TRENDS IN LC-MS AND LC-HRMS ANALYSIS AND CHARACTERIZATION OF POLYPHENOLS IN FOOD.

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Abstract

Polyphenols comprise a large family of naturally occurring secondary metabolites of plant-derived foods and are among the principal micronutrients associated with the health beneficial effects of our diet. Liquid chromatography coupled to mass spectrometry (LC-MS) and, in the last few years, high resolution mass spectrometry (LC-HRMS) is playing an important role in the research of polyphenols, not only for the determination of this family of compounds in food matrices, but also for the characterization and identification of new polyphenols, as well as the classification and authentication of natural extracts in the prevention of frauds. The purpose of this review is to describe recent advances in the LC-MS and LC-HRMS analysis and characterization of polyphenols in food focusing on the most relevant applications published in the last years. Trends regarding sample treatment, chromatographic separation, mass analyzers and chemometric approaches used in the determination and characterization of polyphenols will be addressed.

Keywords: Polyphenols; Liquid Chromatography; UHPLC; Mass spectrometry; High-resolution mass spectrometry; Food analysis; Chemometrics

1. Introduction

Since several years ago, researchers, food manufacturers as well as the public in general, have become very interested in the quality of food products, which are very complex mixtures consisting of naturally occurring compounds (lipids, carbohydrates, proteins, vitamins, organic acids, and volatile organic compounds –VOCs–) and other substances generally coming from technological processes, agrochemical treatments, or packaging materials. The research on the quality of food products is an issue of great importance in our society not only from the point of view of essential nutrients and bioactive compounds with direct beneficial health effects they provide, but also for the presence of not desired compounds (e.g., contaminants) often dangerous to human health despite occurring at very low levels. Although consumer preferences regarding food products are often influenced by organoleptic (e.g. color, taste, aroma…) and socioeconomic factors (e.g. ecological production, guaranteed origin and quality), people are increasingly more interested in the presence of some specific compounds with health beneficial properties, thereby giving rise even to the production of functionalized food products.

Polyphenols consists of a family of bioactive compounds in foods that caught the attention of consumers over the last few years. Polyphenols are aromatic secondary metabolites ubiquitously spread through the plant kingdom comprising more than 8,000 substances with highly diverse structures. Molecular masses range from small molecules (<100 Da) such as phenolic acids to big molecules (>30,000 Da) of highly polymerized compounds. The main reasons for the interest in polyphenols deals with the recognition of their antioxidant properties, the great abundance in our diet, and their probable role in the prevention of various diseases [1-3]. Furthermore, polyphenols, which also constitute the active substances found in many medicinal plants, modulate the activity of a wide range of enzymes and cell receptors [4]. Moreover, the relevance of polyphenols in food products comes also from their contribution to sensorial properties. Regarding organoleptic concerns, it has been pointed out that contents of compounds such as anthocyanins and proanthocyanidins have a strong influence on color attributes [5]. For instance, glycosides of anthocyanins (such as malvidin, petunidin and peonidin) have been identified as specific descriptors of the color of wines [6]. Also, other compounds including phenolic acids, catechins and some flavonoids play an important role in food quality, as they affect flavor and color properties [6]. Other sensorial characteristics such as bitterness and astringency have been found to be dependent on tannin compounds [7]. Food products such as berries, chocolate, tea, wine and

fresh fruits have been recognized as some of the principal dietary sources of polyphenols for humans, with concentrations ranging from few mg/kg to hundreds of mg/kg, depending on the compound. Their presence in high quantities in transformed products, dietetic supplements and pharmaceutical preparations has also been reported [8-10].

The analysis of polyphenols in food samples is relatively complex due to the great variety of compounds that can be present, which differ in polarity and size (from simple phenolic acids to tannins), but also because many of these compounds in food products are found at low concentration levels. The chemical diversity of polyphenols has hindered the sample extraction and treatment as well as their separation, determination and identification. Liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) is the most effective technique for the structural characterization and determination of both low and high molecular weight polyphenols in food samples [11-13]. The determination of such compounds in complex matrices by LC require high resolution and long analysis times, the latter being sometimes an important limitation when high-throughput analysis is intended. In the last years, ultra-high performance LC (UHPLC), either using sub-2µm particle packed columns [14,15] or porous-shell columns (with sub-3µm superficially porous particles) [16,17], has opened up new possibilities for improving the analytical methods for complex sample matrices, being able to achieve 5- to 10-fold faster separations than with conventional LC, while maintaining or even increasing resolution. Today, UHPLC coupled to MS (UHPLC-MS(/MS) is one of the most widely employed techniques in food analysis and the number of works focusing on the determination of polyphenols is increasing [13].

High resolution mass spectrometry (HRMS) and accurate mass measurements have recently gained popularity due to their great ability to provide more comprehensive information concerning the exact molecular mass, elemental composition and detailed molecular structure of a given compound [18]. Among the multiple advantages of HRMS over classical unit-mass-resolution tandem mass spectrometry we can find: (i) differentiation of isobaric compounds (different compounds with the same nominal mass but different elemental composition); (ii) simplification of sample-preparation procedures, thereby leading to faster methodologies requiring less sample manipulation; (iii) information gathered by a single injection that can be used for quantification and screening purposes, including targeted, suspect and non-targeted analysis; (iv) collection of full-scan spectra that can be stored and used in a later stage retrospective analysis, thus permitting

the formulation of a posteriori hypotheses involving structural elucidation of unknown or suspected compounds [18,19]. In the last years, scientists are taking advantage of LC-HRMS methods either employing time-of-flight (TOF) or Orbitrap analyzers for the characterization, determination and identification of polyphenols in foods [13].

It is noteworthy that beyond the qualitative and quantitative studies of polyphenols, an emerging trend relies on the analysis of compositional profiles and fingerprints as a source of information to be exploited for classification and authentication purposes [20,21]. The number of applications involving a chemometric data analysis has increased dramatically in the last years. Both LC-MS and LC-HRMS provide data of exceptional quality to be further analyzed by chemometric methods such as principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA). Data to be analyzed comprise concentrations of polyphenols of interest (profiling approach) or instrumental signals consisting of intensity counts as a function of *m/z* and retention time (fingerprinting approach). Further data treatments have proved to be highly efficient to facilitate the extraction of relevant information on functional and descriptive characteristics of food products to be exploited for characterization, classification and authentication [8,22].

This review aims at presenting the current state-of-the-art in recent advances in LC-MS and LC-HRMS for the identification and determination of polyphenols in food products, as well as further chemometric MS data analysis for featuring, discrimination, evaluation of adulterations, etc. A selection of the most relevant papers recently published regarding instrumental and methodological aspects, and the newest applications is included. The number of applications in this field is huge, so we discuss on representative works published in the last 2-3 years. First, a description of the different families of polyphenols regarding their chemical structures ad presence in food products is given. Next, we address different aspects (e.g. sample treatment procedures, chromatographic separation, mass spectrometry, high-resolution mass spectrometry and chemometric analysis) by means of relevant applications.

2. Types of polyphenols in food

Polyphenols may be classified into four main families as a function of the number of aromatic phenol rings that they contain as well as the structural elements than bind these rings together: (i) phenolic acids, (ii) flavonoids, (iii) lignans, and (iv) stilbenes[1]. The general classification and

194 the chemical structure representative polyphenols belonging to the different families is shown in
195 Fig. 1. Strictly speaking, polyphenols should contain various aromatic rings with one or several
196 hydroxyl (–OH) groups such as in the case of flavonoids and minor non-flavonoid families (e.g.,
197 stilbenes and lignans). Other compounds such as phenolic acids, which do not match with these
198 structural requirements, are often considered in an extended version of polyphenolic matter, thus
199 being benzoic and cinnamic acids other important subfamilies.
200 As shown in Figure 1, apart from C, H and O, polyphenols do not have characteristic atoms
201 that may help to their identification, characterization and quantification. The typical substituents
202 of flavonoid and non-flavonoid families comprise –COOH, –OH, $–CH_3$ or $–OCH_3$ radicals that
203 are placed on different positions of the corresponding hydrocarbon backbone. Regarding
204 derivatives, phenolic acids may occur naturally as the raw form or combined with other organic
205 compounds such as alcohols, sugars or organic hydroxy acids via ester bonds. For flavonoids,
206 although they may be found free as the so-called aglycons, glycoside derivatives (of glucose,
207 galactose, rhamnose, etc.) are very abundant in vegetal matrices. Special attention deserve the
208 group of flavan-3-ols, commonly referred to as catechins, in which monomers, dimmers, trimmers,
209 etc. and higher polymeric structures are formed.
210 Owing to the variety and levels of polyphenols in food products is greatly diverse since, apart
211 from single flavonoid and non-flavonoid compounds, the number of derivative combinations
212 involving glycosides and hydrolyzable and condensed tannins is huge. Hence, regarding
213 complexity, simple samples such as white wines and beers just contain various dozens of
214 compounds at concentrations in the order of magnitude of 10 – 1 mg L^{-1} or below. For richer
215 polyphenolic sample matrices, such as red wines, fruit extracts, tea, cocoa, etc. hundreds of
216 compounds have been described, some of them occurring at concentrations higher than 100 mg
217 kg^{-1}.
218
219 **2.1. Phenolic acids**
220 There are two main classes of phenolic acids, those corresponding to benzoic acid derivatives
221 (hydroxybenzoic acid group) and hydroxycinnamic acid derivatives (hydroxycinnamic acid group).
222 This family of compounds account for almost 30% of total dietary extractable phenolic and
223 polyphenols compounds. In general, the amount of hydroxybenzoic acids in edible plants is low,
224 although in certain red fruits, black radish and onions concentrations up to several tens of

milligrams per kilogram fresh weight can be found [23]. They result in the basic components of more complex molecules known as hydrolysable tannins which are formed by means of esterification between, for instance, ellagic acid with one or several hydroxyl groups of a sugar residue (i.e., ellagotannins in red fruits such as strawberries, raspberries, and black berries) [24]. In contrast, hydroxycinnamic acids are more abundant than hydroxybenzoic acids. In fact, hydroxycinnamic acid occurs naturally in a number of plants as both *cis* and *trans* isomers, although the latter is the most common one. Cinnamic acid is a key intermediate in shikimate and phenylpropanoid pathways. Shikimic acid is a precursor of many alkaloids, aromatic amino acids, and indole derivatives present in plants. It can be found in free form, but also as ester derivatives (ethyl, cinnamyl, benzyl) in various essential oils, resins and balsams, being very important intermediates in the biosynthetic pathways of most of the natural aromatic products. In addition, hydroxycinnamic acids as a group play a vital role in the synthesis of other important compounds. For instance, they can be converted into immensely important compounds including styrenes and stilbenes through decarboxylation reaction in the nature [25].

The main hydroxycinnamic acids are coumaric acids (being *p*-coumaric acid the most abundant isomer), and caffeic, ferulic, and sinapic acids. Among them, caffeic acid and its derivatives generally represents more than 75% the total hydroxycinnamic acids in broad diversity of fruits. Ferulic acid is the most abundant phenolic acid found in cereals. It should be noted that many of these compounds are typically found as glycosylated derivatives or esters of quinic, shikimic and tartaric acids. For instance, one important family comprises the esters of some hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids, in general) with quinic acid. As an example, chlorogenic acid (3-caffeoylquinic acid) is an ester between caffeic and quinic acids that is an important intermediate of the lignin biosynthesis [26]. Oligomeric forms of hydroxycinnamic acids are also very common, and several dimers, trimers and even tetramers of, for instance, ferulic acid have been described [27].

2.2. Flavonoids

Flavonoids mainly consist of two phenyl rings linked by three carbon atoms that form an oxygen heterocycle ring. This carbon structure is usually referred to as C6-C3-C6. This family of compounds account for 60% of total dietary polyphenols and can be divided into six groups: (i)

flavonols, (ii) flavones, (iii) isoflavones, (iv) flavanones, (v) anthocyanidins, and (vi) flavanols. Indeed, more than 5,000 different flavonoids have been reported in the scientific literature [28].

(i) Flavonols

Flavonols are among the most abundant single or monomeric flavonoids in plant-based foods and beverages, being quercetin (Figure 1), kaempferol and myricetin the main representative compounds. The specific amounts of flavonols in foods are dependent on a range of factors including plant type and growth, season, light, degree of ripeness, food preparation, and processing. As an example, high concentrations of flavonols can be found in apples, apricots, beans, broad beans, broccoli, cherry tomatoes, chives, cranberries, kale, leeks, pear, onions, red grapes, sweet cherries, and white currants [29]. Most of the flavonols in plant-based foods are present in glycosylated forms, associated generally with glucose or rhamnose, but other sugars may also be involved (e.g. galactose, arabinose, xylose, glucuronic acid) [30].

(ii) Flavones

Flavones are a group of flavonoids based on the 2-phenyl-1-benzopyran-4- backbone. They are less common than flavanols in fruit and vegetables. The principal natural flavones include apigenin (Figure 1), luteolin, tangeritin, chrysin, 6-hydroxyflavone, baicalein, scutellarein and wogonin. Flavones are mainly present in cereals and several herbs where they can be found as C-glycosides of flavones [31].

(iii) Isoflavones

Isoflavones are a group of flavonoids structurally similar to estrogens although they are not steroids. The presence of hydroxyl groups in positions 7 and 4' in a similar configuration to estradiol confers these compounds pseudohormonal properties. Most of them act as phytoestrogens in mammals with the ability of bind to estrogen receptors. Isoflavones are produced almost exclusively by members of the *Fabaceae* (i.e. *Leguminosae,* or bean) family. Soya and its processed products are the main source of isoflavones in the human diet. Soy isoflavones, when studied in populations eating soy protein, have been related to a lower incidence of breast cancer and other common cancers because of their role in sex hormone metabolism and biological activity. Soy and soy products contain basically three isoflavones: genistein (Figure 1), daidzein and glycitein, and they are usually found as aglycone (molecule not attached to sugar moieties), 7-*O*-glucoside, 6´´-*O*-acetyl-7-*O*-glucoside, and 6´´-*O*-malonyl-7-*O*-glucoside forms [32].

(iv) Flavanones

Flavanones can be present in tomatoes and some aromatic plants such as mint. They are found at high concentrations in citrus fruits [33,34]. The main compounds in the aglycone form are naringenin in grapefruit, hesperetin (Figure 1) in oranges, and eriodictyol in lemons.However, most of these flavanones are generally glycosylated by a disaccharide (i.e. neohesperidose or rutinose) in position 7, being the main compounds hesperidin (hesperitin-7-rutinoside), narirutin (naringenin-7-rutinoside), neohesperidin (hesperitin-7-neohesperidoside), neoeriocitrin (heridictyol-7neohesperidoside) and naringin (naringenin-7-neohesperidoside) [33].

(v) Anthocyanidins

Anthocyanidins are water-soluble pigments occurring in the vacuolar sap of the epidermal tissues of higher plants, including leaves, stems, roots, flowers and fruits. They provide the characteristic red, pink, purple or blue color (depending on the pH) of such tissues. Although odorless and nearly flavorless, anthocyanidins contribute to taste as a moderately astringent sensation [35].They are usually resistant in plants to degradation, preventing it by glycosylation (generating anthocyanins) with, in general, glucose at position 3, and by esterification with various organic acids (citric and malic acids), as well as phenolic acids. They can be found at relatively high concentrations in red wine, certain varieties of cereals, and several leafy and rood vegetables (aubergines, cabbage, beans, onions…). But it is in fruits where they are most abundant, being cyanidin (Figure 1) the most common anthocyanidin in foods [36].

(vi) Flavanols

Flavanols (flavan-3-ols) are derivatives of flavans that exist as monomeric forms (catechins) and condensed polymers, the so-called proanthocyanidins.

Flavanols are phytochemicals found in high concentrations in a variety of plant-based foods and beverages, and include the following compounds: catechin (Figure 1), epicatechin and some derivatives such as epigallocatechin, epicatechin gallate, and epigallocatechin gallate. High concentrations of catechin can be found in red wine, broad beans, black grapes, apricots and strawberries. Epicatechin concentrations are high in apples, blackberries, broad beans, cherries, black grapes, pears, raspberries, and chocolate. Finally, epigallocatechin, epicatechin gallate, and epigallocatechin gallate are found in high concentrations in both black and green tea [37,38].

Proanthocyanidins, also known as condensed tannins, are dimers, oligomers and polymers of catechins that are bound together by links between C4 and C8 (or C6). Apart from lignin, they represent the most abundant class of natural phenolic compounds in our diet. These compounds

317 can also be classified according to the interflavan linkage as A-type and B-type molecules. B-type
318 proanthocyanidins are those in which monomeric units are linked through the C4 position of the
319 upper unit and the C6 or C8 positions of the lower unit (Figure 2). A-type proanthocyanidins
320 contain an additional ether-type bond between the C2 position of the upper unit and the hydroxyl
321 group at C7 or C5 positions of the lower unit (C2-O-C7 or C2-O-C5) (Figure 2).Apart from their
322 chemical structure, the most important difference between the two families is than only the A-type
323 is capable of inhibiting the adhesion of bacteria to urinary tract tissues [39], being one of the most
324 characteristic health benefits of A-type proanthocyanidins.
325 Catechins and proanthocyanidins are found in common foods such as fruits (grapes, peaches,
326 apples, pears, plums, strawberry, cranberry, kiwi, dates, many red fruits…), cereals (sorghum,
327 barley...), seeds and nuts (beans, peas, almonds…), spices, aromatic plants, and more scarcely in
328 vegetables [40]. They can also be found in various foodstuffs of plant origin (wines, tea, ciders,
329 beers, chocolates, jams, puree..) [41]. However, in these processed foods, catechins and PACs are
330 not only present in their native form, but they have sometimes undergone structural changes
331 especially related to their susceptibility to oxidation with a significant impact on their physico-
332 chemical properties. One of the most obvious examples is probably that of black tea catechins that
333 are enzymatically oxidized, forming theaflavins and thearubigins responsible for the color of the
334 infusions [42].
335
336 **2.3. Lignans**
337 Lignans is a minor class of polyphenols that are formed by two phenylpropane units. The
338 main food source of lignans is linseed, which mainly contains secoisolariciresinol, but they are
339 also found at lower concentrations in cereals (rye, wheat, oat and barley), grains, some fruits such
340 as apricots and strawberries, and certain vegetables such as broccoli and cabbage[43].
341 Secoisolariciresinol (Figure 1) and matairesinol were the first plant lignans identified in foods.
342 Pinoresinol and laricresinol, two recently identified plant lignans, contribute substantially to total
343 dietary lignan intakes (about 75%), while secoisolariciresinol and matairesinol contributed only
344 about 25%. It should be noted that plant lignans are among the principal source of phytoestrogens
345 in the diets of people who do not typically consume soy foods [44].
346
347 **2.4. Stilbenes**

Stilbenes, also known as stilbenoids, are characterized by a double bond connecting two aromatic rings in a C6-C2-C6 structure with several hydroxyl groups. Hence, both *cis* and *trans* isomers naturally occur, being the trans comparatively more common. They are usually found in low quantities in the human diet. Resveratrol (Figure 1) and pterostilbene are among the most noticeable components of this family. Resveratrol is found in grape skins, red wine, peanuts, blueberries and cranberries. Although several anticarcinogenic effects have been attributed to resveratrol during screening of medicinal plants, as of 2014 evidences of its effect on cancer in humans seems to be inconsistent [45]. Its glucoside, the so-called piceid, is also relevant because of the antioxidant properties. Pterostilbene, a stilbenoid chemically related to resveratrol, is found in blueberries and grapes. It is also found in age-old darakchasava, an Indian medicine in which the main ingredient is dried *Vitis vinifera* berries, i.e. raisins [46].Other stilbenes worth being mentioned are piceatannol and pinosylvin, and oxyresveratrol, quite characteristic of species of pinaceae and fabaceae, respectively.

3. Sample treatment procedures

Liquid chromatography (LC) is by far the analytical technique of choice for qualitative and quantitative analysis of phenolic compounds. Despite the advancements in chromatographic separations and mass spectrometry technologies that have allowed analytical chemists to achieve superior separation efficiency, sensitivity and resolution, sample treatment (including extraction, sample clean-up, fractionation, and compound purification) is still one of the most essential parts of the whole analytical procedure. Within this context, several sample preparation methods have been developed in recent years to improve the extraction of polyphenols from food samples. The extraction approach obviously depends on the nature of the sample matrix as well as on the chemical properties of the phenolics, including molecular structure, polarity, concentration, number of aromatic rings and hydroxyl groups [47]. Different extraction solvents such as methanol, ethanol, acetone, water, ethyl acetate, diethyl ether and their combinations have been mentioned in the literature [48], with liquid–liquid extraction (LLE) and solid-phase extraction (SPE) being probably the most used techniques for the fractionation/purification step. The selection of appropriate solvents can improve limits of detection (LOD) and reduce matrix effects in LC-MS analysis. The most effective extractants typically are mixed aqueous-organic solvent systems employing methanol, ethanol, or acetone [49], since phenolic compounds are generally more

soluble in polar organic solvents than in pure water. Furthermore, compounds other than phenolics such as water soluble proteins, peptides, carbohydrates, and organic acids may be co-extracted when increasing the water concentration in the extraction solvent or when using water alone [50]. But the use of organic solvents in the extraction mixtures can also provide additional benefits over simply reducing the risk of further matrix effect in LC-MS analysis. For instance, the use of acetone may improve the extraction yield of polyphenolic compounds by inhibiting protein-polyphenol complex formation during extraction or even by breaking down hydrogen bonds formed between phenolic groups and protein carboxyl groups [51]. On the other hand, the use of *n*-hexane (or other apolar solvents) is of primary importance when performing LLE extraction from fatty samples or oils in order to efficiently remove co-extracted lipophilic substances that would lead to subsequent ionization efficiency and/or chromatographic separation problems. For instance, LLE using methanol/water (40:60, v/v) and n-hexane as washing solvent has been recently reported for the simultaneous extraction of various catechins and gallic acid derivatives (e.g., such as catechin gallate, epigallocatechin and epigallocatechin gallate)in vegetable oils including tea seed oil, sunflower seed oil and soya bean oil [52]. LODs in the range of 0.05–1.65 ng on-column with recovery rates ranging from of 96.2 to 100.5% (RSD <3.7%) have been obtained. However, because of the large amounts of solvent usually required by solvent-based extraction together with its limited selectivity, SPE has been extensively used as an alternative to LLE for sample clean-up purposes.

SPE offers the additional advantages of being rapid, economical and simple to use. Furthermore, SPE devices can be easily automated for higher throughput and different SPE cartridges with a great variety of materials are currently available as sorbents. With regard to SPE stationary phases, octadecyl bonded silica reversed phase (RP-C18) cartridges have been by far the most common choice for extracting phenolic compounds in food samples. However, C18 SPE sorbents may lead to low recoveries when dealing with the most polar compounds (i.e., hydroxybenzoic and hydroxycinnamic acids and their derivatives) [53]. Anyway, they can be successfully retained on reversed phase mode when working at acidic pH values to get the neutral (protonated) species of phenolic acids. Therefore, over the last few years, a wide variety of new SPE sorbents has been employed for phenolic determination. For instance, Pérez-Mangariño *et al.* [53] assayed and compared the efficiency of ten different SPE cartridges and XAD-2 resin to C18 SPE sorbent for the isolation of phenolic compounds present in low concentration in wines (i.e.,

simple phenolic acids and alcohols, flavonols, stilbenes, and their derivatives). As a result, polymeric cartridges, mainly the hydrophilic-lipophilic balance (HLB) sorbents with N-vinylpyrrolidone-divinylbenzene copolymer have seemed to be a good alternative to replace C18 cartridges for the isolation of wine phenolic compounds. In fact, HLB sorbent showed a higher sensitivity for the compounds slightly detected with the C18 cartridges (i.e., hydroxycinnamic acids and their derivatives) together with very good reproducibility, and high percentages of recovery. More recently, the effectiveness of QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction and dispersive-SPE (150 mg $CaCl_2$, 50 mg primary-secondary amine, 50 mg C18) for the sensitive quantification of multiclass polyphenols in wines has also been proved [54]. Nine phenolic compounds were determined at concentrations above the method detectable levels ($0.004<LODs<0.079$ µg/mL). On the other hand, some researchers have focused on alternative "intelligent" materials, such as immunosorbents and molecularly imprinted polymers (MIPs) in order to improve and increase the selectivity and specificity while reducing sample matrix interferences [55,56].

It should be noted, however, that during the last few years there has also been a consistent increase in the development of new rapid, economical and environmentally friendly polyphenols extraction techniques aimed to overcome common drawbacks of traditional methods. Prominent among these novel techniques are microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and ultrahigh pressure extraction. For instance, MAE has been successfully used for the extraction of polyphenols from grape seeds [57], and spices [58] while microwave-assisted enzymatic extraction (MAEE) has been shown to be an efficient and environment-friendly option for the polyphenols extraction from waste peanut shells [59]. In this latter case, the extraction yield reached by using MAEE (1.75 ± 0.06%) was significant higher than those obtained by heat-refluxing extraction (1.53 ± 0.03%), ultrasonic-assisted extraction (1.56 ± 0.02%) and enzyme-assisted extraction (1.62 ± 0.04%). The authors attribute these results to the greater contact area between solid and liquid phase and therefore better access of solvent to phenols upon the disruptions of tissues and cell walls by the action of microwave irradiation. As another interesting application of these novel techniques, the extraction of polyphenols from orange peel has been recently conducted by using MAE and ultrasound technology without adding any solvent but only using ''in situ'' water of citrus peels which was recycled and employed as solvent [60]. Compared with the conventional extraction, the optimized

ultrasound-assisted procedure gave an increase of 30% in total phenolic yield, with significant advantages also in terms of time and energy saving, cleanliness and reduced waste water. Previously, UAE has also been effectively applied to the extraction of phenolics in several other food matrices such as black chokeberry [61], *Laurus nobilis* L. [62] and defatted hemp, flax and canola seed cakes [63] while SFE has been successfully used in hazelnut, coffee and grape wastes samples [64]. In fact, SFE, being a green process, has emerged in the last decade as one of the techniques of choice for the extraction and isolation of high-value natural products and phytochemicals, including polyphenols. Interestingly, a comprehensive enzyme-assisted supercritical fluid extraction (EASCFE) of phenolic antioxidants from pomegranate peel has been reported by Mushtaq *et al.*[65]. In this study, the extraction of phenolics from enzyme pretreated pomegranate peel was carried out by supercritical carbon dioxide (SC-CO_2) with ethanol as a co-solvent. The results revealed that the optimized EASCFE not only enhanced the recovery of extractable bioactive components but also that the levels of extracted total phenolics and antioxidant activities in terms of determination of radical scavengers, inhibition of linoleic peroxidation, and trolox equivalent antioxidant capacity were also significantly improved. Finally, pulsed electric field (PEF) treatment have also been explored for the isolation of total polyphenols and flavonoids (naringin and hesperin) from orange peel [66], so demonstrating the potential of PEF technique as a gentle technology for the extraction by pressing of polyphenols without using organic solvents and with reduced extraction times.

4. Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) are among the most widely used techniques for both quantification and structural characterization of low molecular weight polyphenols but also some oligomer (dimers, trimmers,…) tannins. The number of publications dealing with the LC-MS(/MS) analysis of polyphenols is huge, and some reviews and book chapters devoted to this topic can be found in the literature [8,12,13]. In this section we will focus only on recent and representative applications to the analysis of polyphenols in food samples (Table 1). Regarding the chromatographic separation, both conventional HPLC methods and UHPLC methods are being proposed for the analysis of polyphenols in food matrices. In general, peak efficiency and chromatographic resolution provided in UHPLC are higher than in conventional HPLC and, consequently, the coupling of UHPLC with

mass spectrometry is typically less affected by possible matrix effects. Another advantage is that UHPLC methods can be considered more cost-effective because they typically consume around 80% less organic solvents than conventional HPLC methods. For these reasons, UHPLC-MS(/MS) methods are becoming more popular in the analysis of polyphenols in food [10,67-69], although many conventional HPLC-MS methods can still be found in the literature.

Reversed-phase mode mainly using C_{18} stationary phases (Table 1) is the most widely employed chromatographic separation mode for the analysis of phenolic compounds in food samples, although examples using other stationary phases such as C_8[70,71] or even high strength silica (HSS) T3 [72,73] can also be found in the literature. For instance, Y. Sapozhnikova [71] proposed the use of a Luna C8(2) column (100x4.6 mm, 3 µm particle size) for the conventional HPLC-MS/MS determination of polyphenolic compounds in liquid samples of grape juice, green tea and coffee. The sample preparation employed was based on a dimple "dilute and shoot" approach. The detection was performed by using a triple quadrupole mass analyzer with electrospray ionization in negative mode and quantification using genistein-d_4 as internal standard. In general, satisfactory recoveries (70-120%) were obtained for almost all analyzed polyphenols. In contrast, Gosetti *et al.* [73] recently described the use of an Acquity UHPLC HSS T3 column (100x2.1 mm, 1.8 µm particle size) for the UHPLC-MS/MS determination of eight polyphenols and pantothenic acid in extra-virgin olive oil (EVOO) samples. Figure 3 shows, as an example, the UHPLC-MS/MS separation of a mixture of analyzed compounds (a) and the chromatogram of an EVOO sample (b) in which hydroxytyrosol (HT), tyrosol (TYR) and quercetin (QUE) were quantified. Sample treatment was carried out by LLE using ethanol:water 70:30 (*v/v*) solution and defatting with hexane. Detection was carried out in multiple reactions monitoring (MRM) mode by monitoring two selective reaction monitoring (SRM) transitions with a Q-Trap mass analyzer in negative electrospray ionization mode. Satisfactory recoveries (74-100%) were described, with good limits of quantitation (LOQ) values (0.8-28.3 µg/L) and acceptable intra-day and inter-day precisions (%RSD lower than 5.7). The authors demonstrated that no significant matrix effect was found in the investigated samples. Other chromatographic separation modes such as the use of hydrophilic interaction chromatography (HILIC) with amide-bonded stationary phases or pentafluorophenyl (PFP) columns have also been described in the literature for the analysis of some phenolic compounds [74,75]. For instance, Regos *et al.* [75] evaluated and compared the separation performance of a pentafluorophenylpropyl phase for the analysis of different

503 polyphenolics including phenolic acids and flavonoids (both glycosides and aglycones) with those
504 obtained using a bifunctional phase constituted of octadecyl and phenylpropyl bonded silica as
505 well as three conventional C_{18} columns. As a result, all analytes, with the exception anthocyanins,
506 were considerably more retained on the perfluoro phase compared to the other columns, revealing
507 the suitability of pentafluorophenylpropyl bonded phase for the separation of broad range phenolic
508 compounds. More recently, PFP column has also been proven to offer superior resolving power
509 than C_{18} column when dealing with complex anthocyanin-laden matricessuch as those found in
510 hybrid grape cultivars [76]. In such a case, PFP column allowed the identification and
511 quantification of all 10 anthocyanin species (mono- and diglucoside anthocyanins) found in hybrid
512 wines whereas C_{18} column showed poor separation of the diglucosides from each other as well as
513 the other monoglucosides. Therefore, while C_{18} column could be the preferred choice for
514 anthocyanidin andmonoglucoside analysis, the sufficient resolving power provided by
515 pentafluorophenyl column makes PFP stationary phase the most suitable option when separation
516 of complex mixtures of coexisting mono- and diglucoside anthocyanins is required.
517 Generally, for the separation of polyphenols by reversed-phase chromatography acidified
518 water (with small amounts of formic acid or acetic acid) and methanol or acetonitrile as organic
519 solvents (in some cases also acidified with formic acid or acetic acid) are employed as mobile
520 phases (Table 1). Formic acid or acetic acid concentration is usually kept as low as possible in
521 order to ensure a satisfactory reversed-phase separation without compromising ionization when
522 acquiring in negative ionization mode, and typically is kept between 0.05-0.5%. Although several
523 works are proposing the use of isocratic elution for the determination of polyphenols [77],
524 generally the chromatographic separation of phenolic compounds with similar polarity is better
525 accomplished by gradient elution by using methanol [10,69,71,73,78-83], acetonitrile [67,68,84-
526 86] or even methanol/acetonitrile mixtures [87].
527 Regarding the ionization of polyphenols in LC-MS, electrospray in negative mode is, by far,
528 the most generalized ionization source employed (Table 1), usually providing the deprotonated
529 molecule $[M-H]^-$, although ESI in positive ionization mode has also been proposed in some
530 specific applications [68,87]. The most characteristic examples deal with the detection of
531 anthocyanins which consists of species that already contain flavylium cation moiety (see Figure
532 1) that makes possible their detection in positive mode. In the publication by Kim *et al.* [87], LC-
533 MS/MS was used with positive ESI mode in a QTrap MS analyzer working in SRM acquisition

534 mode, which yielded the protonated molecule $[M+H]^+$, for the profiling of flavonoids in several
535 citrus varieties native to the Republic of Korea. Electrospray ionization in the positive mode has
536 also recently been used by Kaliora *et al.* [88] for the characterization of the phenolic profiles of
537 Greek herbal infusions. All the phenolic compounds showed an intense signal corresponding to
538 the pseudo-molecular ion $[M+H]^+$ and, to a lesser extent, water adducts $[M+18]^+$ and sodium
539 adducts $[M+23]^+$ were also observed. Although less common in the analysis of polyphenols, other
540 atmospheric pressure ionization sources such as atmospheric pressure chemical ionization (APCI)
541 [89-91] or even atmospheric pressure photoionization (APPI) [92,93] have also been described.
542 For instance, LC-APCI-MS in positive ionization mode was proposed for the characterization of
543 apple polyphenols, reporting for the first time five isorhamnetin glycosides, two hydroxyphloretin
544 glycosides and quercetin in apple peel [91]. LC-APPI-MS^n using acetone as dopant reagent in
545 negative mode in an ion-trap instrument was employed for the analysis and characterization of
546 stilbenes and derivatives from downy mildew-infected grapevine leaves [92]. The authors
547 analyzed by ESI–MS and APPI–MS resveratrol derivatives induced after UV treatment of
548 Chasselas grapevine leaves. Compared to ESI, the APPI method showed a higher sensitivity for
549 the detection of all the induced resveratrol dimers (Figure 4). For the peaks of known stilbenes as
550 trans-ε-viniferin (peak 12) and trans-δ-viniferin (peak 16) the intensities increased with a factor of
551 ten and five, respectively. Sensitivity increased also for unknown resveratrol dimers (peaks 3, 6,
552 11 and 14), which were better observed by the APPI–MS method. The APPI mass spectra were
553 also cleaner compared to the corresponding ESI spectra. Although not typically employed in the
554 analysis of polyphenols because they are easily ionized by ESI, APPI could be a good alternative
555 in some specific applications because of the increased sensitivity that can be achieved for some
556 polyphenols [92] but also because APPI is in general less affected by matrix effects than ESI. For
557 instance, in a recent application, Parets *et al.* [94] compared the use of UHPLC-ESI-MS/MS and
558 UHPLC-APPI-MS/MS polyphenolic profiles for the characterization and classification of
559 cranberry-based and grape-based natural products and cranberry-based pharmaceutical
560 preparations. APPI(-) using acetone as dopant reagent showed to be more sensitive than ESI(-) for
561 several targeted polyphenols (i.e. gallic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic
562 acid, gyringaldehide, umbelliferon, and quercetin). Besides, UHPLC-APPI-MS/MS polyphenolic
563 profiles allowed a better principal component analysis (PCA) discrimination between samples than
564 the profiles obtained by ESI, fact that was attributed by the authors to the lower matrix effects

observed with APPI. The fact that ESI data were more sensitive to undergo variations due to the coelution of analytesand matrix components was also confirmed by comparing the external calibration slope of both UHPLC-API-MS/MS methods (ESI vs APPI) with thatobtained by matrix-matched calibration using homogentisic acid and resveratrol in blank cranberry extracts. As a result, a slight matrix effect by ion suppression was observed when ESI was employed (although lower than 20%), while APPI provided the most satisfactory results showed almost no matrix effect. Hence, when using ESI sources, possible matrix effects and ionization competition between co-eluted molecules may occur, thus making crucial to maintain reasonably good separations in the chromatographic domain.

LC-MS/MS or UHPLC-MS/MS methods using triple quadrupole (QqQ) mass analyzers are often proposed for the determination of polyphenols in food and plant products because of their high sensitivity in MRM acquisition mode (Table 1). In general, when working with QqQ instruments, the selectivity of the analysis given by such analyzers has prevailed over the possibility to give a general overview of the compounds in the sample due to the limited sensitivity of these instruments when carrying out a full scan acquisition. Thus, in these systems, collision energies are optimized for two SRM transitions for every compound and both SRM transitions are used for confirmation analysis to meet the EU Decision 2002/657/EC [95]. The most sensitive SRM transition is then used for quantitation purposes. In order to achieve confirmation of a given targeted compound in food analysis, the EU Decision 2002/657/EC has established an identification point system in which at least 3-4identification points are required to fulfill confirmation. In general, 2 identification points are obtained with each SRM transition when working with LC-MS/MS, and for this reason triple quadrupole instruments using two SRM transitions are the most frequently used low resolution MS analyzers in food analysis. For example, recently, Puigventós *et al.* [10] proposed the determination of 26 polyphenols in cranberry-based pharmaceutical and natural products by UHPLC-ESI-MS/MS monitoring two SRM transitions. The information achieved by targeting these 26 polyphenols was then exploited for the PCA classification of samples based on the fruit of origin of the analyzed extracts.

The use of single quadrupole mass analyzers has also been described in the determination of polyphenols, for instance, in wine samples [80] or plant extracts (*Cassicum annuum* L. extracts) [69]. Nevertheless, although a general overview of the compounds present in the sample can be

595 obtained with quadrupole MS analyzers when full scan MS acquisition is performed, these
596 instruments lack in sensitivity in comparison to QqQ analyzers.

597 Ion-trap mass analyzers are typically employed when structural information is required to
598 achieve elucidation of target analytes, because typically full scan MS and product ion scan MS
599 acquisition modes are employed, being able to obtain MS^n spectra which are helpful to establish
600 fragmentation patterns and then to elucidate the structure of a given analyte. For example, Du *et*
601 *al.* [85] proposed the use of HPLC-ESI-MS/MS with a ion-trap analyzer for the elucidation of
602 bioactive compounds of five wild *Chaenomeles* fruits. Among the 24 polyphenol compounds
603 identified in the analyzed extracts, 20 were flavan-3-ols (including catechin, epicatechin and
604 procyanidin oligomers).

605 Lately, the use of QTrap mass analyzers, hybrid instruments combining a quadrupole and a
606 liner ion-trap in a similar configuration than a QqQ instrument, is gaining popularity for the
607 analysis of food products. Several applications can be found in the literature dealing with the
608 determination of polyphenols [71,73,78,79,83,87]. For instance, LC-ESI-MS/MS using a QTrap
609 instrument was described for the structural elucidation and the determination of polyphenols in
610 three *Capsicum annuum* L. (bell pepper) varieties [79]. Twenty-eight polyphenol components of
611 the analyzed fruits were profiled via a single LC-MS/MS run. Of these 28 polyphenols, three
612 hydroxycinnamic acid derivatives (feruloyl hexoside and sinapoyl hexoside types) and five
613 flavonoids components (vicenin-2, orientin, isoscoparin, quercetin 3-*O*-hexoside and luteolin
614 malonylpentosyldihexoside) were identified for the first time in the fruits of the three analyzed
615 varieties thanks to the structural information provided by full scan and product ion scan MS
616 acquisition modes. However, although structural information can be achieved with QTrap
617 instruments, many authors continue to work in MRM acquisition mode in a similar way than with
618 a QqQ instrument (by monitoring two SRM transitions) [71,73,78,83], even when it is well known
619 that similar sensitivity can be achieved in both SRM and product ion scan acquisition modes when
620 MS analyzers based on ion-trap technology are employed.

621 Besides the employment of LC-MS and LC-MS/MS methods for the quantitative
622 determination of polyphenols in a variety of food matrices, tandem mass spectrometry analyses
623 are also a powerful technique for the characterization and structural elucidation in the identification
624 of polyphenols, especially when MS^n fragmentation can be achieved by employing ion-trap
625 technology, and many examples can be found in the literature [96-99]. For example, Maul et al.

[96] employed liquid chromatography and gas chromatography techniques hyphenated with tandem mass spectrometry as tools for the characterization of unknown derivatives of isoflavonoids. For LC-ESI(+)-MS/MS experiments, the basic retro-Diels-Alder fragmentation offered information about the substitution pattern in the A- and B-rings of flavonoids and the elimination of a protonated 4-methylenecyclohexane-2,5-dienone (*m/z* 107) fragment can be then proposed as a diagnostic ion for the identification of many isoflavones. Kuhnert and co-workers [97,98] described the use of LC-MS^n for the characterization and quantification of hydroxycinnamate derivatives in *Stevia rebaudiana* leaves by employing an ion-trap mass analyzer in negative electrospray ionization mode. Tandem mass spectral data up to MS^4 was obtained for each compound, and peak compositional assignments were performed on the basis of structure diagnostic hierarchical approaches. Twenty-four hydroxycinnamic acid derivatives of quinic and shikimic acid were detected, and 19 of them were successfully characterized by the authors to regioisomeric levels, being 23 of them described for the first time in the analyzed sample (three monocaffeoylquinic acids, seven dicaffeoylquinic acids, one *p*-coumaroylquinic acid, one feruloylquinic acid, two caffeoyl-feruloylquinic acids, three caffeoylshikimic acids, and two tricaffeoylquinic acids). The authors also observed cis isomers of di- and tricaffeoylquinic acids [97]. In another interesting work, Chen et al. [99] achieved the structural identification of theaflavin trigallate and tetragallate from black tea by employing LC-ESI-MS/MS fragmentation in an ion-trap mass instrument. The structural identification was addressed by obtaining MS^n spectra (n = 1-4) of suspected compounds and comparing the MS/MS spectra of the product ions to the MS/MS spectra of (-)-epigallocatechin-3-gallate, (-)-epicatechin-3-gallate and theaflavin-3,3'-digallate standards. This work allowed the authors to confirm for the first time the presence of theaflavin trigallate and tetragallate in black tea samples.

Despite the fact that MS/MS fragmentation is a powerful tool for the structural characterization and identification of polyphenols, the low resolution attainable with QqQ and ion-trap instruments makes sometimes difficult the differentiation between isomeric compounds, as well as the unequivocal assignation of fragment compositions. For these reasons, high resolution mass spectrometry, especially when combined with tandem mass spectrometry experiments, also appear as a powerful tool to achieve polyphenolic characterization and identification, and some examples will be addressed in the next section.

5. High resolution mass spectrometry

When dealing with complex sample matrices, such as food, adequate mass resolution is often essential. Consequently, in the past few years, high-resolution mass spectrometry (HRMS) has also gained wide acceptance as a highly sensitive and selective technique for the determination of polyphenols in food matricesby virtue of its numerous and significant advantages over low-resolution mass spectrometry [13]. HRMS, in fact, achieving a high mass resolution and hence a high accuracy of mass measurement, enhances the possibility to unambiguously determine the elemental composition of known and new constituents with a high level of accuracy, typically below 5 ppm, which allow the analyst to also distinguish between target analytes and other co-eluting isobaric compounds [100]. It is worth mentioning that the high accuracy of mass measurements achieved in HRMS is based on the exact mass being measured correctly, and this will depend on the stability and the accuracy of the mass calibration of the HRMS instrument. Unlike TOF-systems, where frequent calibrations are needed, the calibration of the Orbitrap mass analyzers is stable for several days. Even these instruments have available some features such as The Check Mass Calibration tool that enables users to check the mass accuracy after an user-defined time period to see if re-calibration is required or not. Usually, Orbitrap mass calibration is performed by employing text mix solutions (depending on the mass range to be calibrated) provided by the instrument supplier. In contrast, for more accurate mass measurements, TOF and Q-TOF instruments frequently used a lock mass correction, which consists of the constant infusion of a reference compound selected by the users (which could be a polyphenol perfectly characterized) and the correction of the experimental *m/z* values with that of the reference. External mass calibration by employing a reference compound can also be used with Orbitrap mass analyzers if required.

Furthermore, HRMS enable collection of full-scan spectra that can be stored and used for retrospective analysis allowing the formulation of a posteriori hypotheses with further detection and structural elucidation of unknown or suspected polyphenol compounds [19]. The recent widespread use of LC-HRMS, which is clearly exposed when examining the number of publications using the coupling of LC to HRMS throughout the years, is largely due, however, to the recent development and availability of more rugged, sensitive, and selective instrumentations able to operate at reduced costs [18]. From the different HRMS instrumentation available [magnetic sector, time-of-flight (TOF), Orbitrap, and Fourier transform ion cyclotron (FT-ICR)],

688 TOF and Orbitrap are the most-commonly used analyzers for both LC and UHPLC analysis of
689 phenolic compounds in food matrices. In fact, classic HRMS instrumentation (sector or FT-ICR)
690 are too slow, too complex to handle, and probably too expensive to buy and to maintain [101]. On
691 the contrary, recent advances in both TOF and Orbitrap mass analyzers have reduced power
692 requirements, size and instrument costs (especially when compared to FT-ICR) while maintaining
693 high resolving powers of approximately 10.000-40.000 FWHM (full width at half maximum) and
694 10.000-140.000 FWHM for TOF and Orbitrap, respectively [102].
695 For all these reasons, here we decided to review the use of LC-HRMS based on Orbitrap and
696 TOF mass analyzers, by discussing some recent and representative applications to the analysis of
697 polyphenols in food samples (Table 2). As mentioned above, in almost all of these works, LC
698 separation of polyphenols has been performed in the reversed-phase mode, mostly using water
699 with methanol/acetonitrile as organic modifier and small amounts of formic/acetic acid. It is also
700 evident that, in recent applications (2013-2015), UHPLC technology has almost replaced
701 conventional HPLC, thus becoming the chromatographic method of choice in modern laboratories
702 for separating polyphenols in foods when using TOF of Orbitrap mass spectrometers. Finally, after
703 LC separation, detection is mainly performed by negative electrospray ionization [ESI or heated
704 ESI (HESI)], being an excellent tool for identifying phenolic compounds. In fact, although
705 chromatographic separation requires acidic conditions, the response of polyphenols (with the
706 exception for anthocyanins and isoflavonoids) has been proven to be better in the negative ion
707 mode than in the positive one [13,103].
708
709 **5.1. Orbitrap mass analyzer**
710 Until a few years ago, there were still few applications of Orbitrap MS to analyze phenolic
711 compounds in the food field and, when a single analyzer was used, TOF was the most commonly
712 reported [13]. The panorama, however, has deeply changed in the last two/three years and from
713 Table 2 it can be seen that Orbitrap mass analyzer has now become the mainstream mass
714 spectrometry technique for the analysis of food polyphenols. For instance, Orbitrap-based mass
715 spectrometer methodologies have been successfully employed to the analysis of phenolic
716 compounds in fruit products [9], artichoke [104], barberry herb [105], *Pistacia lentiscus* var. *chia*
717 leaves [106], and alcoholic fermented strawberry products [107]. However, at present, there are
718 only a few papers reporting polyphenols HRMS analysis based on single stage Orbitrap mass

719 spectrometer. In fact, hybrid mass spectrometers, which are devices resulting from the combination
720 of two or more analyzers of different types, are undoubtedly today's methods of choice for modern
721 analytical chemists since they combine different performance characteristics (i.e., mass resolving
722 power, speed of analysis, and dynamic range of mass accuracy) offered by the various types of
723 analyzers in one mass spectrometer [18]. And among Orbitrap hybrid instruments, hybrid mass
724 spectrometers using linear ion trap technology, such as LTQ-Orbitrap, has evolved into the most
725 common mass spectrometers currently used in this field. In fact, LTQ-Orbitrap offers the
726 possibility of screening, identification and structural characterization of unknown polyphenolic
727 compounds by using, for instance, exact mass to calculate the most favorable elemental
728 composition and accurate mass of the MS^n product ions in the data dependent scan. As an example,
729 polyphenolic profiles of 44 unifloral Serbian honeys obtained by means of UHPLC coupled with
730 LTQ-Orbitrap mass spectrometer have been recently used to perform a PCA statistical analysis for
731 selecting and defining floral markers of the botanical origin of Serbian honey [108]. The authors
732 showed how the use of high sensitivity accurate mass scan together with automatic data-dependent
733 capability allowed the identification of four different phenolics with almost identical masses
734 (apigenin and galangin at 269.0459 *m/z*, alpinetin and pinostrobin at 269.0822 *m/z*). Furthermore,
735 exact mass search and different fragmentation patterns also permitted the identification of different
736 co-eluting compounds such as chrysin and prenyl caffeate or pinobanksin-3-*O*-acetate and caffeic
737 acid phenylethyl ester. In fact, the use of a very narrow mass could compensates for a lack of
738 chromatographic resolution, thus providing the possibility to discriminate co-eluting compounds
739 as well as to cut off disturbing interferences with a significant increase in the method's selectivity
740 (Figure 5). In some cases, however, even HRMS cannot individually determine and quantify
741 compounds characterized by the same exact mass (similar elemental composition) and retention
742 time (RT). For instance, López-Gutiérrez *et al.* [109] developed a method based on single-stage
743 Orbitrap high resolution mass spectrometry for the identification of phytochemicals in
744 nutraceutical products obtained from green tea. In this case, for some compounds with the same
745 exact mass such as homoorientin and orientin (*m/z* 447.09328) or quercetin-3-*O*-glucoside and
746 quercetin-3-*O*-galactoside (*m/z* 463.08820) that also showed the same retention times, the high
747 similarity between the structures of these pair of compounds also provides similar fragments
748 during all-ion fragment (AIF) experiments. Therefore, the use of characteristic fragments strategy
749 to distinguish these analytes was ineffective in this type of situation. Nevertheless, LTQ-Orbitrap

mass spectrometer obviously remains a promising and powerful tool for the identification, structural elucidation and quantitative analyses of food polyphenols. Recently, the combination of LTQ-Orbitrap data-dependent scan and MS^n experiments allowed to tentatively identify 47 phenolic compounds in beer, seven of which have never been determined before in this type of matrix: feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside [110]. In another study, 120 phenolic compounds, including hydrolysable and condensed tannins, flavonoids and phenolic acids, have also been identified tentatively in walnuts on the base of their accurate mass measurement and subsequent mass fragmentation data from LTQ-Orbitrap [111]. In conclusion, the Orbitrap mass analyzer, especially in the hybrid configuration, has become a powerful addition to the arsenal of mass spectrometric techniques for polyphenols analysis in food. In fact, it offers significant advantage over low-resolution QqQ technology by permitting the use of HRMS applications in complex matrices such as food samples, where significantly higher sensitivity and selectivity are often required. Furthermore, the continuing evolution of Orbitrap technology toward increased acquisition speed, higher resolving power, mass accuracy, and sensitivity will undoubtedly give rise to new applications into the fields of polyphenols determination, thus permitting, in the near future, even more widespread use of Orbitrap mass analyzers for both routine and research analysis of these compounds in food.

5.2. Time-of-flight (TOF) mass analyzer

In recent years, several hybrid TOF instruments have been developed such as quadrupole-time of flight (Q-TOF), ion trap-time of flight (IT-TOF) and TOF-TOF, among others. However, from Table 2, we can see that hybrid Q-TOF instrument is currently the most popular HRMS TOF-based device used for food polyphenol analysis since it is capable of tandem MS experiments and additional scanning type such as ion product and selected reaction monitoring. The use of LC-Q-TOF HRMS methods has been recently reported for the analysis of flavonoids and hydroxycinnamic acid derivatives in rapeseeds (*Brassica napus* L. var. *napus*) [112] as well as for the evaluation of the influence of cross-breeding segregating populations on the phenolic profile of virgin olive oils [113]. In the same way, Capriotti *et al.* [114] developed a UHPLC-Q-TOF method for the analysis of polyphenols in virgin olive oil while Jerman Klen *et al.* [100] employed

781 an UHPLC system with diode-array (DAD) and electrospray ionization quadrupole time-of-flight
782 high resolution mass spectrometry (ESI-Q-TOF-HRMS) for assessing the phenolic profile of
783 olives and olive oil process-derived matrices. In the latter study, two new diastereoisomers of
784 verbascoside derivatives were first discovered in olive extracts. The use of Q-TOF-HRMS allowed
785 their tentative identification by calculating the possible molecular formula from experimental *m/z*
786 and MS fragment data interpretation, yielding $C_{30}H_{38}O_{16}$ with a high mass accuracy (< 5 ppm) for
787 all matrices. The HRMS spectra and fragmentation pattern for the parent ion at *m/z* 653.2082
788 revealed an identical fragmentation profile (*m/z* 621, 459, 179, 161) which is typical for
789 verbascoside derivatives. Furthermore, the data provided by the hybrid Q-TOF MS also permitted
790 to tentatively assign the identities of two new compounds: methoxynüzhenide, and
791 methoxynüzhenide 11-methyl oleoside. These results undoubtedly offer another example of the
792 main benefits of hybrid HRMS mass spectrometry compared to triple quadrupole MS and/or to
793 low-resolution MS, in general. In another paper, the use of HPLC-ESI-Q-TOF-MS with negative
794 ion detection has also shown to be a powerful technique for the characterization of phenolic
795 compounds of peel and seed extracts of three mango varieties (Keitt, Sensation and Gomera 3)
796 produced in Spain [115]. MS and MS/MS spectra and data obtained by Q-TOF MS analysis
797 provided essential information for the characterization of the structures of the phenolic compounds
798 present in different vegetable products. Comparison of Q-TOF data with the literature and online
799 database (i.e., Phenol-Explorer, ChemSpider, MassBank, METLIN, LIPID MAPS, Metabo
800 Analyst, and Spectral Database for Organic Compounds) allowed the tentative identification of
801 thirty phenolic compounds including gallates, gallatannins, flavonoids, xanthones, benzophenones,
802 gallic acid and derivatives, eight of which, had not been reported before in mango peels and seeds.
803 It is worth mentioning that the match probabilities of these databases is based on the exact mass
804 being measuredcorrectly and compared against the one in the database, and this will depend on the
805 stability and the accuracy of the mass calibration of the HRMS instrument, as previously
806 commented. Database software then correct the *m/z* values for any targeted or non-targeted
807 polyphenol, taking into account the variation obtained between the reference standard exact mass
808 and the experimental one. Moreover, someskill in MS is also required when using databases in
809 order to use the M + 1 and M + 2 isotopic information to achieve a correct formula, and to lower
810 the probability of erroras much as possible to low ppm values. To help in this process, today several
811 database programs also give the isotope table and the isotope abundances for each match, and

812 many instruments provide software to help with isotope identification and matching, which is quite
813 useful for correct formula identification.
814 LC-HRMS results in an excellent technique for the tentative identification of unknown
815 components from the interpretation of data such as the exact mass, and MS and MS/MS spectra.
816 Although this kind of studies may be sufficient in some cases, it should be noted that the final
817 confirmation of the identity of the compounds will require additional assays using standards of the
818 candidates. The full concordance of chromatographic and spectral data may be used as the criterion
819 of positive identification. Finally, regardless of the type of MS analyzer, the use of high resolution
820 and accurate mass will surely become routine in food polyphenol analysis as instrument resolving
821 power, accuracy, and sensitivity continue to improve.
822
823 **6. Chemometrics**
824 Chemometrics applies mathematics, statistics and logic to design and optimize the
825 experimental conditions, and to facilitate the recovery of the relevant underlying information from
826 a given data set [116]. Regarding the topic of this review, chemometrics will be used in the
827 preliminary steps of the development of analytical methods to facilitate the optimization of sample
828 treatments and chromatographic separations. Chemometrics will be also fundamental for the
829 analysis of the great amount of data provided by LC-MS, thus offering excellent possibilities in
830 characterization, classification and authentication of food products [8].
831
832 **6.1. Optimization**
833 A comprehensive optimization of sample extraction and chromatographic conditions is
834 crucial when dealing with complex food samples containing a great diversity of components. This
835 is obviously the case of the determination of polyphenols in food matrices. Recently, however, the
836 introduction in our laboratories of more advanced and powerful instruments such as LC-RHMS
837 platforms may entail a certain carelessness around the optimization issues. Anyway, in our opinion,
838 despite the great resolution performances offered by these massive techniques, optimization issues
839 should not be underestimated to avoid unwanted interferences and matrix effects.
840 Often, the optimization is conducted by trial-and-error in which the study carried out without
841 a pre-established plan of experiments. Although very popular, such a strategy is quite inefficient
842 and time-consuming so that alternative approaches for a more satisfactory optimization are

welcome. In this regard, the design of experiments (DOE) has demonstrated to be highly effective to find out the best sample treatment and chromatographic conditions from a reduced set of experiments [117]. When working with DOE approaches, the two following issues deserve our attention: (i) the optimization criterion to be used and (ii) the experimental variables to be explored.

It should be also noted that the concept of "what is optimal" is not trivial. Frequently, multiple objectives need to be reached simultaneously. In DOE, we may define an optimization criterion that refers to the overall suitability or quality of the experimental results. For instance, in LC some important objectives to be attained include good resolution of those relevant compounds closely eluted, separation of as many components as possible and reduction of the run time to speed up the analysis. Under these circumstances, a single objective may be insufficient to express the optimal situation of the separation. Hence, in order to take into account all desired objectives simultaneously multicriteria approaches are recommended. For such a purpose, multicriteria response functions can be implemented as mathematical expressions involving the combination of weighted contributions of each individual objective. This is often accomplished from product functions written as the following generic expression: $D = \Pi(d_i)^{1/n}$, where D is the overall response and di represents each individual objective. A very popular case of such expressions is based on Derringer desirability functions [118] (see below for an illustrative example). Analogous considerations could be taken into account for the optimization of sample treatments.

The diversity of variables that are involved in the different steps of the LC-MS methods, including pretreatment, separation and detection, often involves DOE as a more effective way to gain key information from a reduced set of runs. In the case of the separation, the systematization of the optimization of the LC gradient profile may result in a quite complex task, especially for dealing with multi-step profiles. Some typical factors to be considered around the composition of the mobile phase often comprise pH, organic solvent percentage, organic modifier concentration, etc. If necessary, various gradient sections can be connected to get an overall gradient valid of a wide variety of analytes belonging to the different polyphenol families.

Following DOE, those factors found relevant are candidates to be investigated in a more comprehensive optimization while those irrelevant can be obviated. Commonly, the evaluation of the intensity of main effects and interactions is carried out by full factorial design. The experimental cost depends on the number of levels L and the number of variables f of the design, being L^f the number of runs to be performed. Then, if the number of variables involved in processes

874 is high, preliminary screening by fractional or Plackett-Burman designs may be recommendable.
875 It should be highlighted that when interactions of factors are detected, simultaneous optimization
876 of such variables should be conducted to assess the final conditions. For this purpose, methods
877 such as central composite and grid designs can be used and the resulting data can be fitted to a
878 response surface.
879 The treatment of food samples is mainly focused on attaining a quantitative (or high)
880 recoveries of analytes, polyphenols in our case, as well as obtaining clean extracts free of
881 interferences from the sample matrix [8]. As mentioned above, the sample treatment to be applied
882 will depend on the characteristics of the food matrices. For instance, for simple matrices such as
883 cold drinks, juices, beer, wine and spirits, sample filtration prior analysis may be sufficient. When
884 dealing with solid samples, however, solvent extraction is used to recover polyphenols. Apart from
885 solvent composition, other chemical variables such as pH, solvent volume, time or temperature
886 may be also relevant to enhance the recovery yield. The wide range of physicochemical
887 characteristics (e.g., molecular mass, solubility, polarity and acid-base properties) of compounds
888 belonging to the diverse families of polyphenols entails important differences in the extraction
889 procedures. Below, some recent applications of experimental design to the treatment of food
890 samples will be discussed.
891 The optimization of the extraction of some phenolic acids and flavonoids, with special
892 attention on flavanols, in apple was based on a 3-factor 2-level design with 3 replicates in the
893 central point [119]. The objectives to be optimized consisted of individual contents of selected
894 compounds (e.g., flavanol monomers, phloridzin, chlorogenic acid, hyperoside, etc.) as well as the
895 total phenolic content expressed as catechin equivalent kg^{-1} fresh food. The effect of four variables,
896 namely solvent composition, sample mass, time and number of extraction cycles was statistically
897 evaluated. Factors and interactions found as significant were used as independent variables to
898 establish multilinear models to fit the extraction data. Authors pointed out the difficulty to find a
899 consensus among optimal conditions for all components, so the extraction procedure to be applied
900 depended on the polyphenol family of interest. In another example, central composite design was
901 used to investigate the extraction of phenolic acids in star fruit pulp [120]. The influences of
902 temperature and ethanol concentration on overall phenolic content, antioxidant capacity and
903 scavenging activity were evaluated. Second-order polynomials were fitted to build the
904 corresponding response surfaces. Makris and coworkers reported the optimization of the extraction

of polyphenols in pomegranate by a three-factor central composite design [121]. Variables considered were pH, ethanol concentration and extraction time taking the total phenolic yield as the objective response to be maximized. Extracts from each run were further analyzed by LC-MS. Some relevant polyphenols such as punicalins and ellagic acid were successfully recovered under the optimal conditions. Another application from Makris group presented the optimization of the extraction of phenolic compounds from olive leaves [122]. Glycerol concentration in the extracting solvent and time were optimized by response surface methodology. Teng *et al.* developed the microwave-assisted extraction of anthocyanins and other phytochemicals from raspberry using 3-factor central composite designs [123]. In this case, irradiation power, process time and ethanol percentage were screened. Total polyphenol content and anthocyanin recovery were taken as objective responses of interest. Data was adjusted to second order expressions including only those significant contributions of effects, quadratic terms and interactions. Selected experimental conditions to perform the extraction were different depending on the type of analyte (response) to be considered.

As a summary of papers published, the focus of the extraction may be different depending on the analytes of choice. Some studies have been addressed to specific target compounds while other are intended to maximize the overall polyphenol recovery or other general properties such as antioxidant capacity. Variables under study may be diverse although solvent composition and time seems to be of general interest. In order to represent visually the results, data is typically fitted by multilinear regression considering those significant factors and interactions as independent variables. Because of the number of experiments is, in general, limited the use of more data demanding modeling methods such as partial least square regression has not been considered yet.

Regarding the chromatographic separation, the role of optimization will be especially remarkable in the case of UV-Vis spectroscopic detection but it should not be underestimated in MS. Indeed, despite the great performance of modern HRMS instruments, some severe drawbacks that may hinder the reliability of results remain unsolved. In particular, the occurrence of isomeric compounds and ionic suppression/enhancement effects may induce interferences on MS detection. Under these circumstances, the chromatographic separation of all the analytes of interest appears as an undeniable concern.

DOE has hardly been applied to optimize the separation in liquid chromatography because of the difficulty of factorizing the gradient profile, especially when dealing with complex food

samples that require multi-ramp elution gradients. Some strategies have been proposed elsewhere to tackle multistep gradient optimization by factorial design [124]. Recently, Pérez-Ràfols and coworkers optimized the separation of polyphenols in beer [125]. In particular, the resolution of syringic acid and epicatechin ($Rs_{s/e}$), and ferulic and salicylic acids ($Rs_{f/s}$) were considered as the objectives of interest to define a multicriteria approach based on Derringer desirability functions. The mathematical expression of overall desirability was as follows, $D = (d_{Rss/e} \times d_{Rsf/s} \times d_{tR})^{1/3}$, being the individual desirabilities $d_{Rss/e}$ for the resolution syringic acid epicatechin peaks, $d_{Rsf/s}$ for the resolution of ferulic and salicylic acid peaks and d_{tR} for the retention time of the last compound eluted. Resolution data was transformed into desirabilities considering that $Rs > 1.3$ corresponded to an excellent separation ($d = 1$) and $Rs < 1$ were unacceptable ($d = 0$). For retention time, limits of optimal (fast) and unacceptable (too time-consuming) were set to 10 and 25 min, respectively. Under these criteria, the response surface describing the overall desirability is shown in Figure 6, in which the best separation was obtained at the surface maximum (see arrow) as a reasonable compromise between chromatographic separation and speed. A similar approach was followed by Raja *et al.* to develop a new method for the determination of polyphenols in pear pulp relying on solvent extraction and liquid chromatography [126]. Both sample treatment and separation were optimized by experimental design. A multi-step gradient profile was required to deal with the great diversity of compounds to be determined. First of all, working with standards of prominent compounds, a multicriteria function was created considering the resolution of problematic peaks acid and analysis time as the objective responses. Regarding factors, methanol percentage (three levels) and initial gradient time (two levels) were chosen to design the gradient profile. The simultaneous occurrence of similar benzoic, hydroxycinnamic and flavonoid compounds required three isocratic steps at different MeOH percentages. Once the separation of the standard mixture was successfully accomplished, it was validated on pear extracts. In that case, the desirability function was redefined considering the separation of the higher number of peaks in the minimum analysis time as the objectives considered. Final LC conditions provided an excellent separation without noticeable interferences nor matrix effects.

6.2. Data analysis

The application of LC-MS to the analysis of food samples provided huge amounts of data of exceptional quality that can be exploited for characterization, classification and authentication

purposes [8,127,128]. The implementation of excellent user-friendly software platforms for data treatment has encouraged many researchers to apply chemometrics in their current studies. Anyway, users should be aware of limitations of the performance of chemometrics. In our opinion, the main risks to be considered are related to representativeness of the sample sets, the quality of data and validation of the models.

Regarding representativeness, large sets of samples should be used to extract robust conclusions that could be generalized to other (new) samples of similar characteristics. On the contrary, the validity of conclusions drawn might be inappropriate for other similar samples. This is the case, for instance, of the search of class descriptors found relevant from a given working set that cannot be encountered on independent samples because the dimension of the study is too reduced. In a similar way, if the sources of variation among the samples under study are excessive conclusions may be wrong. This means that for the investigation of potential polyphenol markers of different wine origins, other variables such as grape variety, ageing, vintage, winemaking practices should be controlled.

Data to be treated chemometrically often consists of chromatographic or spectroscopic fingerprints. Raw data may be affected by some reproducibility issues such as baseline drift, peak shifting, background interferences, etc. These data imperfections can be corrected mathematically using, for instance, drift de-trending, peak alignment, smoothing procedures. Besides, the addition of internal standards may be of great interest to minimize the variability of in the sensitivities of signals. In order to check the overall reproducibility of the chemometric models, samples should be analyzed by triplicate so that replicates should appear together in the map of scores. An excellent way to track the robustness of the models is based on the analysis of one (or several) quality control (QCs) which consists of a representative pool of all the set of samples. Typically, the QC is analyzed periodically (e.g., every 10 samples) throughout the series. As a result, QCs should appear in a compact group in the center of the model. On the contrary, dispersions or trends in the QC behavior may indicate changes in the separation performance, detection sensitivity, etc. throughout the series of measurements.

Another important point to be considered is the validation of the chemometric models. This issue should be treated by external validation using an independent set of samples to confirm that results and conclusions extracted from one set can generalized to a bigger group of samples. Unfortunately, this aspect is often undervalued an internal cross validation is commonly applied

998 for validation purposes. In this regard, in the paper by Gallart-Ayala et al. polyphenolic profiles
999 by LC-MS were used to classify beers according to the brewing procedure [129]. Data from a set
1000 of lager and ale was analyzed by PCA and PLS-DA to identify potential markers of each of the
1001 classes. Although various features were found to be discriminant, some of them could not be
1002 confirmed on an independent set of analysis of new beers. These results point out that conclusions
1003 extracted from such a models may be overoptimistic and should require a thorough confirmation.
1004 A proper validation is even more crucial in untargeted metabolomics in which features retained
1005 for analysis are highly dependent on experimental conditions and instrumental platform used [130].
1006 Compositional profiles of naturally occurring polyphenols have recently been proposed as a
1007 rich source of analytical information that needs to be studied and interpreted. The description and
1008 discrimination of samples can be also tackled from the analysis of the so-called fingerprints, i.e.,
1009 complex instrumental signals that may contain mixed contributions from several known or
1010 unknown components. When several samples are analyzed simultaneously, the corresponding data
1011 is arranged in a data matrix, in which each row corresponds to a given sample and each column to
1012 the concentration of a given chemical species (profiling) or an intensity features (fingerprinting)
1013 [22].
1014 By far, PCA is the most popular method for an exploratory study of food properties. Anyway,
1015 occasionally, cluster analysis (CA) is used to complement the information regarding the
1016 distribution of samples into groups. PCA relies on the concentration of the relevant variance into
1017 new mathematical variables, the so-called principal components (PCs) [116,131]. The data matrix
1018 is decomposed into matrices of scores (coordinates of the samples) and loadings (eigenvalues),
1019 providing information on samples and variables, respectively. The scatter plot of scores of PCs is
1020 often used to show the distribution of samples, that may reveal patterns and differences attributed
1021 to features such as origin, manufacturing practices, product varieties and so on. The plot of
1022 loadings explain the behavior of variables and their correlations so the most descriptive ones can
1023 be identified and studied. Besides, relationships between samples and variables can also be
1024 investigated from the simultaneous study of scores and loadings, from the so-called bi-plots.
1025 The classification of food products into pre-established categories can be carried out by
1026 Discriminant Analysis (DA) and related methods, often combined with Partial Least Square
1027 regression (DA-PLS), and Soft Independent Modeling of Class Analogy (SIMCA) methods
1028 [116,131]. In classification and authentication, a set of well-defined samples belonging to the

1029 classes of interest (e.g., variety 1 and variety 2, authentic and fake, etc.) are used to create
1030 prediction models to further assign unknown samples to each class. The classification performance
1031 can be evaluated by external validation using a test set of new samples to account the ability to
1032 correctly assign the samples to their actual classes. PLS-DA models are interpreted, in a similar
1033 way as indicated for PCA, to try to find markers of each class. Relationships of physicochemical
1034 variables with agricultural, manufacturing or sensorial attributes can be thus established.
1035 Anton *et al.* determined the polyphenolic composition of tomato cultivars by LC-DAD-MS
1036 as well as the antioxidant capacities by the Folin-Ciocalteau method [132]. Results from several
1037 tomato varieties and cultivated under conventional and organic conditions were compared using
1038 PCA. Although there was not a marked sample discrimination according to the growing conditions,
1039 some compounds such as apigenin acetylhexoside and caffeic acid hexoside occurred at
1040 significantly higher concentrations in all organic samples. In another study, PCA and CA were
1041 used to investigate the recovery of phenolic acids in mango by-products like peels and seed [115].
1042 Data consisted of levels of 30 compounds including gallates, gallatannins, flavonoids and
1043 derivatives as well as 8 peaks of unknown compounds. Results pointed out the key role of the
1044 extraction procedure in the recovery of richer extracts. Phenolic profiling was also exploited to
1045 explore the influence of breeding and cropping methods on the characteristics of Sicilian wines
1046 using PCA and canonical discriminant analysis [133]. Another work evaluated the influence of
1047 alcoholic fermentation of strawberry products on the polyphenol composition and the antioxidant
1048 activity. Results by linear discrimination analysis (LDA) revealed significant changes in the
1049 composition as a function of the process [107]. Also, some up- (and down-) regulated compounds
1050 such as homovanillic and p-hydroxyenzoic acids were proposed as tentative markers of the
1051 alcoholic fermentation. The metabolomic approach was applied to investigate changes in the
1052 polyphenolic fingerprints of seeds and sprouts of a type of Asian bean depending on the
1053 germination process [134]. Samples were clearly distributed sequentially over time and several
1054 flavonoids were related to markers of the germination process.
1055 Studies classification and authentication of citrus fruit juices were conducted by Abad-García
1056 *et al.* using LDA and PLS-DA [135]. Data consisting of contents of 49 polyphenols revealed
1057 various markers that might be characteristic of the different products. Samples were correctly
1058 assigned to their corresponding classes. Besides, PLS models allowed the successful determination

of adulterations of sweet orange juices by tangerine ones when present in percentages between 10 to 70%.

In the study by Puigventós and coworkers, LC-ESI-MS/MS was applied to the analysis and authentication of fruit-based products and pharmaceutical preparations [10]. Different kinds of cranberry and grape samples were analyzed, including fruits, fruit juices, and raisins, as well as commercial natural extracts, powder capsules, syrup and sachets. 26 polyphenolic compounds belonging to different families (stilbenes, phenolic acids, and flavonoids) were determined. PCA suggested that levels of polyphenols resulted in a suitable source of potential descriptors for the authentication of fruit-based products. Samples were clustered according to type of fruit (Figure 7).

Conclusions and future trends

The high numbers of works dealing with polyphenol studies in foods that have been conducted to date provide a good indication of the relevance of this family of compounds for society, researchers and food producers. It is well known that polyphenols are an important source of natural antioxidants with a great variety of positive health effects. However, detailed intake values for all kind of polyphenols are missing because of the complexity of food matrices and the lack of validated and standardized methods for their determination.

The analysis of polyphenols in food samples is relatively complex due to the great variety of compounds that can be present, which differ in polarity and size (from simple phenolic acids to condensed tannins), but also because many of these compounds in food products are found at low concentration levels. The chemical diversity of polyphenols has hindered the sample extraction and treatment as well as their separation, determination and identification. Regarding sample treatment, liquid–liquid extraction and solid-phase extraction continue to be among the most used techniques for the fractionation/purification step in polyphenolic analysis, although other extraction approaches such as QuEChERS, microwave-assisted extraction, ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and ultrahigh pressure extraction have also been described. The future trend in polyphenolic sample treatment will focus on simple and rapid sample procedures able to isolate a great variety of polyphenols specially when dealing with the characterization, classification and authentication of natural products.

Liquid chromatography-mass spectrometry or liquid chromatography-tandem mass spectrometry are among the most widely used techniques for both quantification and structural characterization of polyphenols. Chromatographic separation is commonly achieved by reversed-phase mode using C18 columns and acidified water and methanol or acetonitrile mobile phases in gradient elution. Regarding ionization, ESI is the most widely used ionization source because polyphenols can easily be ionized by ESI under negative ionization mode. However, APCI and APPI have also been described for the determination of polyphenols, and the few results published in the literature have proven APPI to be a better ionization source for some specific polyphenols with the advantage of presenting lower matrix effect. So much attention will need to be paid on alternative ionization sources such as APPI in the analysis of polyphenols, especially when dealing with the classification and authentication of natural products to prevent frauds. As regards to low resolution mass analyzers, triple quadrupole MS in MRM acquisition mode continue to be the most widely employed instruments because of their highest sensitivity, although ion-trap based analyzers, such as QTraps, are also being selected especially when elucidation and structural characterization is intended.

In the past few years, high-resolution mass spectrometry, mainly using time-of-flight and Orbitrap mass analyzers, has also gained wide acceptance as a highly sensitive and selective technique for the study of polyphenols in food matrices. This is mainly due to the fact that the high mass resolution and hence the high accuracy on mass measurements achieved with this kind of instruments enhances the possibility to unambiguously determine the elemental composition of known and new constituents with a high level of accuracy, which will be essential when dealing with the characterization and elucidation of new polyphenols in food complex matrices.

Finally, it is noteworthy that beyond the qualitative and quantitative studies of polyphenols, an emerging trend relies on the analysis of compositional profiles and fingerprints as a source of information to be exploited for classification and authentication purposes. The number of applications involving a chemometric data analysis has increased dramatically in the last years. Both LC-MS and LC-HRMS provide data of exceptional quality to be further analyzed by chemometric methods such as principal component analysis and partial least square-discriminant analysis. Chemometrics can also be a powerful tool to be used in the preliminary steps of the development of analytical methods to facilitate the optimization of sample treatments and chromatographic separations. A comprehensive optimization of sample extraction and

chromatographic conditions is crucial when dealing with complex food samples containing a great diversity of components, and several relevant applications dealing with both chemometric optimizations in the determination of polyphenols and the authentication of natural extracts have been addressed in the present review. Taking into account the high number of polyphenols present in the plant-kingdom, the complexity of food matrices, and the amount of data provided, specially, with HRMS instruments, the number of publications requiring chemometric studies in the analysis of polyphenols will increase in the future.

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Figure Captions

Figure 1. Classification and chemical structures of some phenolic acids and polyphenols.

Figure 2. Representative structure of a trimeric proanthocyanidin with both A-type and B-type linkages. Reproduced with permission from reference [9]. Copyright (2014) American Chemical Society.

Figure 3. (a) UHPLC-MS/MS chromatographic separation of a mixture of analytes (each at 100.0 µg/L) in the optimal experimental conditions: both traces of the quantifier and qualifier transitions are shown for each peak. (b) UHPLC-MS/MS chromatogram of an EVOO sample. Peak assignation: B5, pantothenic acid; HT, hydroxytyrosol; CAT, catechin; TYR, tyrosol; EGCG, epigallocathechin gallate; EPI, epicatechin; RUT, rutin; OLE, oleuropein; QUE, quercetin; and EMO, emodin. Reproduced with permission from reference [73]. Copyright (2015) John Wiley and Sons.

Figure 4. Comparison of resveratrol dimers (471 and 453 m/z) base peak chromatograms from optimized APPI and ESI methods for UV treated grapevine leaves. Reproduced with permission from reference [92]. Copyright (2006) Elsevier.

Figure 5. (a) A total ion chromatogram of a honey sample; (b) A chromatogram extracted from TIC with 200 ppm mass precision; chromatograms of the sample extracted from TIC with 1 ppm mass precision (c) apigenin (39) and galangin (22); (d) alpinetin (34) and pinostrobin (35). Reproduced with permission from reference [108]. Copyright (2013) Elsevier.

Figure 6. Example of optimization of the chromatographic gradient profile for the separation of polyphenols based on resolution and analysis time as the objective responses.

Figure 7. PCA results using normalized concentrations as the analytical data. (a) Scatter plot of scores of PC1 and PC2; grape samples in green circles, cranberry samples in red circles. *F* fruit, *J* juice, *R* raisin (dried sample), *E* extract, *S* sachet, *P* pill, *Sy* syrup. (b) Scatter plot of

1635 loadings of PC1 and PC2. Dashed line indicates the separation among cranberry- and grape-based
1636 samples. Reproduced with permission from reference [10]. Copyright (2014) Springer.
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1638

Table 1.Recent applications of LC-MS(/MS) analysis of polyphenols in food samples

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	MS instrument	Ref.
Vitis vinífera L. (Grapevine) leaves	0.02% HCl in 80% aqueous methanol	-	Gemini RP-18column (100 × 2.0 mm, 3 μm); Solvents: (A) water with 0.5% formic acid and (B) methanol with 0.5% formic acid; Flow: 400 μL min^{-1}	-	ESI (-)	QTrap SRM acquisition mode	[78]
Herba lycopi (aerial part of *Lycopus lucidus* Turcz)	Methanol	-	Agilent Elipse Plus C_{18}column (100 × 4.6 mm, 3.5 μm); Solvent: 0.1% acetic acid:methanol 20:80 (*v/v*); Flow: 300 μL min^{-1}; V injected: 20 μL	-	ESI (-)	QqQ SRM acquisition mode	[77]
Capsicum annuum L. (bell pepper)	Methanol	*n*-hexane; extraction with ethyl acetate; silica gel column and elution with methanol:dichlorometane	Zorbax SB-C_{18}column (250 × 4.6 mm, 5 μm); Solvents: (A) water with 1% acetic acid and (B) methanol; Flow: 500 μL min^{-1}	-	ESI (-)	QTrap Product ion scan mode	[79]
Kudiezi injection (*Ixeris sonchifolia* (Bunge) Hance)	Dilution with acetonitrile	-	-	Acquity BEH C_{18} column (100 × 2.1 mm, 1.7 μm); Solvents: (A) water with 0.5% formic acid and (B) acetonitrile with 0.5% formic acid; Flow-rate: 400 μL min^{-1}; V injected: 10 μL	ESI (-)	QqQ SRM acquisition mode	[84]
Red wine	-	-	Diamonsil C_{18} column (250 × 4.6 mm, 5 μm); Solvents: (A) water with 0.1% acetic acid and (B) methanol; Flow: 800 μL min^{-1}; V injected: 10 μL	-	ESI (-)	Quadrupole Full scan MS acquisition mode (*m/z* 100-1000)	[80]
Chaenomeles (Rosaceae) fruits	Acetone:water 80:20 (*v/v*)	Defatting with petroleum ether; ENVI-18 SPE cartridges; Elution with methanol	ODS 80Ts QA C_{18} column (150 × 4.6 mm, 5 μm); Solvents: (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid; Flow-rate: 400 μL min^{-1}; V injected: 10 μL	-	ESI (-)	Ion-trap Product ion scan mode (*m/z* 50-1000)	[85]

Table 1.Recent applications of LC-MS(/MS) analysis of polyphenols in food samples (cont.)

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	MS instrument	Ref.
Vitis vinífera L. (Grapevine) leaves	Reflux extraction with hexane, ethylacetate and 80% methanol	-	Agilent Eclipse XDB-C_{18} column (250 × 4.6 mm, 5 µm); Solvents: (A) water with 0.1% formic acid and (B) acetonitrile; Flow-rate: 500 µL min^{-1}; V injected: 10 µL	-	ESI (-)	QqQ SRM acquisition mode	[86]
Brazilian cherry seeds (*Eugenia uniflora* L.)	Pressurized fluid extraction (PFE)	Purification by extraction with 2.5 mL methanol and 32.5 mL chloroform	LiChrospher 100 RP-C_{18} column (100 × 4.6 mm, 5 µm); Solvents: (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid; Flow-rate: 1 mL min^{-1}; V injected: 10 µL -Flow rate split to 300 µL min^{-1} for LC-MS experiments	-	ESI (-)	QqQ SRM acquisition mode	[81]
Urtica dioica L. extracts	80% aqueous methanol	-	-	Zorbax Eclipse XDB-C_{18} column (50 × 4.6 mm, 1.8 µm); Solvents: (A) water with 0.05% formic acid and (B) methanol; Flow-rate: 1 mL min^{-1}	ESI (-)	QqQ SRM acquisition mode	[82]
Black chokeberry (*Aronia melanocarpa*)	70% aqueous methanol	-	Zorbax Eclipse XDB-C_{18} column (250 × 4.6 mm, 5 µm); Solvents: (A) water with 0.1% formic acid and (B) methanol:water 6:4 (*v:v*) with 0.1% formic acid; Flow-rate: 500 µL min^{-1}; V injected: 10 µL	-	ESI (-)	QTrap SRM acquisition mode	[83]
Citrus samples (*Citrus leiocarpa* Hort., *Citrus aurantium* L. and *Citrus erythrosa* Hort.)	70 % aqueous methanol	Silica gel colum and elution with methanol-dichloromethane 1:5 (*v/v*)	Zorbax SB-C_{18} column (250 × 4.6 mm, 5 µm); Solvents: (A) water with 0.1% formic acid and (B) methanol:acetonitrile 1:1 (*v:v*); Flow-rate: 500 µL min^{-1}; V injected: 10 µL	-	ESI (+)	QTrap Product ion scan mode (*m/z* 100-1000)	[87]

Table 1.Recent applications of LC-MS(/MS) analysis of polyphenols in food samples (cont.)

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	MS instrument	Ref.
Grape juice, green tea and coffee	-	-	Luna C_8(2) column (100 × 4.6 mm, 3 µm); Solvents: (A) 0.2 mM ammonium formate buffer at pH 4.7 (B) Methanol; Flow-rate: 400 µL min^{-1}; V injected: 20 µL	-	ESI (-)	QTrap SRM acquisition mode	[71]
Mulberry (*Morus alba* L.) fruits	Methanol with 0.1% HCl	Fractionation with SPE C18 cartridges	-	Syncronis C_{18} column (100 × 2.1 mm, 1.7 µm); Solvents: (A) Water with 0.2% formic acid (B) Acetonitrile; Flow-rate: 400 µL min^{-1}; V injected: 5 µL	ESI (-)	QqQ SRM acquisition mode	[67]
California-style black ripe olives and dry salt-cured olives	Methanol:water 4:1 (*v/v*)	Oil removal with hexane	-	Poroshell 120 EC-C_{18} column (150 × 2.1 mm, 2.7 µm); Solvents: (A) Water with 0.1% formic acid (B) Acetonitrile; Flow-rate: 400 µL min^{-1}; V injected: 1 µL	ESI (+/-)	QqQ SRM acquisition mode	[68]
Cranberry-based and grape-based natural products; cranberry-based pharmaceutical preparations	Acetone:water: HCl 70:29.9:0.1 (*v/v/v*)	-	-	-Kinetex C_{18} column (100 × 4.6 mm, 2.6 µm); Solvents: (A) Water with 0.1% formic acid (B) Methanol; Flow-rate: 1 mL min^{-1}; V injected: 10 µL -Flow rate split to 500 µL min^{-1} for LC-MS experiments	ESI (-)	QqQ SRM acquisition mode	[10]
Capsicum annuum L. extracts	Methanol, ethyl acetate of both with 0.05% hydrochloric acid	-	-	Ascentis Express C_{18} column (150 × 4.6 mm, 2.7 µm); Solvents: (A) Water with 0.075% acetic acid (B) Methanol with 0.075% acetic acid; Flow-rate: 1 mL min^{-1}; V injected: 5 µL -Flow rate split to 350 µL min^{-1} for LC-MS experiments	ESI (-)	Quadrupole Full scan MS acquisition mode (*m/z* 100-800)	[69]

Table 1.Recent applications of LC-MS(/MS) analysis of polyphenols in food samples (cont.)

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	MS instrument	Ref.
Extra-virgin olive oil	Ethanol:water 70:30 (*v/v*)	Oil removal with hexane	-	Acquisty UPLC HSST3 column (100 × 2.1 mm, 1.8 µm); Solvents: (A) Water with 0.01% acetic acid (B) Methanol with 0.01% acetic acid; Flow-rate: 400 µL min^{-1}; V injected: 5 µL	ESI (-)	QTrap SRM acquisition mode	[73]

Table 2. Recent applications of LC-HRMS analysis of polyphenols in food samples

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	HRMS instrument	Mass range	Resolving power	Ref.
Fermented Strawberry Products	Methanol	-	Phenomenex Luna C_{18} column (150 × 2.0 mm, 3 µm); Solvents: (A) water with 0.1% formic acid and (B) methanol; Flow-rate: 250 µL min^{-1}; V injected: 20 µL		ESI (-)	Orbitrap Fusion Tribrid (Q-OT-qIT)	-	-	[107]
Pistacia lentiscus var. *chia* leaves	Soxhlet extraction (SE), Microwave-assisted extraction (MAE), Ultrasound-assisted extraction (UAE)	-	-	Ascentis Express Fused-Core™ C_{18} column (100 × 2.1 mm, 2.7 µm); Solvents: (A) water with 0.1% acetic acid and (B) methanol; Flow-rate: 400 µL min^{-1}; V injected: 10 µL	ESI (-)	LTQ-Orbitrap XL	100-1000 *m/z*	30.000	[106]
Serbian polyfloral honeys	Water adjusted to pH 2 with 0.1% HCl	SPE C18	-	Hypersil gold C_{18} column (50 × 2.1 mm, 1.9 µm); Solvents: (A) water containing 1% formic acid and (B) acetonitrile; Flow-rate: 300 µL min^{-1}; V injected: 5 µl	HESI (-)	LTQ-OrbiTrap	100-900 *m/z*	-	[136]
Rorippa indica (Cruciferae)	Methanol/water (60:40, v/v); Acidic hydrolysis (HCl)	-	-	Hypersil Gold AQ RP-C_{18} column (200 × 2.1 mm, 1.9 µm); Solvents: (A) water containing 0.1% formic and (B) acetonitrile containing 0.1% formic). Flow-rate: 300 µL min^{-1}; V injected: 2 µl	ESI (-)	LTQ-Orbitrap XL	100-1500 *m/z*	15.000	[137]
Plant products (jujube fruit, Fuji apple, fruit pericarps of litchi and mangosteen, dark chocolate, and grape seed and cranberry extracts)	Methanol/water (60:40, v/v)	SPE C18 (powdered chocolate samples)	-	Hypersil Gold AQ RP-C_{18} column (200 × 2.1 mm, 1.9 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid; Flow-rate: 300 µL min^{-1}; V injected: 1 µl	ESI (-)	LTQ-Orbitrap XL	50-2000 *m/z*	15.000	[138]

Table 2. Recent applications of LC-HRMS analysis of polyphenols in food samples (cont.)

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	HRMS instrument	Mass range	Resolving power	Ref.
Royal jelly products	Water	Turbulent flow chromatography (TurboFlow™)	-	Zorbax Eclipse Plus C_{18} column (100 × 2.1 mm, 1.8 µm); Solvents: (A) ammonium acetate 30 mM, pH 5 and (B) methanol; Flow-rate: 200 µL min^{-1}; V injected: 5 µL	HESI (-)	Orbitrap Exactive	100-1000 *m/z* (Full Scan); 70-700 *m/z* (MS/MS)	25.000 (Full scan); 10.000 (MS/MS)	[139]
Nutraceutical products from green tea	Ethanol:acidified water at pH = 4 (80:20, v/v)	-	-	Acquity C_{18} column (100 × 2.1 mm, 1.7 µm); Solvents: (A) ammonium acetate 30 mM, pH 5 and (B) methanol; Flow-rate: 200 µL min^{-1}; V injected: 10 µL.	HESI (-) HESI (+)	Orbitrap Exactive	100-1000 *m/z* (Full Scan); 70-700 *m/z* (MS/MS)	25.000 (Full scan); 10.000 (MS/MS)	[109]
Plantains pulp and peel	Acetone:water:acetic acid (50:49:1; v/v/v)	SPE C18	XSelect CSH C_{18} column (100 × 3 mm, 2.5 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile 0.1% formic acid; Flow-rate; 750 µL min^{-1}; V injected: 20 µL	-	ESI (-)	LTQ-Orbitrap XL	-	-	[140]
Beer	-	SPE Oasis® MAX	Luna C_{18} column (50 × 2.0 mm, 3 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic; Flow-rate: 400 µL min^{-1}; V injected: 5 µL	-	ESI (-)	LTQ-Orbitrap Velos	100-1000 *m/z*	30.000 (Full scan); 15.000 (MS/MS)	[110]
Walnut	Acetone/water (60:40, v/v)	-	Atlantis T3 C_{18} column (100 × 2.1 mm, 3 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. Flow-rate: 350 µL min^{-1}; V injected: 10 µL	-	ESI (-)	LTQ-Orbitrap Velos	-	60.000 (Full scan); 30.000 (MS/MS)	[111]

Table 2. Recent applications of LC-HRMS analysis of polyphenols in food samples (cont.)

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	HRMS instrument	Mass range	Resolving power	Ref.
Serbian poplar type propolis	Ethanol/water (80:20, v/v)	NH_2 HPTLC	-	Hypersil gold C_{18} column (50 × 2.1 mm, 1.9 µm); Solvents: (A) water containing 1% formic acid and (B) acetonitrile; Flow-rate: µL min^{-1}; V injected:5 µL	HESI (-)	LTQ-Orbitrap	100-900 *m/z*	-	[141]
Strawberry	Methanol/water/formic acid (60:40:1 v/v/v)	-	-	Hypersil Gold C_{18} column (200 × 2.1 mm, 1.9 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid; Flow-rate: 300 µL min^{-1}; V injected: 2 µL	ESI (-) ESI (+)	LTQ-Orbitrap XL	100-1500 *m/z*	15.000	[142]
Red wine and zebrafish embryos	Water with 0.1% formic acid (zebrafish embryos)	-	Luna C_{18} column (50 × 2.0 mm, 5 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid; Flow-rate: 400 µL min^{-1}; V injected: 5 µL	-	ESI (-)	LTQ-Orbitrap Velos	100-1000 *m/z*	30.000 (Full scan); 15.000 (MS/MS)	[143]
Appe fruit	Ultrasound-assisted solid–liquid extraction (USLE)	-	-	Acquity BEH SHIELD C_{18} column (150 × 3.0 mm, 1.7 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic Acid; Flow-rate: 500 µL min^{-1}; V injected: 5 µL	ESI (-)	Orbitrap Exactive	90-1800 *m/z*	50.000	[144]
Serbian unifloral honeys	Ethyl acetate	-	-	Hypersil Gold C_{18} column (50 × 2.1 mm, 1.9 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid; Flow-rate: 400 µL min^{-1}; V injected: 5 µL	HESI (-)	LTQ-OrbiTrap XL	100-900 *m/z*	-	[108]

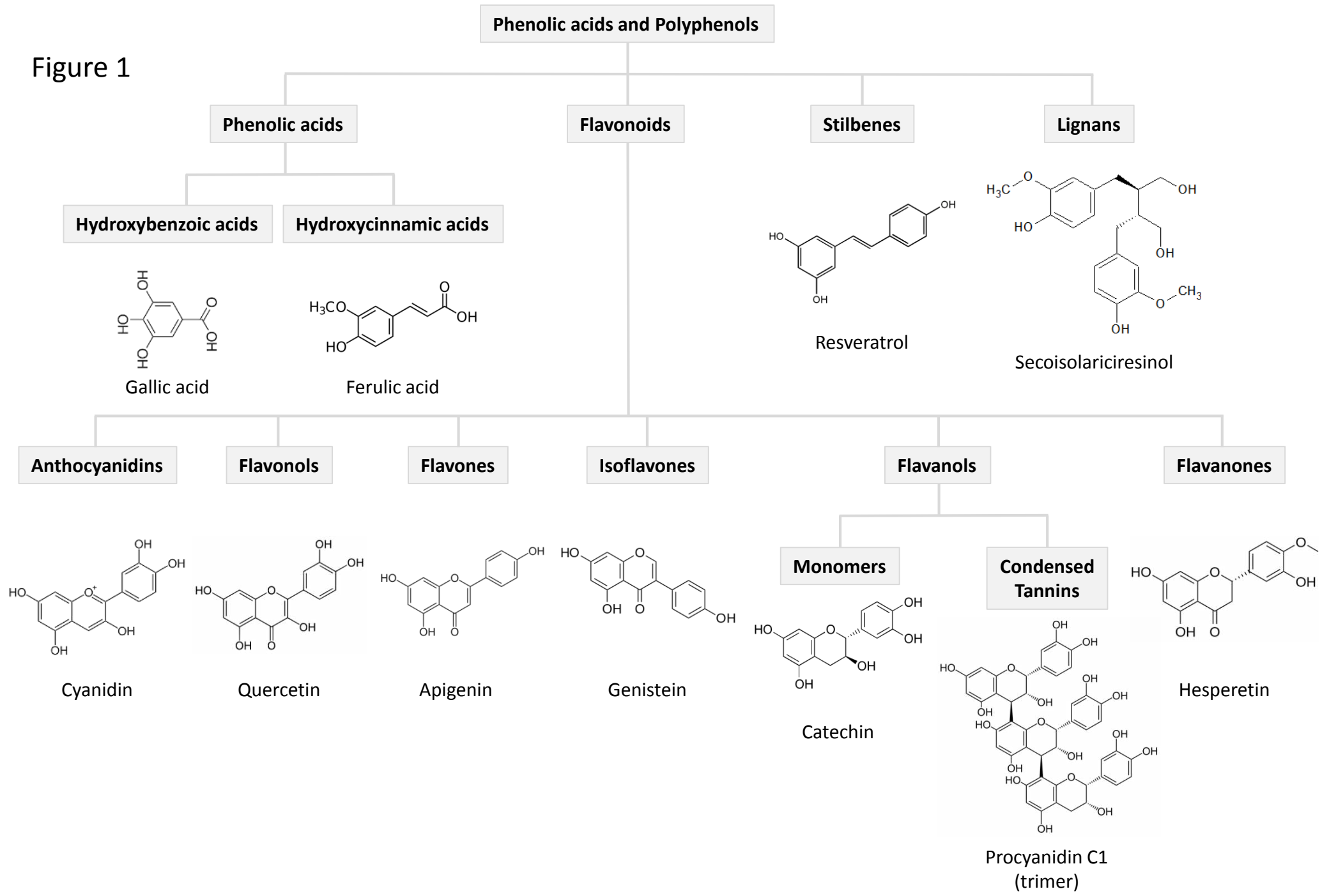
Table 2. Recent applications of LC-HRMS analysis of polyphenols in food samples (cont.)

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	HRMS instrument	Mass range	Resolving power	Ref.
Cretan barberry herb	Accelerated solvent extraction (ASE); Supercritical fluid extraction (SFE); SFE coupled with ASE	-	-	Hypersil Gold C_{18} column (50 × 2.1 mm, 1.9 µm); Solvents: (A) water containing 0.1% formic acid and (B) methanol; Flow-rate 200 µ min^{-1}	ESI (-)	LTQ -Orbitrap Discovery	50-1000 *m/z*	30.000	[105]
Artichoke	Methanol/water (70:30, v/v)	-	Gemini C_{18}-110A column (150 × 2 mm, 5 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. Flow-rate: 200 µL min^{-1};V injected: 20 µl	-	HESI (-)	Orbitrap Exactive	65-1000 *m/z*	25.000	[104]
Virgin olive oil	Methanol	Dispersive C 18 SPE (for methanolic extract); Diol SPE (for oil/hexane mixture)	-	Zorbax Eclipse Plus C_{18} column (100 × 2.1 mm, 1.8 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic; Flow-rate: 600 µL min^{-1}; V injected: 5 µL	ESI (-) ESI (+)	Q-ToF	100-1100 *m/z*	-	[114]
Mango	Microwave-assisted extraction (MAE)	Sephadex LH-20 column chromatography	C_{18} Hypersil ODS column (250 × 4.6 mm, 5 µm); Solvents: water containing 1% formic acid and (B) acetonitrile containing 1% formic acid; Flow-rate: 1 mL min^{-1}; V injected: 10 µL	-	ESI (-)	Q-ToF	100-1000 *m/z*	-	[115]
Olives and Olive Oil Process-Derived Matrices	Ultrasound-assisted solid-liquid extraction (USLE) (Destoned fruits, stones, paste, pomace, defatted wastewater); Ultrasound-assisted liquid-liquid extraction (USLLE) (olive oil)	Freeze-based fat precipitation (olive oil)	-	Kinetex PFP column (100 × 4.6 mm, 2.6 µm); Solvents: (A) water-acetic acid (95:5, v/v) and (B) methanol. Flow-rate: 1 mL min^{-1}; V injected: 10 µL	ESI (-) ESI (+)	Q-TOF	50-3000 *m/z*	-	[100]

Table 2. Recent applications of LC-HRMS analysis of polyphenols in food samples (cont.)

Sample	Extraction	Clean-up	HPLC conditions	UHPLC conditions	Ionization mode	HRMS instrument	Mass range	Resolving power	Ref.
Virgin olive oil	Methanol/water (60:40, v/v)	-	-	C_{18} Pursuit XRs Ultra column (50 × 2.0 mm, 2.8 µm); Solvents: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid; Flow-rate: 400 µl min^{-1}; V injected: 5 µL	ESI (-)	Q-TOF	100-1700 *m/z*	25.000-45.000	[113]
Rapeseeds	Methanol/water (80:20, v/v)	Sephadex LH-20 column chromatography	Ultimate XB-C_{18} column (150 × 2.1 mm, 3.5 µm); Solvents: water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid; Flow-rate: 200 µL min^{-1}; V injected: 10 µL	-	ESI (-)	Q-TOF	100-2000 *m/z*	-	[112]

Figure 1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Lignans
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Secoisolariciresinol
Anthocyanidins
Flavonols
Flavones
Isoflavones
Flavanols
Flavanones
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Procyanidin C1 (trimer)
Hesperetin

Figure 2

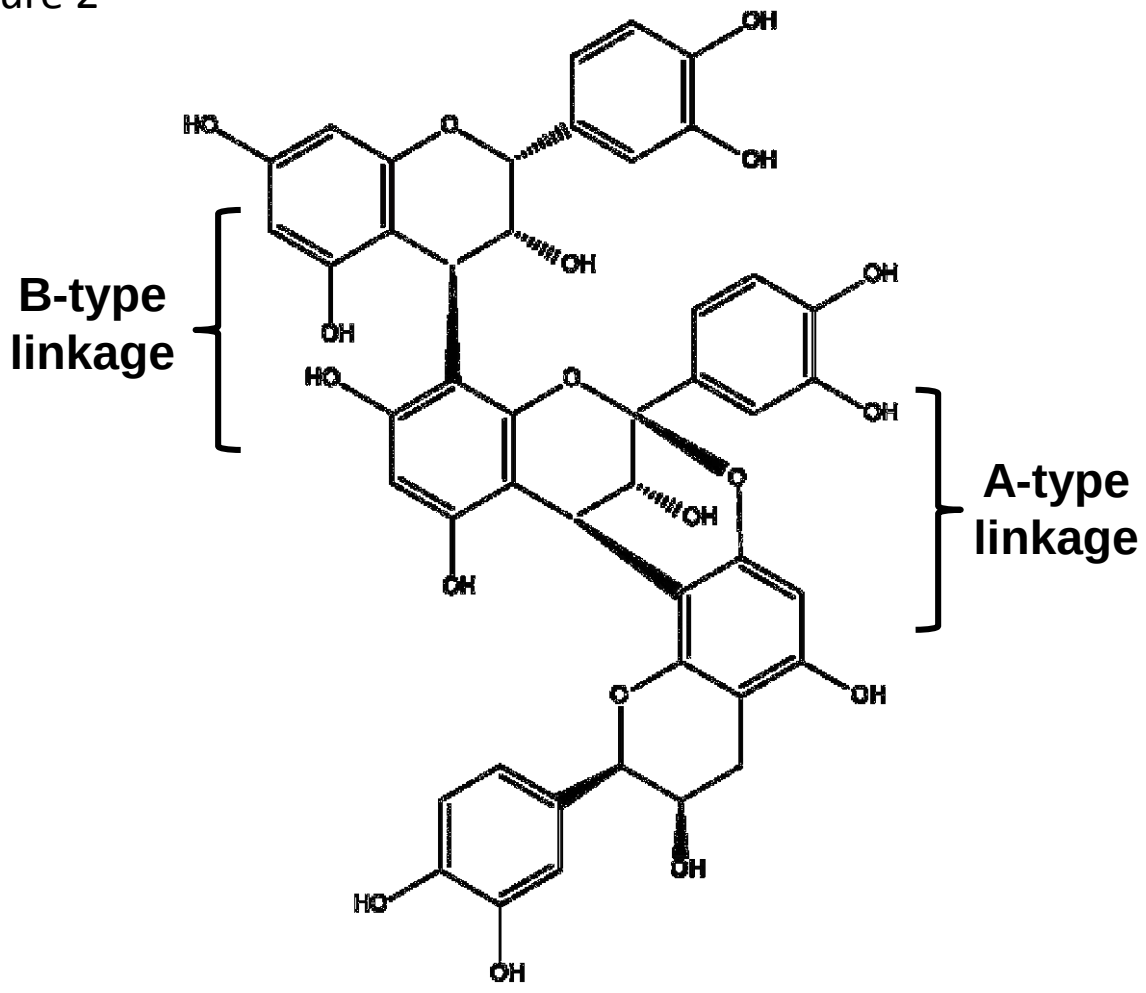
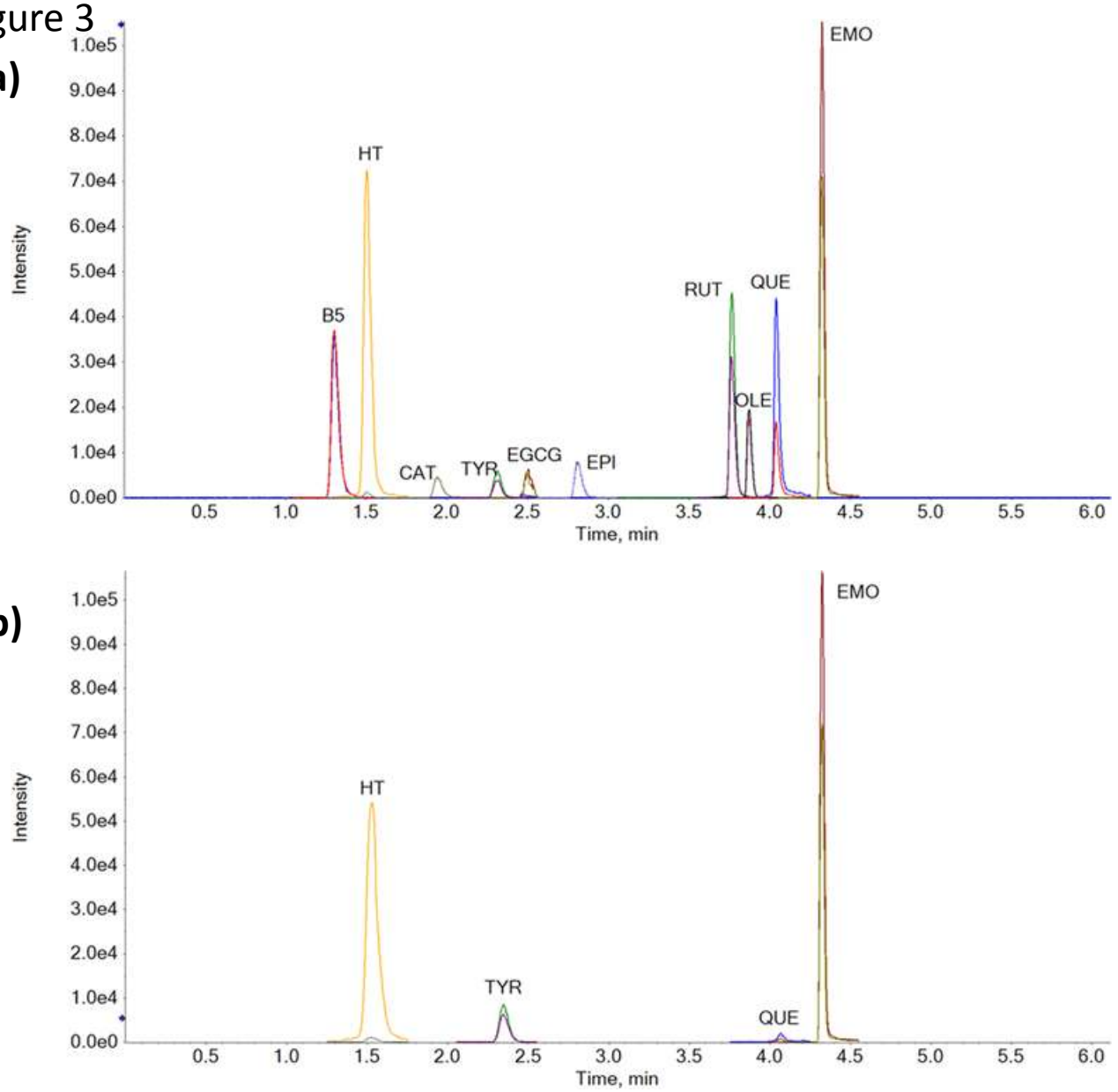


Figure 3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2.0e4
1.0e4
0.0e0
B5
HT
CAT
TYR
EGCG
EPI
RUT
QUE
OLE
EMO
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
Time, min
(b)
Intensity
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8.0e4
7.0e4
6.0e4
5.0e4
4.0e4
3.0e4
2.0e4
1.0e4
0.0e0
HT
TYR
QUE
EMO
0.5
1.0
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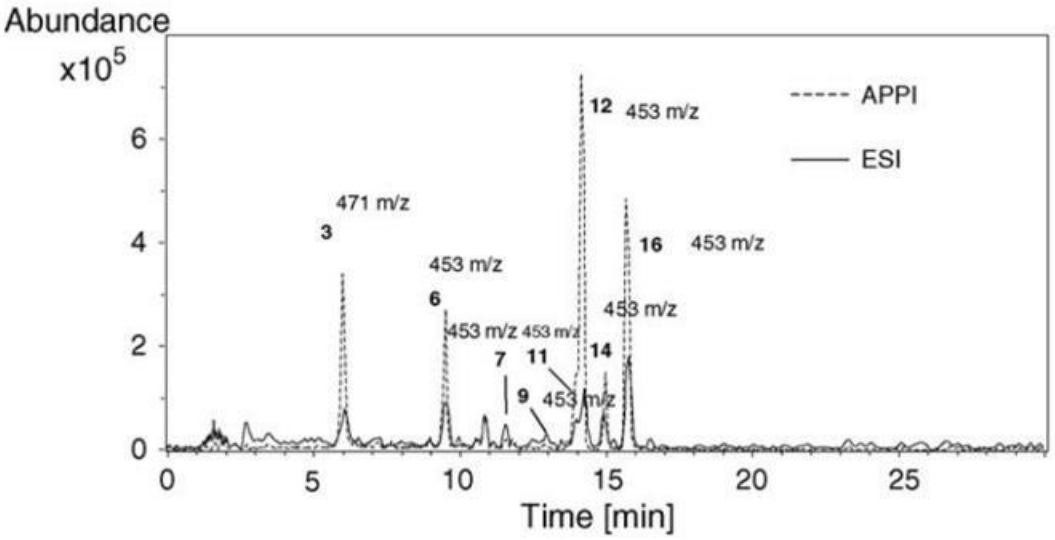
6.0
Time, min

Figure 4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ESI
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453 m/z
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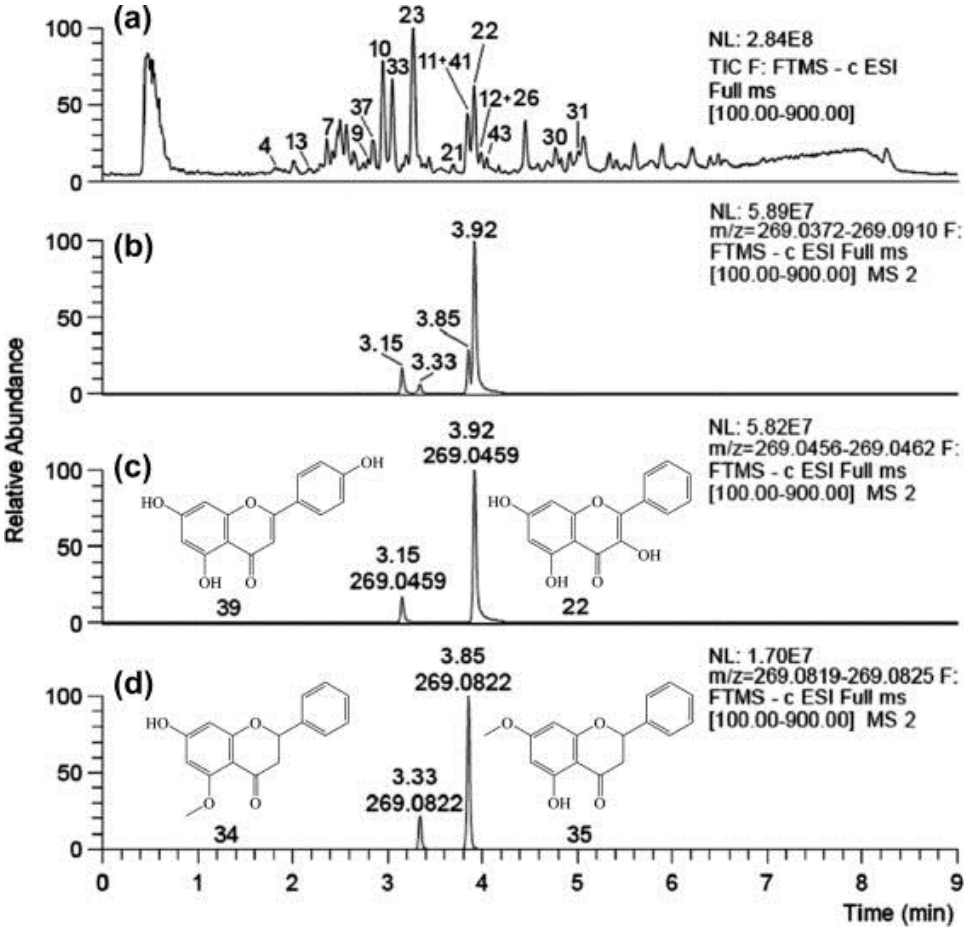
16
453 m/z

Figure 5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FTMS - c ESI Full ms
[100.00-900.00] MS 2
(c)
NL: 5.82E7
m/z=269.0456-269.0462 F:
FTMS - c ESI Full ms
[100.00-900.00] MS 2
(d)
NL: 1.70E7
m/z=269.0819-269.0825 F:
FTMS - c ESI Full ms
[100.00-900.00] MS 2
Relative Abundance
Time (min)
39
22
34
35

Figure 6

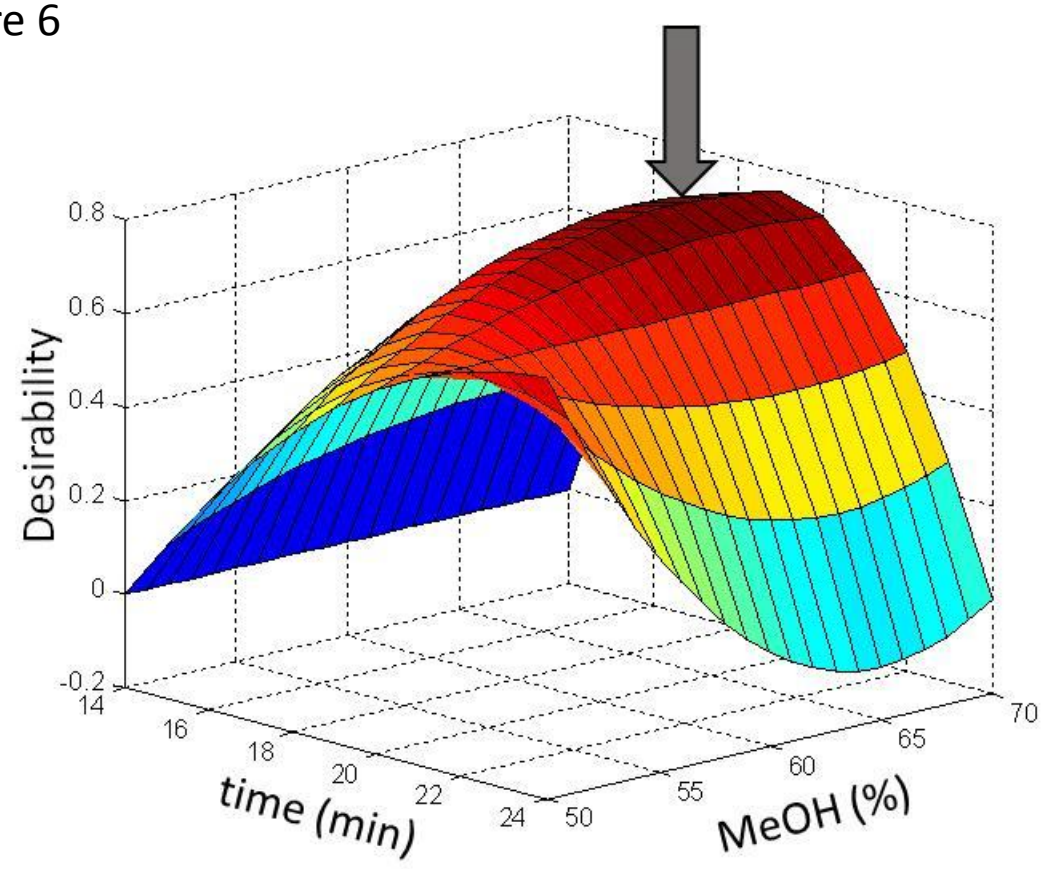
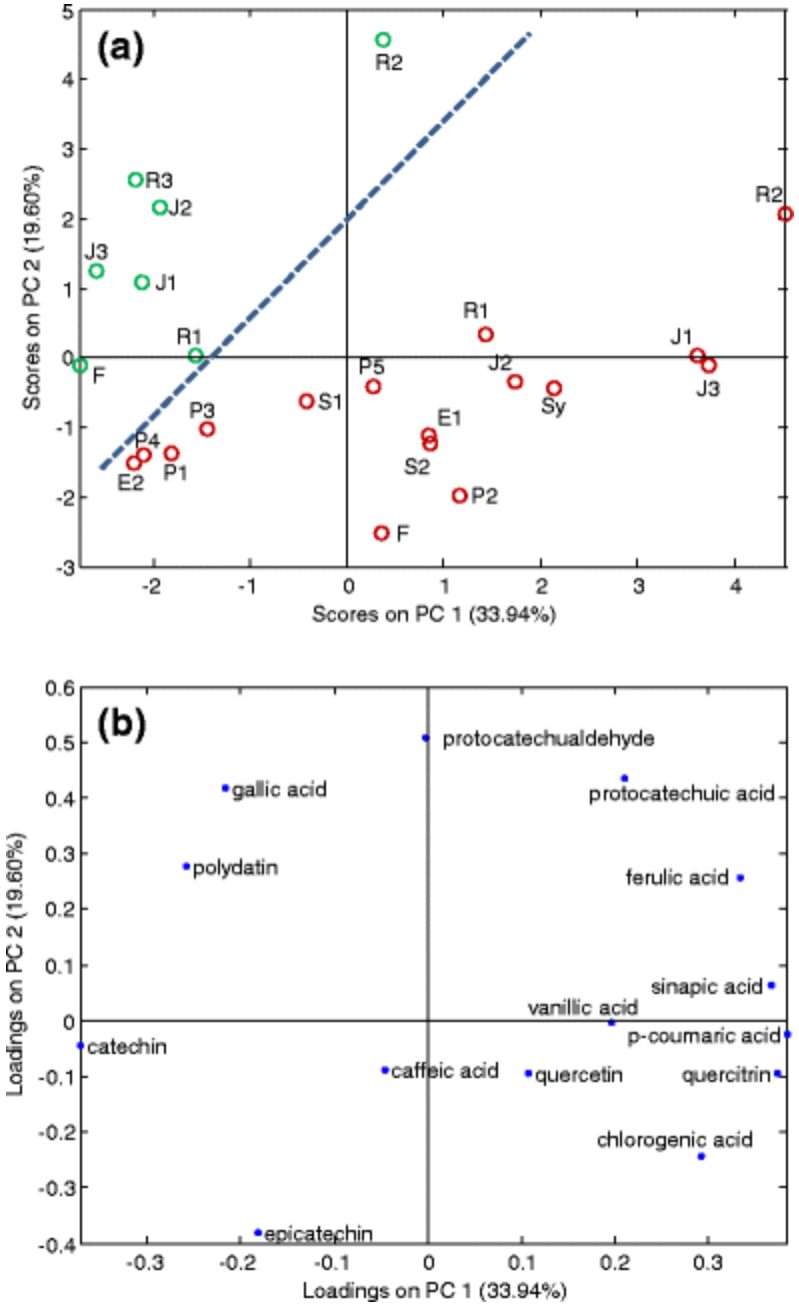

Desirability
0.8
0.6
0.4
0.2
0
-0.2
14
16
18
20
22
24
time (min)
50
55
60
65
70
MeOH (%)

Figure 7


(a)
R2
R3
J2
J3
J1
R1
F
P3
P4
P1
E2
S1
P5
J2
Sy
E1
S2
P2
F
R2
R1
J1
J3
Scores on PC 2 (19.60%)
Scores on PC 1 (33.94%)
(b)
protocatechualdehyde
gallic acid
protocatechuic acid
polydatin
ferulic acid
sinapic acid
vanillic acid
p-coumaric acid
catechin
caffeic acid
quercetin
quercitrin
chlorogenic acid
epicatechin
Loadings on PC 2 (19.60%)
Loadings on PC 1 (33.94%)