentrations only in citrus fruit, but they are also found in tomatoes and certain aromatic plants such as mint. The main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons.

Isoflavones have structural similarities to estrogens, i.e. hydroxyl groups in the C7 and C4, positions, like estradiol molecule. They are phytochemicals that are found in many plants and plant-derived foods in both native ("aglycon") form and as acetyl-, or malonyl-, etc., β-glucosides. Important health effects are attributed to them, and so it has been suggested that they should be used for the prevention or cure of prevalent diseases such as atherosclerosis or cancer. Some physiological effects are attributed to their structural similarities to β-estradiols,

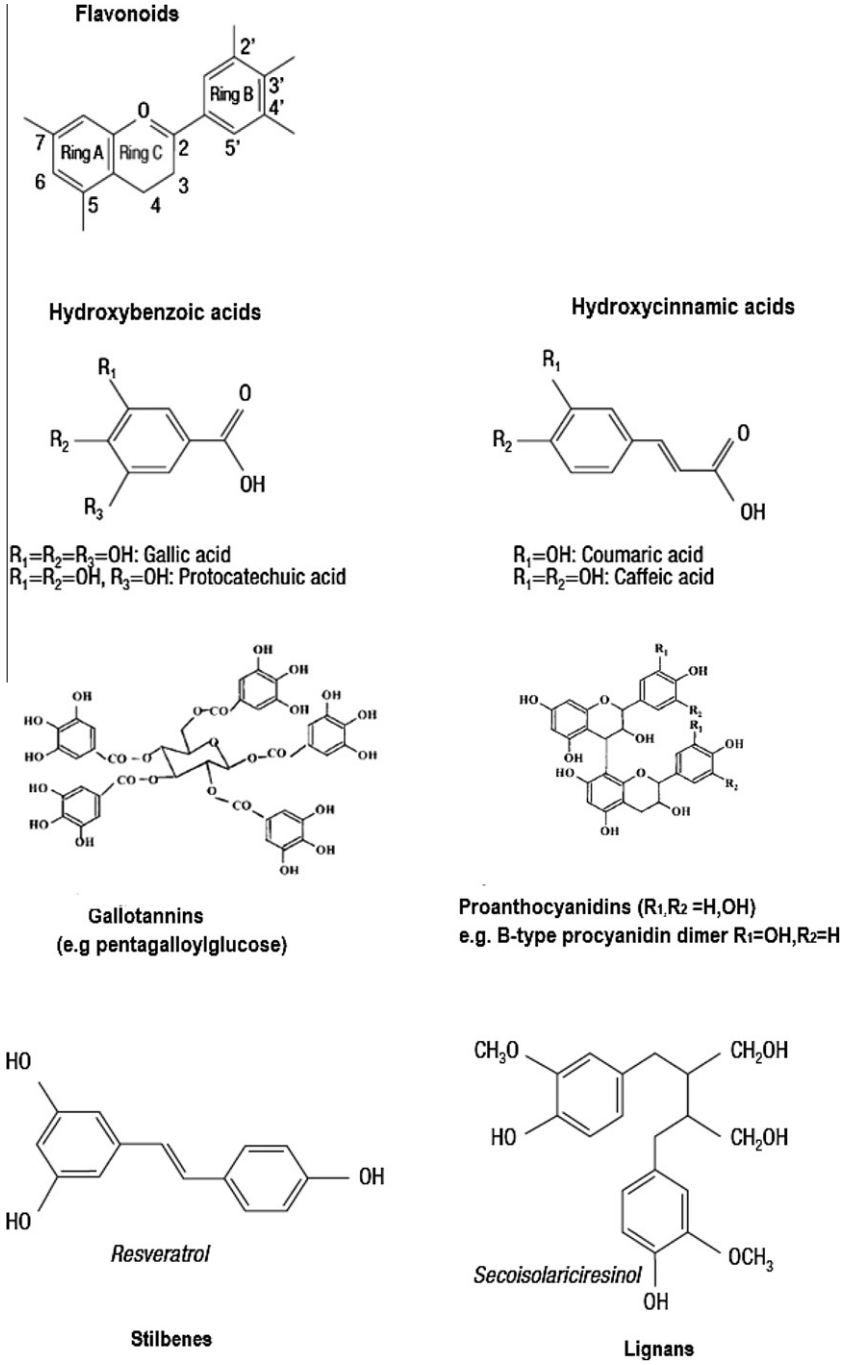

Fig. 1. Chemical structures of the main classes of phenolic compounds.

and they are occasionally referred to as "phytoestrogens" (Klejdus et al., 2007; D'Archivio et al., 2007).

Anthocyanins are water-soluble vacuolar pigments that may appear as red, purple, or blue depending on pH. They belong to a parent class of molecules called flavonoids, synthesised *via* the phenylpropanoid pathway. Anthocyanins occur in all plant tissues, including leaves, stems, roots, flowers, and fruits.

The anthocyanidins are the basic structures of the anthocyanins. The anthocyanidins (or aglycons) consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring B (Konczak & Zhang, 2004). When the anthocyanidins are found in their glycoside form (bonded to a sugar moiety) they are known as anthocyanins.

The glycoside derivatives of the three non-methylated anthocyanidins (pelargonidin-*Pg*, cyaniding-*Cy*, delphinidin-*Dp*) are the most common in nature, being found in 80% of pigmented leaves, 69% in fruits and 50% in flowers (Dey & Harborne, 1993).

Six anthocyanidins occur most frequently in plants: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. The sugars commonly linked to anthocyanidins are monosaccharides (glucose, galactose, rhamnose and arabinose), and di- or tri-saccharides formed by combination of the four monosaccharides (Bureau, Renard, Reich, Ginies, & Audergon, 2009). Moreover, many anthocyanins have sugar residues acylated with aromatic or aliphatic acids (Mazza & Miniati, 1993). The isolated anthocyanins are highly instable and very susceptible to degradation (Giusti & Wrolstad, 2003). Their stability is affected by several

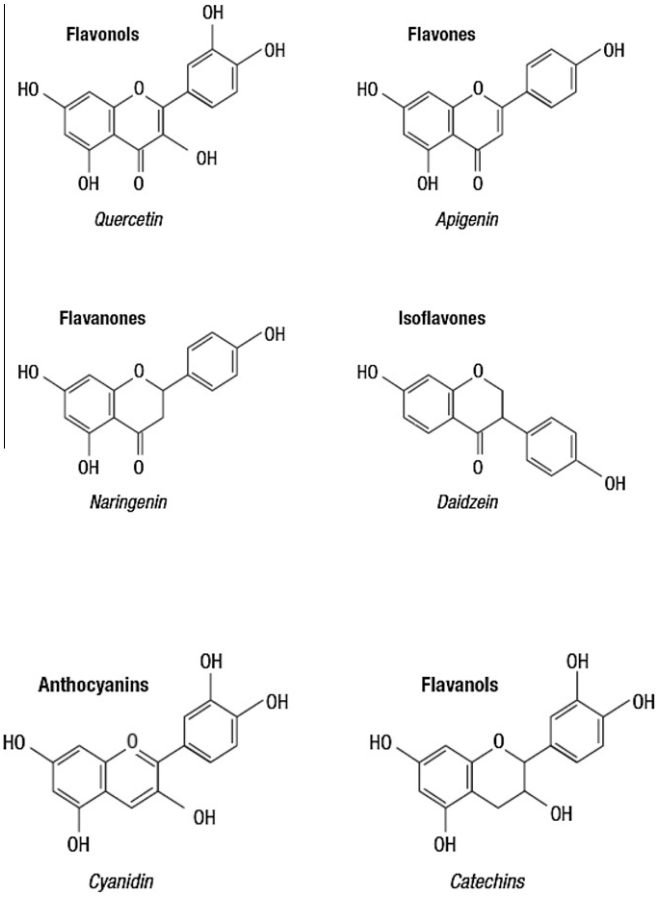

Fig. 2. Chemical structures of flavonoids.

factors such as pH, storage temperature, chemical structure, concentration, light, oxygen, solvents, the presence of enzymes, flavonoids, proteins and metallic ions (Castañeda-Ovando et al., 2009). Anthocyanins, as well as other phenolics, can act as antioxidants by donating hydrogen to highly reactive radicals, thereby preventing further radical formation (Iversen, 1999). Their antioxidant potential is dependent on the number and arrangement of the hydroxyl groups and the extent of structural conjugation, as well as the presence of electron-donating and electron-withdrawing substituents in the ring structure (Lapornik et al., 2005). Anthocyanins possess well-known pharmacological properties and strong biological functions such as anti-inflammatory and antioxidant activities (Kong, Chia, Goh, Chia, & Brouillard, 2003). Phenolic compounds including anthocyanins, flavonoids and phenolic acids, are known to be responsible for antioxidant capacities in fruits, the fruits with higher phenolic contents generally showing stronger antioxidant capacities (Fang et al., 2009). In recent years, synthetic food dyes have been banned in many countries because of their toxicity and carcinogenicity. Anthocyanins, coloured natural compounds easily obtained from fruits and vegetables, can be considered potential substitutes for the banned food dyes: they have, in fact, bright attractive colours, while their high water solubility in water of these compounds allows their easy incorporation into aqueous food systems (Kammerer, Carle, & Schieber, 2004). Moreover, the proved antioxidant activity of anthocyanins, related to the prevention of a number of degenerative diseases (Ames, Shigena, & Hagen, 1993; Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005) provides additional benefits to the food dyed with these natural substances (Bleve et al., 2008).

2.2. Phenolic acids

Phenolic acids constitute about one-third of the dietary phenols, which may be present in plants in free and bound forms (Robbins, 2003). Bound-phenolics may be linked to various plant components through ester, ether, or acetal bonds (Zadernowski, Czaplicki, & Naczk, 2009). The different forms of phenolic acids result in varying suitability to different extraction conditions and different susceptibilities to degradation (Ross, Beta, & Arntfield, 2009). Phenolic acids consist of two subgroups, the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have in common the C6–C1 structure. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C6–C3), caffeic, ferulic, *p*-coumaric and sinapic acids being the most common representatives (Bravo, 1998).

2.3. Tannins

Tannins, relatively high molecular compounds which constituting the third important group of phenolics, may be subdivided into hydrolysable and condensed tannins (Porter, 1989). Proanthocyanidins (condensed tannins) are polymeric flavonoids. Although the biosynthetic pathways for flavonoid synthesis are well understood, the steps leading to condensation and polymerisation have not been elucidated. The most widely studied condensed tannins are based on flavan-3-ols (−)-epicatechin and (+)-catechin.

Hydrolysable tannins are derivatives of gallic acid (3,4,5 trihydroxyl benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolysable tannins (Hagerman, 2002).

A third subdivision, the phlorotannins consisting entirely of phloroglucinol, has been isolated from several genera of brown algae (Porter, 1989), but these are not significant in the human diet (Bravo, 1998).

Tannins have diverse effects on biological systems since they are potential metal ion chelators, protein precipitating agents and biological antioxidants. Because of the varied biological roles that tannins can play and because of the enormous structural variation, it has been difficult to develop models that would allow an accurate prediction of their effects in any system. An important goal of future work on the biological activities of tannins is the development of structure/activity relationships so that biological activities can be predicted (Hagerman, 2002).

2.4. Stilbenes and lignans

Low quantities of stilbenes are present in the human diet, and the main representative is resveratrol, that exists in both *cis* and *trans* isomeric forms, mostly in glycosylated forms (Delmas, Lancon, Colin, Jannin, & Latruffe, 2006) It is produced by plants in response to infection by pathogens or to a variety of stress conditions (Bavaresco, 2003). It has been detected in more than 70 plant species, including grapes, berries and peanuts.

Lignans are produced by oxidative dimerisation of two phenylpropane units; they are mostly present in nature in the free form, while their glycoside derivatives are only a minor form. The interest in lignans and their synthetic derivatives is growing because of potential applications in cancer chemotherapy and various other pharmacological effects (Saleem, Kim, Ali, & Lee, 2005).

3. Natural source of polyphenols

Polyphenols are widely distributed in plants, such as fruits, vegetables, tea, olive oil, tobacco and others. The plant kingdom offers

Table 1
Dietary sources of plant phenolics (Naczk & Shahidi, 2006).

Phenolic compounds	Dietary sources
Phenolic acids	
Hydroxycinnamic acids	Apricots, blueberries, carrots, cereals, pears, cherries, citrus fruits, oilseeds, peaches, plums, spinach, tomatoes, eggplants
Hydroxybenzoic acids	Blueberries, cereals, cranberries, oilseeds
Flavonoids	
Anthocyanins	Bilberries, black and red currants, blueberries, cherries, chokecherries, grapes, strawberries
Chalcones	Apples
Flavanols	Apples, blueberries, grapes, onions, lettuce
Flavanonols	Grapes
Flavanones	Citrus fruits
Flavonols	Apples, beans, blueberries, buckwheat, cranberries, endive, leeks, lettuce, onions, olive, pepper, tomatoes
Flavones	Citrus fruits, celery, parsley, spinach, rutin
Isoflavones	Soybeans
Xanthones	Mango, mangosteen
Tannins	
Condensed	Apples, grapes, peaches, plums, mangosteens, pears
Hydrolysable	Pomegranate, raspberries

a wide range of natural antioxidants. Consequently, antioxidants have become an essential part of the preservation technology and contemporary health care. The potential toxicity of some synthetic antioxidants, however, has intensified research efforts to discover and utilise antioxidants from natural sources, such as fruits and vegetables (Popa, Danaila, Volf, & Popa, 2007; Zhang et al., 2009).

The most common sources of plant phenolics are presented in Table 1.

3.1. Agro-industrial by-products as a source of phenolic compounds

Amongst fruits, vegetables and different herbs, agricultural and industrial residues are attractive sources of natural antioxidants (Moure et al., 2001; Volf & Popa, 2004; Volf, Mamaliga, & Popa, 2006). Special attention is focused on the extraction from inexpensive or residual sources from agricultural industries. By-products, remaining after processing fruits and vegetables in the food processing industry, still contain a huge amount of phenolic compounds. Some studies have already been done on by-products, which could be potential sources of antioxidants.

One of the richest sources are berry skins, which during wine and juice making remain as husks and are usually made into compost (Larrosa, Llorach, Espin, & Tomas-Barberan, 2002; Lapornik et al., 2005).The olive mill wastes are also a major potential source of phenolics. The phenolic content of the olive mill waste water (OMWW) is reported to fluctuate between 1.0% and 1.8% (Visioli & Galli, 2003) depending on varietals factors and processing effects. The major components in OMWW include hydroxytyrosol, tyrosol, oleuropein, and a variety of hydroxycinnamic acids (Obied et al., 2005). Besides OMWW, olive leaves are another by-product of the olive industry that has been explored as a source of phenolics (Benavente-Garcia, Castillo, Lorente, Ortuno, & Del Rio, 2000).

The citrus industry produces large quantities of peel and seed residues, which may account for up to 50% of the total fruit weight (Bocco, Cuvelier, Richard, & Berset, 1998). Citrus industry by-products, if utilised optimally, could be major sources of phenolic compounds as the peels, in particular, have been found to contain higher amounts of total phenolics compared to the edible portions (Balasundram et al., 2006). Sreenath, Crandall, and Baker (1995) also studied citrus by-products, while by-products obtained after artichoke, cauliflower, carrot, celery and onion processing were investigated by Larrosa et al. (2002).

The peels of several other fruits have also been found to contain higher amounts of phenolics than the edible fleshy parts. Apple peels were found to contain up to 3300 mg/100 g dry mass of phenolics (Wolfe & Liu, 2003), while the lypholisate recovered from apple pomace was found to contain about 118 mg/g of phenolics (Schieber et al., 2003).

The peels and seeds of tomatoes have been also found to be richer sources of phenolic compounds than the fleshy pulp (George, Kaur, Khurdiya, & Kapoor, 2004).

Also many researches have been done on obtaining polyphenols from grape marc. Saura-Calixto (1998) and Loo and Foo (1999) studied grape seeds and grape pomace peels. Louli, Ragoussis, and Magoulas (2004) investigated the effect of various process parameters such as: solvent type, and feed pre-treatment (crushing, removal of stems), on the efficiency of the extraction of phenolic antioxidants from grape marc, whereas Negro, Tommasi, and Miceli (2003) investigated the content of total polyphenols and antioxidant activity of grape marc extracts (Lapornik et al., 2005).

3.2. Beverages

Beverages such as fruit juices, tea and wines are important sources of phenolics in the human diet. Over the recent few decades, green tea has been subjected to many scientific and medical studies to determine the extent of its long-purported health benefits, with some evidence suggesting regular green tea drinkers may have lower chances of developing heart disease and certain types of cancer. The main phenolic compounds present in tea are catechins. Their content is quite diversified depending on the type of the raw material used and on the technology of its preservation. Generally, green tea contains more of such compounds than black or red tea and thanks to this it shows over twice higher antioxidant activity (Sikora, Cieslik, & Topolska, 2008).

As widely accepted by the scientific community, wine is one of the most important sources of dietary polyphenolic antioxidants including a large variety of both flavonoid (flavonol, flavan-3-ol and anthocyanin) and non-flavonoid compounds (phenolic acids, phenolic alcohols, stilbene, hydroxycinnamic acid), (Makris, Boskou, & Andrikopoulos, 2007).

The polyphenolic profile of red wines differs essentially from that of white wines due to differences in the composition of red and white grapes, and also due to those in the vinification technology used (Bravo, 1998; Alén-Ruiz, García-Falcón, Pérez-Lamela, Martínez-Carballo, & Simal-Gándara, 2009). Red wine has been deemed more protective on health than are other alcoholic beverages (Gronbaek, Henriksen, & Becker,1995; Alén-Ruiz et al., 2009), possibly because the polyphenols it contains help prevent oxidative stress-related diseases.

Coffee also provides a significant source of dietary antioxidants. The content of phenolic compounds in roasted coffee reaches 8%, from which chlorogenic acid is dominant. An infusion of 5 g of

Table 2
Total polyphenols content of different beverages.

Beverage type	Total phenolics content	References
Commercial juices		
Apple	339 ± 43[a]	Gardner, White, McPhail, and Duthie (2000)
Grapefruit	535 ± 11[a]	Gardner et al. (2000)
Orange	755 ± 18[a]	Gardner et al. (2000)
Pineapple	358 ± 3[a]	Gardner et al. (2000)
Fresh juices		
Grape (red)	1728[a]	Sanchez-Moreno et al. (1999)
Grape (white)	519[a]	Sanchez-Moreno et al. (1999)
Tea		
Black tea	80.5–134.9[b]	Khokhar and Magnusdottir (2002)
Green tea	65.8–106.2[b]	Khokhar and Magnusdottir (2002)
Green tea	61–200[b]	
Coffee		
Instant coffee	146–151[b]	Schulz et al. (1999)
Ground coffee	52.5–57.0[b]	
Red wines		
Argentine	1593–1637[a]	Lakenbrink, Lapczynski, Maiwald, and Engelhardt (2000)
Brazilian	1947–1984[a]	
Spanish	1869[a]	Lakenbrink et al. (2000)
French	1847–2600[a]	Sanchez-Moreno et al. (2004)
White wines		
Argentine	216[a]	Minussi et al. (2003)
Brazilian	256–353[a]	Sanchez-Moreno et al. (1999)
French	245[a]	Minussi et al. (2003)
Spanish	292[a]	Minussi et al. (2003)Sanchez-Moreno et al. (1999)

[a] mg gallic acid equivalents/L.
[b] mg gallic acid equivalents/g dry matter.

ground roasted coffee can contain even about 140 mg of this compound, which can be responsible for the possible acrid effect of this drink (Sikora et al., 2008). Klatsky, Morton, Udaltsova, and Friedman (2006) studied the interrelation between the consumption of coffee as a dietary source of polyphenolic compounds and the apparent reductions in the risks of Alzheimer's disease, Parkinson's disease, heart disease, diabetes mellitus type 2 and livercirrhosis.

Fruits juices like grapefruit, orange, apple juice are also abundant sources of natural phenolic compounds. Generally, commercial or natural fruit juices provide vitamin C and an abundance of phytonutrients. Most of the data available on the phenolic contents of commonly consumed juices are for commercial samples.

The following table (Table 2) provides some information about the total polyphenols content (TPC) of some beverages.

4. Extraction

In the last several years, works regarding the extraction of phenolic compounds occurring in natural products have attracted a special interest (Pinelo et al., 2005).

Extraction is a very important step in the isolation, identification and use of phenolic compounds and there is no single and standard extraction method. Solvent extraction (Baydar, Ozkan, & Sagdic, 2004; Bucic-Kojic, Planinic, Tomas, Bilic, & Velic, 2007) and extraction with supercritical fluid (Bleve et al., 2008; Fredj & Francois, 1990; Nahar & Sarker, 2005; Palma & Taylor, 1999) are the most common used techniques for the isolation of phenolic compounds.

A large number of articles in the literature focus on the extraction and analysis of polyphenols from plant derived materials, including fruits, vegetables, wines, coffee, tea, herbs, cereals and pulse crops such as beans (Balasundram et al., 2006; Luthria & Pastor-Corrales, 2006; Naczk & Shahidi, 2006).

The phenolic compounds have been extracted by grinding, drying or lyophilising fruits, vegetables and herbs or only by soaking fresh plants with subsequent solvent extraction (Merken & Beecher, 2000). These methodologies imply the co-extraction of non-phenolic substances, such as sugars, organic acids and proteins, requiring subsequent purification processes (for example extraction in solid phase, SPE) (Castañeda-Ovando et al., 2009). Solvent extraction, as a function of the biomass status may be liquid–liquid extraction or solid–liquid extraction.

4.1. Liquid–liquid extraction

Liquid–liquid extraction is a mass transfer operation in which a liquid solution (the feed) initially containing one or more solutes is thoroughly mixed with an immiscible or nearly immiscible liquid (solvent).The solvent exhibits preferential affinity or selectivity towards one or more of the components in the feed and has different density. Two streams result from this contact: the extract, which is the solvent rich solution containing the desired extracted solute, and the raffinate, the residual feed solution containing little solute. (Müller, Berger, Blass, Sluyts, & Pfennig, 2008). Extraction becomes a very useful tool if a suitable extraction solvent is chosen.

For the separation of phenolic compounds, liquid–liquid extraction is frequently used with industrial liquid by-products, such as those resulting from the beverage industry.

4.2. Solid–liquid

Solid–liquid extraction, or leaching can be defined as a mass transport phenomenon in which solids contained in a solid matrix migrate into a solvent brought into contact with the matrix. Mass transport phenomena can be enhanced by changes in concentration gradients, diffusion coefficients or boundary layer (Corrales, Fernández García, Butz, & Tauscher, 2009). It is a unit operation extensively used to recover many important food components: sucrose in cane or beets, lipids from oilseeds, proteins in oilseed meals, phytochemicals from plants, functional hydrocolloids from algae and polyphenolic compounds from plants, fruits, vegetables, etc.

Table 3
Organic solvents used for polyphenols extraction.

Polyphenolic compounds	Solvent	References
Phenolic acids, flavonols, antocyanins	Ethyl acetate	Pinelo et al. (2005); Russell et al. (2008)
Anthocyanins, Phenolic acids, catechins, flavanones, flavones, flavonols, procyanidins, ellagic acids, Rutin, chlorogenic acids	Methanol and different aqueous forms (50–90%, v/v)	Bleve et al. (2008); Caridi et al. (2007); Ross et al. (2009); Mattila and Kumpulainen (2002)
Anthocyanins, flavonols, free phenolic acids	Ethanol and different aqueous forms (10–90%, v/v)	Altiok et al. (in press); Balas and Popa (2007); Wang et al. (2009); Bleve et al. (2008), Bucić-Kojić et al. (2006); Corrales et al. (2009); Ross et al. (2009)
Flavonols, free phenolic acids	Chloroform	Sharififar, Dehghn-Nudeh, and Mirtajaldini (2009)
Flavonols, phenolic acids	Dietyl ether	Ross et al. (2009)
Proantocyanidins, phenolic acids	Hot water 80–100°	Diouf, Stevanovic, and Cloutier (2009)
Tannins, bound phenolic acids	NaOH (2 N–10 N)	Nardini et al. (2002); Popa et al. (2008)); Ross et al. (2009)
Phenolic compounds, phenolic acids	Petroleun ether	Zhang et al. (2009)
Flavonols, phenolic acids, hydroxycinamic acids, coumarins, Flavonols xanthones	Acetone/water 10–90% (v/v)	Altiok et al. (in press); Naczk & Shahidi (2006); Sharififar et al. (2008); Schieber et al. (2003)
Flavonols, phenolic acids, simple phenolics,anthocyanins	*n*-Hexane, isooctane, ethyl acetate	Alonso Garcia et al. (2004)
Polyphenols from olive leaves, oleuropein and rutin	Acetone, ethanol and their aqueous forms (10–90%,v/v)	Altiok et al. (in press)
Flavonols, quercetin 3,4′-diglucoside and quercetin 4′-monoglucoside.	Methanol/water 70% v/v	Caridi et al. (2007)

Extraction efficiency is known to be a function of process conditions. Several factors affect the concentration of the desired components in the extract: temperature, liquid–solid ratio, flow rate and particle size. For instance, the phenolic content of almond hull extracts was found to be three times higher when a batch liquid–solid extraction was performed at 50 °C in comparison with that at 25 °C. Time contact and liquid–solid ratio were also reported to be significant variables (Hayouni, Abedrabba, Bouix, & Hamdi, 2007; Pinelo, Rubilar, Sineiro, & Nunez, 2004; Rubilar, Pinelo, Franco, Sineiro, & Nunez, 2003).

The most common solvents extraction methods are those using acidified methanol or ethanol as extractants (Amr & Al-Tamimi, 2007; Awika, Rooney, & Waniska, 2005; Caridi et al., 2007; Lapornik et al., 2005). From these methods, the extraction with methanol is the most efficient (Kapasakalidis, Rastall, & Gordon, 2006); in fact, it has been found that in anthocyanin extractions from grape pulp, the extraction with methanol is 20% more effective than that with ethanol, and 73% more effective than water extraction (Castañeda-Ovando et al., 2009) nevertheless, in food industry ethanol is preferred due to the methanol toxicity.

Amongst ethanol and methanol extractions, a multitude of other extraction solvents have been noted in literature, some of them being presented in Table 3.

Usually, the extraction procedure is sequential and systematically releases the phenolic compounds from their respective forms. When we talk about phenolic acids (free or bound acids), the first step of the procedure typically involves the use of an aqueous organic solvent to extract soluble/extractable phenolic acids (free, soluble esters, and soluble glycosides) (Escarpa, Morales, & Gonzalez, 2002; Mattila & Kumpulainen, 2002; Russell, Scobbie, Labat, Duncan, & Duthie, 2008).

Phenolic acids also exist as insoluble bound complexes, which are coupled to cell wall polymers through ester and glycosidic links and are not extractable by organic solvents. Bound phenolic acids are typically liberated by base hydrolysis, acid hydrolysis or both (Mattila & Kumpulainen, 2002). The main step in most procedures involves base hydrolysis with NaOH ranging from 2 to 10 M, using incubation time up to 16 h, sometimes under nitrogen (Popaet al., 2008; Nardini et al., 2002). Following base hydrolysis, acid hydrolysis is sometimes performed to liberate bound phenolics that have not been previously hydrolysed (Ross et al., 2009). Mattila and Kumpulainen (2002) showed that acid hydrolysis liberated significant amounts of gallic acid from red raspberries and strawberries along with significant amounts of protocatechuic acid from carrots, crisp-bread, red raspberries, and strawberries. Substantial amounts of gallic and ellagic acids from mango seeds are released by acid hydrolysis according to Soong and Barlow's (2006) study. In other cases (apples or apple juice and potatoes), acid hydrolysis was unnecessary as base hydrolysis was sufficiently aggressive (Luthria & Pastor-Corrales, 2006; Mattila & Kumpulainen, 2002). Base and acid hydrolyse were also assayed on mangosteen fruits. Zadernowski, Czaplicki, and Naczk (2009) showed that bound phenolic acids were the predominant phenolic acids in mangosteen fruits especially hydroxybenzoic acid derivatives. Phenolic acids liberated from soluble esters comprised from 41.4% (peel) to 76.5% (aril) of the total phenolic acids present in the fruits. Hydroxybenzoic acid derivatives comprised from 91.5% (rind) to 100% (aril) of phenolic acids identified in this fraction.

4.3. Supercritical fluid extraction

Supercritical fluid extraction (SFE) could be an environmentally beneficial alternative to the conventional organic solvent extraction of biological compounds: SFE methods are rapid, automatable, selective and avoid the use of large amounts of toxic solvents. In addition, the absence of light and air during the extraction reduces the degradation processes that can occur during the traditional extraction techniques (Bleve et al., 2008). Supercritical Fluid Extraction (SFE) is based on the fact that, close to the critical point, the solvent changes its properties rapidly with only slight variations of pressure (Palenzuela et al., 2004).

Supercritical fluids (SCFs) are increasingly replacing organic solvents such as *n*-hexane, dichloromethane, chloroform, and others which are conventionally used in industrial extraction, purification, and recrystallisation operations because of regulatory and environmental pressures on hydrocarbon and ozone-depleting emissions. SCF have solvating powers similar to liquid organic solvents, but with higher diffusivity, lower viscosity, and lower surface tension.

By far the most utilised critical fluid has been supercritical carbon dioxide (SC–CO_2), due to its benign effect on the environment, low toxicity, nonflammability and compatibility with processed foodstuffs. Furthermore, it has modest critical conditions, it can be readily separated from solutes and it is inexpensive. In natural product extraction and isolation, supercritical fluid extraction (SFE), especially that employing supercritical CO_2, has become the method of choice. Sophisticated modern technologies allow precise regulation of changes in temperature and pressure, and thus manipulation of the solvating property of the SCF, which helps the extraction of natural products of a wide range of polarities

(Nahar & Sarker, 2005). By adding modifiers to a SCF (like methanol to CO_2) its polarity can be changed for obtaining more selective separation power. Therefore, supercritical carbon dioxide (SC–CO_2) methods are ideal for the extraction of natural products from plant materials and are particularly recommended for the extraction of thermolabile compounds, when low temperatures are required. In addition, SC–CO_2 methods allow obtaining extracts without remaining solvent traces and without using a cleaner, as the degradation of certain compounds by lengthy exposure to high temperatures or oxygen is avoided.

However, previous studies have shown that the extraction of anthocyanins by using SC–CO_2 methods required high pressures and the presence of an organic co-solvent (methanol, ethanol) in high percentage due to the polarity of anthocyanins (Bleve et al., 2008). These factors seemed to prohibit the use of SC–CO_2 for the extraction of these compounds. In contrast to that, SC–CO_2 has been employed, instead, for the purification of a primary anthocyanin extract obtained from red fruits by extraction with organic solvents, in order to improve its properties without causing any thermal or chemical degradation (Fredj et al., 1990). Bleve et al. (2008) described a new and innovative method for the purification of anthocyanins from grape skin extracts as liquid matrix (LM), by using CO_2 under liquid and sub-critical conditions. The CO_2 purification process under optimised conditions gave rise to a desired fraction containing pure anthocyanins.

4.4. Other extraction methods

Conventional extraction as heating, boiling, or refluxing can be used to extract natural phenolic compounds, however, the disadvantages are the loss of polyphenols due to ionisation, hydrolysis and oxidation during extraction, as well as the long extraction time (Hui, Bo, & Shouzhuo, 2005).

In recent years, various novel extraction techniques have been developed for the extraction of nutraceuticals from plants, including ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and high hydrostatic pressure extraction (HHP) (Wang & Weller, 2006). Amongst these, ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction techniques (Jing, Baoguo, Yanping, Yuan, & Xuehong, 2008). This method describes a procedure for extracting nonvolatile and semivolatile organic compounds from solids such as soils, sludges, and wastes. The ultrasonic process ensures an intimate contact of the sample matrix with the extraction solvent. Ultrasonication is often used to improve the extraction of lipids, proteins and phenolic compounds from plants. Huang, Xue, Niu, Jia, and Wang (2009) assayed a ultrasound-assisted extraction of phenolic compounds from *Folium eucommiae,* which seems to be more efficient than extractions by heating, microwave-assisted and enzyme-assisted extractions.

The extraction of anthocyanins from grape skins was also achieved using an ultrasonics bath at a frequency of 35 kHz for 30 min, followed by stirring at a temperature of 70 °C in a water bath for 2.5 h according to Corrales et al. (2009).

Extraction using microwave energy is a largely unexplored area, although by using microwaves to mediate the extraction, it is possible to maintain mild conditions and achieve a superior effect of the extraction.

Enzymatic release of phenolic compounds is another useful technique for the extraction natural polyphenols. A process for enzyme-assisted extraction of polyphenols from grape pomace was developed on a laboratory and pilot-plant scale by (Maier, Goppert, Kammerer, Schieber, & Carle, 2008). Min et al. (2006) investigated the ability of three commercial enzymes – Ultraflo L, Viscozyme L, and α-Amylase – to induce the release of ferulic acid from the *Ipomoea batatas* L. (sweet potato) stem. The rate of ferulic acid release was optimal when Ultraflo L (1.0%) was used, compared to the other enzymes, whereas Viscozyme L was the most effective for the release of vanillic acid and vanillin. Thus, these enzymes may be useful for the large-scale production of ferulic acid and other phenolic compounds from sweet potato stems.

High hydrostatic pressure (HHP) is a novel method to enhance mass transport phenomena (Rastogi, Angersbach, & Knorr, 2003). Higher caffeine extraction yields from coffee and a higher carotenoid content in tomato puree have been demonstrated when extractions were assisted by high hydrostatic pressure (Sanchez-Moreno, Plaza, de Ancos, & Cano, 2004).

More recently, studies undertaken by Shouqin, Jun and Changzhen (2005) have also demonstrated the benefits of HHP for the extraction of flavanols from propolis, while Corrales et al. (2009) studied the extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. During HHP assisted extraction, the air gaps present in fruit tissues are partially filled with liquid. When the pressure is subsequently released, the occluded air in the pores exits causing plant cell membrane damage (Fernandez Garcia, Butz, & Tauscher, 2001). HHP can also cause deprotonation of charged groups and disruption of salt bridges and hydrophobic bonds, resulting in conformational changes and denaturation of proteins making the cellular membranes less and less selective, thereby rendering the compounds more accessible to extraction up to equilibrium (Corrales et al., 2009).

5. Quantification and separation of polyphenols

There is an increasing demand for highly sensitive and selective analytical method for the determination of polyphenols (Liu et al., 2008). Despite a great number of investigations, the separation and quantification of different polyphenolics remain difficult, especially the simultaneous determination of polyphenolics of different groups (Tsao & Yang, 2003).

5.1. Spectrophotometric methods used in quantification of total phenolics and its classes

A number of spectrophotometric methods have been developed for the quantification of plant phenolics. These assays are based on different principles and are used to determine different structural groups present in phenolic compounds. The Folin–Ciocalteu assay (Tsao & Yang, 2003; Lapornik, Prosek, and Golc, 2005) is widely used for determining total phenolics, while the vanillin and proanthocyanidin assays have been used to estimate total proanthocyanidins (Naczk & Shahidi, 2006).

Spectrophometric assays for the quantification of total anthocyanins using pH differential method are based on their characteristic behaviour under acidic conditions. The principle of this method is the decrease of the extracts pH, to values ranging between 0.5 and 0.8, which causes all anthocyanins to transform to red-coloured flavilium cation (Lapornik et al., 2005).

Spectrophotometric methods provides very useful qualitative and quantitative information; actually, spectroscopy is the main technique used for the quantification of different classes of polyphenols due to its simplicity and low cost. Giusti and Wrolstad (2003) published excellent reviews of the main methods used in the characterisation and quantification of anthocyanins by UV–Vis.

On the other hand, the total flavonoids content can be determined using a colorimetric method based on the complexation of the phenolic compounds with Al(III) (Huang et al., 2009; Naczk & Shahidi, 2006). The main disadvantage of the spectrophotometric assays is that they only give an estimation of the total phenolic content. It does not separate nor does it give quantitative measurement of individual compounds.

Table 4
HPLC procedures in separation of different classes of polyphenolic compounds.

Compounds	Stationary phase	Mobile phase		T (°C)	Flow rate (mL/min)	λ (nm)	References
		A	B				
Catechines	C18 4.6 mm × 250 mm, 5 μm	15% ACN 2% acetic acid	80% ACN 2% acetic acid	35	1	278	Chen et al. (2008)
Antocyanidines	C18 SS Wakosil 4.6 mm × 150 mm, 5 μm	0.1% TFA in water	0.1% TFA in ACN	32	1	250–600 520	Bleve et al. (2008)
Antocyanins, flavone	C18 Diamonsil™ 4.6 mm × 150 mm, 5 μm	0.1% Formic acid in water	80% ACN in water	–	0.5–1	360	Fang et al. (2009)
Phenolic acids, flavonoids	C18 4.6 mm × 150 mm, 5 μm	4% Acetic acid	Methanol	–	1	210–400	Fang et al. (2009); Wang et al. (2009)
Antocyanins (cyanidin-3-O-glucoside cyanidin-3-O-rutinoside)	C18 Lichrosorb 4.6 mm × 250 mm, 5 μm	Water:formic acid 90:10	Water:ACN:formic acid 60:30:10	35	0.8	200–600	Bureau et al. (2009)
Polyphenols, flavonoids, phenolic acids	C18 SunFire™ 4.6 mm × 150 mm, 5 μm	Methanol	Acetic acid:water (1:99)	–	1	327–370	Liu et al. (2008)
Phenolic acids	C18 Phenomenex Gemini 4.6 mm × 150 mm, 5 μm	0.1% Formic acid	100% methanol	25	0.7	270, 325	Ross et al. (2009)
Antocyanins	C18 Aqua, Phenomenex 4.6 mm × 250 mm, 5 μm	Water:formic acid:ACN 87:10:3	Water:formic acid: ACN 40:10:50	20	1	520	Corrales et al. (2009)
Antocyanins, flavonols	Mediterranean Sea $_{18}$ 4.6 mm × 250 mm, 5 μm	5% Formic acid in water	Methanol	30	1	520, 360	Guerrero, Sineiro, and José Nunez (2008)
Antocyanins, Polyphenols	Superspher 100 RP, 250 × 4.6 mm 18.5 μm (Merck)	10% Formic acid in water	Methanol:water:formic acid (45:45:10, v/v/v)	30	0.8	530	Lapornik et al. (2005)
Flavonoids, phenolic acids	LiChroCart, 250-4, Hypersil ODS (5 μm) Merck	2.5% acetic acid	Acetonitrile/2.5% acetic acid 80:20 v/v	–	1	280	Svedstrom et al. (2006)
Hydroxybenzoic acid derivatives, flavan-3ols,dihydrochalcones, hydroxycinnamic acid derivatives, flavonols, anthocyanins	C18 Phenomenex®Luna 250 mm × 4.6 mm 5 μm	6% Acetic acid in 2 mM sodium acetate	Acetonitrile	–	1	280, 320, 360, 520	Tsao and Yang (2003)
Flavonols, quercetin 3,4′-diglucoside and quercetin 4′-monoglucoside	C18 Alltech Prevail 2.1 × 150 mm 3 μm	0.1% Formic acid in water 0.1% formic acid in water	0.1% formic acid in methanol 0.1% formic acid in acetonitrile	30	0.2	280, 346, 364 and 370	Caridi et al. (2007)
	C18 Hypersil 2.1 × 150 mm 3 μm			30	0.2		
Rosmarinic acid	C8 Hypersil Gold column 250 × 4.6 mm 5 μm	0.1% (v/v) TFA in water	0.1% (v/v) TFA in methanol	40		200–400 280, 360	Achamlale, Rezzonico, and Grignon-Dubois (2009)
Quercetin; gallic acid, (+)-catechin and (−)-epicatechin; caffeic acid, *p*-coumaric acid, salycilic acid; and gentisic acid.	C18 Waters Symmetry 150 × 4.6 mm 5 μm	Acid methanol (1% acetic acid)	Acid water (1% acetic acid)		1	253 280 306 330	Alonso Garcia et al. (2004)
Hydroxytyrosol, tyrosol, rutin, luteolin-7-glucoside, verbascoside, apigenin-7-glucoside, oleuropein, luteolin, caffeic acid, vanillic acid, catechin	C18 LiChrospher 250 × 4.6 mm 5 μm	Acetic acid/water (2.5:97.5)	Acetonitrile	30	1		Altiok et al. (in press)

5.2. Chromatographic techniques used in separation, qualitative and quantitative analysis

5.2.1. High performance liquid chromatography (HPLC)

Amongst the different methods available, HPLC is preferred for the separation and quantification of polyphenolics in fruits. The chromatographic conditions of the HPLC methods include the use of, almost exclusively, a reversed-phase C18 column; UV–Vis diode array detector, and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). Reverse phase (RP) HPLC has become a dominating analytical tool for the separation and determination of polyphenols with different detection systems, such as diode array detector (DAD), mass or tandem mass spectrometry. Sakakibara, Honda, Nakagawa, Ashida and Kanazawa (2003), determined all polyphenols in foodstuffs simultaneously with HPLC–DAD and constructed a library comprising respective calibration curves for 100 standard chemicals. Various supports and mobile phases are available for the analysis of different classes of polyphenols like anthocyanins, procyanidins, flavonones, flavonols, flavan-3-ols, procyanidins, flavones and phenolic acids. The introduction of reversed phase columns has considerably enhanced the HPLC separation of phenolic compounds (Naczk & Shahidi, 2006).

Some HPLC procedures for determining some classes of phenolic compounds are presented in Table 4.

Nevertheless, due to the disadvantages in detection limit and sensitivity, HPLC methods present limitations especially in complex matrix, such as crude plant extracts and environmental samples. Thus, an initial preconcentration and purification of the polyphenols from complex matrix is crucial prior to the instrumental analysis by HPLC. The aim of preconcentration is to simplify the chromatograms obtained so that they can be reliably identified and quantified. The purification stage is the critical part of a method, the removal of potential interfering components varies according to the vegetal matrix to be analysed. The procedure includes liquid–liquid partitioning with a immiscible solvent and open column chromatography on Sephadex LH-20, polyamide, Amberlite, prep-HPLC and solid phase extraction (SPE) using commercially available cartridges.

Polyphenols can usually be purified by adsorption–desorption processes by using highly efficient sorbents, of which C18 and highly crosslinked styrene–divinylbenzene (S–DVB) copolymers are very popular (Liu et al., 2008). Silva, Pompeu, Larondelle, and Rogez (2007), tested the adsorption on macroporous resins for purifying the phenolic compounds from crude extracts of *Inga edulis* leaves. Different types of adsorbents (XAD-7, XAD-16, EXA-90 and EXA-118) has been used.

Michalkiewicz, Biesaga, and Pyrzynska (2008) also used different solid sorbents such as Bond Elut octadecyl C_{18}, Oasis HLB, Strata-X and Amberlite XAD-2, for isolation and preconcentration of phenolic acids and some flavonols from honey samples prior to their determination by HPLC.

In other studies, the preconcentration was accomplished by the adsorption–desorption method with a styrene–divinylbenzene resin (XAD-4) or XAD-16 and the results demonstrated that both resins are capable of successfully adsorbing polyphenols (Li, Wang, Ma, & Zhang, 2005; Liu et al., 2008). However, in most of the works for determining polyphenols, solid phase extraction (SPE) was used for purification, and the analytes were usually eluted with methanol, ethanol or their aqueous form (Lalaguna, 1993; Liu et al., 2008; Michalkiewicz et al., 2008).

Lower molecular mass polyphenols can be analysed by HPLC on reversed-phase or normal phase columns. However, these techniques are time consuming and can have poor resolution as the polymer chain length and structural diversity increase. The detection of higher molecular weight compounds, as well as the determination of molecular mass distributions, remain major challenges in the analysis of polyphenol (Fulcrand et al., 2008).

Liquid Chromatography–Mass Spectrometry (LC–MS) techniques are nowadays the best analytical approach to study polyphenols from different biological resources, and are the most effective tool in the study of the structure of phenolic compounds (Bureau et al., 2009).

5.2.2. HSCCC (High Speed Counter Current Chromatography)

Polyphenols are sometimes difficult to separate in classical liquid chromatography. Counter current chromatography (CCC) uses a biphasic liquid system to separate the components of a mixture. A centrifugal field allows to use a liquid stationary phase in an open tube. The phase density difference and the centrifugal field are the only parameters allowing the equilibrium between the two liquid phases. The high advantage of the technique in preparative separation is the dual-mode capability of CCC. The role of the phases can be switched during a run. The mobile phase becomes stationary and vice versa. Then no injected material can be left in the machine (Berthod, Billardello, & Geoffroy, 1999). Cao, Wang, Pei, and Sun (2009) applied two methods of separation and purification of polyphenols from apple pomace extract, methods that were established by the combination of gel chromatography with high speed counter current chromatography (HSCCC) and solvent extraction with HSCCC, respectively. The optimal separation was performed on a Sephadex LH-20 column using gradient aqueous ethanol as eluting solvent from 0% to 100% in increments of 10%. HPLC analysis indicated that main polyphenols existed in fractions eluted between 40% and 50% aqueous ethanol. The fractions of interest from the column were separated by HSCCC with the hexane–ethyl acetate-1% aqueous acetic acid (0.5:9.5:10, v/v/v) solvent system. Ethyl acetate fractionation of the apple pomace extract, followed by direct HSCCC separation by the same solvent system in the volume ratio of 1:9:10, also produced a good separation of the main polyphenols of interest.

High speed counter current chromatography (HSCCC) using the J-type coil planet centrifuge was applied to compositional analysis of tea catechins and separation of other food-related polyphenols. The HSCCC separation of nine different standard compounds and those from extracts of commercial tea leaves was performed with a two-phase solvent system composed of *tert*-butyl methyl ether–acetonitrile-0.1% aqueous trifluoroacetic acid (TFA) (2:2:3, v/v/v) by eluting the upper organic phase at a flow rate of 2 mL/min. The main compounds in the extract of non-fermented green tea were found to be monomeric catechins, their galloylated esters and caffeine. In addition to these compounds, oxidised pigments, such as hydrophobic teaflavins (TFs) and polar thearubigins (TRs) were also separated and detected from the extracts of semi-fermented oolong tea and fermented black tea. Furthermore, several food-related polyphenols, such as condensed catechin oligomers (procyanidins), phenolic acids and flavonol glycosides were clearly separated under the same HSCCC conditions. These separation profiles of HSCCC provide useful information about the hydrophobic diversity of these bioactive polyphenols present in various types of tea and food products (Yanagida et al., 2006). High-speed countercurrent chromatography (HSCCC) for the separation of polyphenols from tea leaves (*Camellia sinensis* L.) was also applied by Degenhardt, Engelhardt, Lakenbrink, and Winterhalter (2000). The ability of HSCCC to isolate pure tea polyphenols from complex mixtures on a preparative scale was demonstrated for catechins, flavonol glycosides, proanthocyanidins, and strictinin from green and black tea.

5.2.3. Supercritical fluid chromatography (SFC)

Supercritical fluid chromatography (SFC) is a relatively recent chromatographic technique used in the separation and identification of phenolic compounds. What differentiates SFC from other

chromatographic techniques [gas chromatography (GC) and high performance liquid chromatography (HPLC)] is the use of a supercritical fluid as the mobile phase. Supercritical fluid chromatography is more versatile than high performance liquid chromatography, more cost-efficient, user friendly, with higher output, better resolution and faster analysis times than general liquid chromatographic methods. The instrumentation that is required for supercritical fluid chromatography is versatile because of its multi-detector compatibility. Kamangerpour, Ashraf-Khorassani, Taylor, McNair, and Chorida (2002) used supercritical fluid chromatography for the separation and identification of eight polyphenols in grape seed extract. Carbon dioxide modified with methanol, which contained less than 1% (w/w) citric acid as a secondary additive, served as the mobile phase. Various components in the extract could be identified by retention time and ultraviolet spectral comparison with a synthetic mixture of polyphenols.

5.3. Other chromatographic techniques

Other chromatographic techniques have been also employed to purify and separate food phenolics. Of these, paper chromatography (PC) and thin-layer chromatography (TLC) techniques are still widely used for the purification and isolation of anthocyanins, flavonols, condensed tannins and phenolic acids using different solvent systems (Naczk & Shahidi, 2006). Years ago, the development of partition chromatography as a preparative method has enabled further progress to be made in the elucidation of polyphenols. The success of preparative partition chromatography suggested that useful information might be obtained by applying the methods of paper chromatography to study catechins and other polyphenols in tea. Roberts and Wood (1951) describe the provisional identification of the main polyphenols in the tea leaf. Other studies showed that PC on Whatman No. 3 has been employed to separate anthocyanins using butanol/acetic acid/water, chloroform/acetic acid/water, or butanol/formic acid/water as possible mobile phases (Jackman, Yada, & Tung, 1987).

TLC is a technique with large applicability in the fields of plant material analysis and stability tests of extracts and final products. The implementation of a modern standardised methodology led to an increasing acceptance and recognition of (HP) TLC as a competitive analytical method. (HP) TLC has many advantages, such as lower costs, short analysis time, the possibility of multiple detection, and specific derivatisation on the same plate, etc.

The separation of polyphenols from each other and from other components of the plant extracts can be carried out by a great number of (HP) TLC developed techniques. Mostly, complex crude plant extracts are screened for antioxidant activity or for distinguishing the components of plant extracts with antioxidant character or radical-scavenging properties. (HP) TLC has been used to determine individual antioxidant capacity of target compounds and might be of interest to the routine chemical or biological screening, the method offering solutions to real analytical problems (Cimpoiu, 2006). TLC on silica gel plates is useful for the rapid and low-cost separation and identification of the polyphenols present in wine. Densitometric quantitative analysis of polyphenols in wine extracts is usually performed by scanning the TLC plates with UV light at wavelengths of 350–365 nm or 250–260 nm (Rastija & Medić-Šarić, 2009).

Gas chromatography is another technique that has been employed for separation and identification of different phenolic compounds. Gas chromatography (GC) methods developed for the analysis of polyphenols require the derivatisation to the volatile compounds by methylation, trifluoroacetylation, conversion to trimethylsilyl derivatives and mass-spectrometric detection in the selective ion monitoring mode (GC/MS–SIM), (Naczk & Shahidi, 2006; Rastija & Medić-Šarić, 2009; Zadernowski et al., 2009). The trimethylsilyl derivatives of phenolic acids from mangosteen fruits were identified using GC–MS methodology as described by Zadernowski et al. (2009). GC has a great separation capacity, and offers high sensitivity and selectivity when combined with mass spectrometry. However, the preparation of samples for GC is very troublesome, including the removal of lipids from the extract, the liberation of phenolics from ester and glycosidic bonds, and derivatisation for low volatile polyphenols (Liu et al., 2008).

Centrifugal partition chromatography (CPC) has been also applied to the separation and purification of bioactive polyphenols in extracts from an oriental crude drug, licorice, and also of oligomeric hydrolysable tannins extracted from *Heterocentron roseum*. The separation was achieved by normal-phase CPC using as a solvent system, $CHCl_3$–MeOH–H_2O (Okuda, Yoshida, & Hatano, 1988).

6. Other methods for separation and quantification of polyphenols

6.1. Capillary electrophoresis (CE)

Capillary electrophoresis (CE), which is an alternative separation technique to HPLC, is especially suitable for the separation and quantification of low to medium molecular weight polar and charged compounds, the resultant separations being often faster and more efficient than the corresponding HPLC separations (Caridi et al., 2007; Frazier & Papadopoulou, 2003). Capillary electrophoresis (CE) is increasingly becoming a versatile analytical tool for the routine determination of a wide variety of phenolic compounds in different types of samples due to its high separation efficiency, high resolution power, short analysis time and low consumption of sample and reagents. On the other hand, one of the major limitations of CE, compared to other techniques like GC or HPLC, is its low sensitivity in terms of solute concentration, and worse reproducibility compared to chromatographic techniques which is caused by the short optical path-length of the capillary used as detection cell and also by the small volumes that can be introduced into the capillary (normally, a few nanoliters) (Molina-Mayo, Hernandez-Borges, Borges-Miquel, & Rodrıguez-Delgado, 2007; Liu et al., 2008).

There are a few examples of CE used to separate and determine the levels of naturally occurring flavonols in plant material (Caridi et al., 2007; Chen, Zhang, & Ye, 2000; Vaher & Koel, 2003; Wang & Huang, 2004). The use of CE in the separation of anthocyanins is a quite recently developed technique, scarce, but promising due to the high hydrosolubility of these compounds. CE is suitable technique for the separation, identification and quantification of anthocyanins. CE has also been used to create correlations between the content of anthocyanins content and the ageing of red wine (Saenz-Lopez, Fernandez-Zurbano, & Tena, 2004). CE with ESI–MS coupling has been used for monitoring anthocyanins and flavonoids in wine (Castañeda-Ovando et al., 2009).

Micellar electrokinetic capillary chromatography (MECC) has extended the utility of capillary electrophoresis to the separation of neutral analytes under the influence of an electric field. The fractionation of monomeric and polymeric pigments of higher molecular mass by gel permeation chromatography (GPC) improved the analysis of these compounds by CE (Rastija & Medić-Šarić, 2009).

7. Spectral methods used in structure elucidation and characterisation of phenolic compounds

7.1. NMR spectroscopy

NMR spectroscopy is nowadays being used more and more to analyse foods. Advantages such as simplicity of the sample preparation and measurement procedures, the instrumental stability and the ease with which spectra can be interpreted have contrib-

uted to the growing popularity of the technique. Standard ^{1}H, ^{13}C and now high resolution magic angle spinning (HR/MAS) NMR spectra can give a wealth of chemical information on liquid foodstuff and even semi-solid foods.

NMR spectra of vegetal samples can act as "fingerprints" that can be used to compare, discriminate or classify samples. Selected variables (NMR peak heights or integrals) that characterise the samples in specific way are also used instead of the whole spectra. Chemometric techniques are often employed to analyse the data as the information contained in the spectra is of a high degree of complexity.

The preparation of the food sample is actually simple, depending on the nature of the sample (liquid, solid). In some cases a previous extraction or fractionation step is required while other samples may be used as they are. For high resolution ^{1}H, ^{13}C, ^{31}P NMR of aqueous liquids (fruit juices, degassed beer, wine, etc.) the samples are often prepared simply by adding 5–10% of D_2O to the liquid (Le Gall and Colquhoun,2003). Deuterated solvents provide a signal for magnetic field stabilisation and allow optimisation of the resolution of the NMR peaks.

Solid samples (fruits, vegetables, green tea) are freeze-dried, ground and then extracted in a deuterated solvent.

Other samples, such as oils or instant coffees are simply dissolved at the desired concentration in a suitable deuterated solvent. Standard procedures should be followed to ensure repeatability and comparability when preparing a series of samples.

The first limitation in using NMR for food analysis (and the most prohibitive one) is the cost of the equipment. A new 500 MHz NMR spectrometer might cost 7–8 times as much as a new HPLC/UV–DAD system. The second limiting reason is the relatively low sensitivity of NMR compared to other techniques such as HPLC or GC. However, the versatility of the technique means that the initial high cost may well be overridden by a number of advantages that other techniques may not provide. The first of them is obviously the power of structural elucidation of the technique. The second advantage is that NMR is probably the best non-target technique to use for the screening of food extracts: all the main metabolites (fatty, amino and organic acids, sugars, aromatic compounds) can be detected in a single spectrum with minimal and non-destructive sample preparation (Le Gall & Colquhoun, 2003).

Various NMR techniques have been employed for the structural elucidation of complex phenolics isolated from foods without previous separation into individual components. These include ^{1}H and ^{13}C NMR, two-dimensional homonuclear (^{2}D ^{1}H–^{1}H) correlated NMR spectroscopy (COSY), heteronuclear chemical shift correlation NMR (C–H HECTOR), totally correlated NMR spectroscopy (TOCSY), nuclear overhauser effect in the laboratory frame (NOESY) and rotating frame of reference (ROESY) (Naczk & Shahidi, 2006). Caridi et al. (2007) acquired NMR spectra (^{1}H and ^{2}D spectra) at 25 °C in d6-DMSO and referenced to residual ^{1}H signals in the deuterated solvent for profiling and quantifying quercetin glucosides in onion.

For identifying *Walnut kernel* antioxidants, 1H and 13C NMR spectra were obtained by Zhang et al. (2009) using deuterated dimethyl sulphoxide (DMSO-d6) or methanol (CD_3OD) as solvents. Therefore, nuclear magnetic resonance (NMR) of ^{1}H and ^{13}C has become a preferred technique for identifying anthocyanins. NMR has also been very useful in identifying the reaction products of anthocyanins with other compounds such as cinnamic acid derivatives, peroxyl radicals, catechins and flavonols (Castañeda-Ovando et al., 2009).

7.2. Mass spectrometry (MS)

Mass spectrometry (MS) is an analytical technique that is also used for elucidating the chemical structures of molecules, such as peptides, polyphenols and other chemical compounds.

Mass spectrometry, had and still has, a very important role for research and its analytical power is relevant for structural studies on polyphenolic compounds. The MS principle consists in ionising chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios (Sparkman, 2000). The main sources used to analyse phenolic compounds are: fast atom bombardment (FAB), electrospray ionisation (ESI), atmospheric pressure ionisation (API) including atmospheric pressure chemical ionisation (APCI), atmospheric pressure photo-ionisation (APPI) and in parallel to the advent of electrospray advent, matrix-assisted laser desorption ionisation (MALDI). Thermospray analysis (TSP) has also been proposed but has proven to be unsuitable for the analysis of oligomers and polymers, due to thermal degradation (Fulcrand et al., 2008).

Direct flow injection electrospray ionisation (ESI) mass spectrometry analysis can be used to establish polyphenol fingerprints of complex extracts.

The matrix-assisted-laser-desorption-ionisation-time-of-flight (MALDI–TOF) technique is suitable to determine the presence of molecules of higher molecular weight with high accuracy, and it has been applied with success to study procyanidin oligomers up to heptamers in the reflectron mode, and up to nonamers in the linear mode (Fulcrand et al., 2008).

The structural heterogeneity of the polyphenols from cranberries, grape seed extracts, sorghum and pomegranate was characterised by MALDI–TOF MS. Polyphenolics were isolated by liquid chromatography and subjected to MALDI–TOF MS using *trans*-3-indoleacrylic acid as matrix. The spectrometric analysis gave information on the degree of polymerisation, monomeric substitution, and the nature of intermolecular bonds (Reed, Krueger, & Vestling, 2005).

Chromatography–Mass Spectrometry (LC–MS) techniques are nowadays the best analytical approach to study polyphenols in vegetal samples, and are the most effective tool in the study of the structure of anthocyanins. The MS/MS approach is a very powerful tool that permits anthocyanin aglycone and sugar moiety characterisation.

LC–MS allows the characterisation of complex structures such as procyanidins, proanthocyanidins, prodelphinidins, and tannins, and provides experimental evidence for structures that were previously only hypothesised (Flamini, 2003).

The levels of resveratrol in wine, an important polyphenol well-known for its beneficial effects, have been determined by SPME (solid-phase microextraction) and LC–MS, the former approach having led to the best results in terms of sensitivity (Flamini, 2003).

7.3. Near infrared (NIR)

Near infrared (NIR) spectroscopy is another powerful, fast, accurate and non-destructive analytical tool that can be considered as a replacement of the older chemical analysis. Hall, Robertson, and Scotter (1988) applied the NIR spectroscopy technique to predict the tea flavin content and moisture content of black tea. Schulz, Engelhardt, Wengent, Drews, and Lapczynski (1999) attempted by NIR spectroscopy to predict simultaneously the presence of alkaloids and phenolic substances in green tea leaves. Further studies on the quantitative analysis of total antioxidant capacity of green tea using NIR spectroscopy, were carried out by Luypaert, Zhang, and Massart (2003). Chen, Zhao, Chaitep, and Guo (2008) also reported the results of simultaneous analysis of main catechins (EC, (−)-epicatechin; ECG, epicatechin-3-gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate) contents in green tea by the Fourier transform near infrared reflectance (FT-NIR) spectroscopy. Recently, NIR spectroscopy was applied for the simultaneous analysis of the content of free amino acids,

caffeine, total polyphenols and amylose in green tea (Chen et al., 2008).

8. Conclusions

Phenolic compounds are a much diversified group of phytochemicals that are widely distributed in plants, such as fruits, vegetables, tea, olive oil, tobacco and so on.

Nowadays, there is a growing interest in substances exhibiting antioxidant properties, which are supplied to human organisms as food components or as specific preventive pharmaceuticals.

Consequently, antioxidants have become an essential part of preservation technology and contemporary health care.

It is well known that plants which possess antioxidative and pharmacological properties are related to the presence of phenolic compounds, especially phenolic acids and flavonoids.

Many researchers have suggested that polyphenols may play an important role in preventing obesity, coronary heart disease, colon cancer, gastrointestinal disorders and can also reduce the risk of diabetes (Altiok, Baycin, Bayraktar, & Ulku, in press; Jitaru et al., 2005; Luthria & Pastor-Corrales, 2006; Ross et al., 2009).

Polyphenols are also known for their ability to prevent fatty acids from oxidative decay, and provide a defence against the oxidative stress of oxidising agents and free radicals (Slusarczyk, Hajnos, Skalicka-Wozniak, & Matkowski, 2009).

The biological properties of polyphenols and their health benefits have intensified research efforts to discover and utilise methods for the extraction, separation and identification of these compounds from natural sources. These methods must be comprehensive, rapid, and rich in spectral information.

This paper provides information on phenolic compounds found in vegetal resources, the advanced methods that are widely used for the isolation of bioactive phytochemicals, as well as other procedures that enable further progress in the separation and identification of these compounds. This review shows that the necessary technology is available to achieve the desired analytical goals concerning the separation and quantification of polyphenols.

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