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Review

Advanced separation methods of food anthocyanins, isoflavones and flavanols

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ABSTRACT

In recent years, increasing knowledge of the positive health effects of food polyphenols has prompted the need to develop new separation techniques for their extraction, fractionation and analysis. This article provides an updated and exhaustive review of the application of counter-current chromatography, high performance liquid chromatography, capillary electrophoresis, and their hyphenation with mass spectrometry to the study of food polyphenols. Flavonoids constitute the largest class of polyphenols, widely spread in the plant kingdom and common in human diet which has been the most widely studied with respect to their antioxidant and biological activities. The main subgroups are anthocyanins, catechins, isoflavones, flavonols and flavones. They are reported to exhibit antioxidant, anti-carcinogenic, anti-inflammatory, anti-atherogenic, anti-thrombotic, and immune modulating functions, among others. Since red fruit anthocyanins, soy isoflavones and flavanols from grapes and teas are currently the most used phenolic compounds for producing new nutraceuticals and functional foods, this review is focused on these three flavonoid groups.

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Abbreviations: APCI, atmospheric-pressure chemical ionization; C, catechin; CCC, counter-current chromatography; CD, cyclodextrin; CE, capillary electrophoresis; CEC, capillary electrochromatography; CGE, capillary gel electrophoresis; CIEF, capillary isoelectric focusing; CITP, capillary isotachophoresis; CMC, critical micellar concentration; CPC, centrifugal partition chromatography; Cy, cyanidin; CZE, capillary zone electrophoresis; DAD, diode array detector; DCCC, droplet counter-current chromatography; DP, degree of polymerization; Dp, delphinidin; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; ELISA, enzyme-linked immunoabsorbent assay; EOF, electroosmotic flow; ESI, electrospray ionization; HILIC, hydrophilic interaction chromatography; HPLC, high pressure liquid chromatography; HSCCC, high-speed counter-current chromatography; ICP, induced coupled plasma; IL, ionic liquids; ITMS, ion-trap mass spectrometers; ITP, isotachophoresis; LOD, limit of detection; LOQ, limit of quantification; MALDI, matrix-assisted laser desorption ionization; MEKC, micellar electrokinetic chromatography; MLCCC, multilayer coil counter-current chromatography; MRM, multiple reaction ion monitoring; MS, mass spectrometry; Mv, malvidin; NMR, nuclear magnetic resonance; PA, proanthocyanidin; Pg, pelargonidin; Pt, petunidin; QqQ, triple-quadrupole mass spectrometer; RP, reversed phase; SDS, sodium dodecyl sulphate; SIM, selected ion monitoring; SRM, selected reaction monitoring; TLC, thin layer chromatography; TOF, time-of-flight mass spectrometer; UPLC, ultra-high pressure liquid chromatograph; UV, ultraviolet.

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1. Introduction

Polyphenols are found ubiquitously in a variety of fruits, vegetables, nuts, seeds, flowers, bark, beverages and manufactured foods, as a component of the natural ingredients used. Cocoa, apples, tea, berries, coffee, wine, jams, chocolates, or onion are common sources for polyphenols in human diets [1].

Although traditionally their interest has mainly been related to their organoleptic properties, such as colour [2] (anthocyanins or curcumin, for example), astringency [3] (tannins), bitterness (flavanols) or taste [4], in recent decades they are increasingly being recognised for their nutritional value, since they may help reduce the risk of chronic disease and, in general, have a positive effect on health [5,6]. They have been reported to have anti-carcinogenic [7], anti-atherogenic [8], anti-ulcer [9], anti-thrombotic [10], anti-inflammatory [11], anti-allergenic, immune modulating, anti-aggregative [12], anti-microbial [13], vasodilatory [14], and estrogenic [15] effects. They can accomplish these roles as antioxidants, chelators of divalent cations, or as modulators or inhibitors of the activity of such enzymes as topoisomerases, protein kinases, or cyclo-oxygenase [9,16,17].

These recently discovered properties of phenolic compounds have been exploited for cosmetics, medicines, pharmaceuticals, nutritional supplements or functional foods. The food industry has launched numerous new functional products, the health functionality of which is closely connected with their polyphenols content, which is usually higher than the content of the traditional products. Milks enriched with soy isoflavones, chocolates enriched with procyanidins, beverages with higher amounts of anthocyanins, functional drinks enriched with tea extracts, and many others are all part of the functional foods revolution [18]. On the other hand, the use of synthetic antioxidants in the food industry is severely restricted as to both application and level. This is the reason why more attention is now being paid to natural antioxidants extracts from plants.

All these healthy properties are strongly dependent on the polyphenols chemical structure [6]. Because the number of phytochemicals already identified is only a small part of those that exist in nature, there is a considerable interest in new methods of separation, isolation and characterization of polyphenol structures from foods.

For the purpose of this review, we aim to give a detailed description of three advanced separation techniques that are currently applied for food analysis and new food polyphenols identification.

(a) Counter-current chromatography is a technique that allows the fractionation and isolation of pure compounds, to yield the large amounts required for identification by MS and NMR methodologies, or for a further utilization as standards in analytical methods or as bioactive compounds for biological studies.
(b) High pressure liquid chromatography is the classic separation technique for analyzing polyphenols. Its hyphenation with mass detectors is being crucial for detecting and identifying minor and unknown polyphenols in complex food samples.
(c) Capillary electrophoresis is an interesting alternative to HPLC, its main advantage being that it takes less time and uses smaller quantities of solvent. Recent advances in hyphenation with mass detectors make this technique a promising field of application.

2. Polyphenol structures

The identification of polyphenols has blossomed during the last decades [19–21]. The development of new isolation, separation and identification techniques has made it possible to constantly increase the database of phenolic compounds with new structures, and to provide a better understanding of the mechanisms that govern their effects. The more recent advances have been related with the identification and quantifications of polyphenols in food complex matrices and in biological fluids and tissues [22–25].

However, several difficulties arise, because the term "polyphenols" includes a lot of different families with widely differing structures and properties, and every year a large number of new polyphenol structures are identified. This means that no universal method can be used with all the phenolic compounds: different approaches must be used depending on the specific foods and polyphenols of interest.

Table 1 shows the most important families of polyphenols in foods, classified by their skeleton structure [26]. Flavones, flavonols, flavanones, flavanols, isoflavonoids and anthocyanins are known as flavonoid compounds and all of them share the same basic structure. Fig. 1 shows this basic structure and the numbering system of

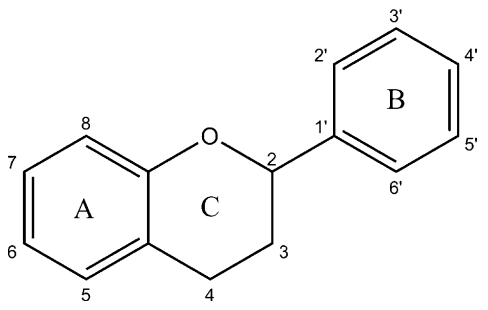
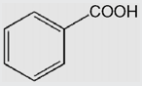
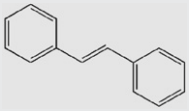
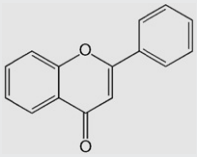
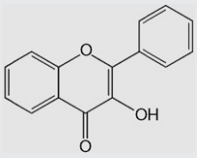
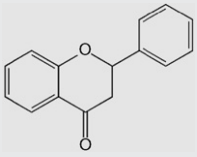
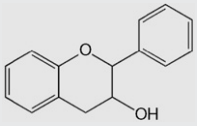
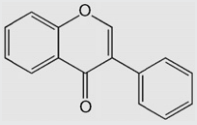
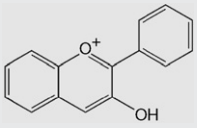


Fig. 1. Basic structure and numbering system of flavonoids.

Table 1
Main phenolic structures found in foods (adapted from Bravo [26]).

Class	Structure	Examples	Occurrence in foods
Phenolic and hydroxycinnamic acids		Gallic acid Caffeic acid Coumaric acid	Coffee Grapes Wine
Stilbenes		Resveratrol Piceid Viniferin	Red wines Grapes
Flavones		Apigenin Luteolin	Parsley Celery White wines
Flavonols		Quercetin Myricetin Kaempferol Rutin	Onions Cherries Apples
Flavanones		Naringenin Hesperidin Eriodictiol	Orange juice, Lemon juice
Flavanols, Proanthocyanidins and Tannins		Catechin, Epicatechin Epigallocatechin Procyanidin B1, trimer C1	Cocoa and chocolate, wine, tea, apple
Isoflavonoids		Genistein Daidzein Glycitein	Legumes (soybean)
Anthocyanins		Malvidin Cyanidin Malvidin-3-glucoside	Berries (grape, bilberry, cranberry, cherry) Eggplant

flavonoids. The three classes of flavonoids that have attracted most attention in the area of nutraceutical and functional foods are the anthocyanins, the flavanols or procyanidins, and the isoflavonoids. Because of their importance, in this review we will focus on these three families.

2.1. Anthocyanins

Anthocyanins (from the Greek anthos = flower and cyan = blue) are one of the flavonoid groups that have been most widely studied in recent decades. Anthocyanins are found in many foods because they are responsible for the cyan and red colours of several fruits such as red berries (grape berries, elderberries, cranberries, raspberries, black currant, mulberries, etc.), cherries, pomegranates, or plums, and also of eggplants, beetroots or pink potatoes. All these fruits are regularly consumed in diets, and consequentially they have also been used for a long time by the food industry to produce juices, soft drinks, alcoholic beverages or pies, among other products [27]. This traditional use has exploited the organoleptic characteristics of anthocyanins to create colourful attractive products. Red wine is the classic example that first comes to mind, and the role of anthocyanins and their interactions in the colour intensity and the stability of wine have been widely studied, through their spectroscopic characteristics, copigmentation

effects, and pyranoanthocyans formation by condensation reactions [2].

Food industry first used anthocyanins as natural food colourants, but has now started to focus on possible health applications as nutritional supplements, functional food formulation, medicines, etc. Their health effects have been linked to their antioxidant properties and to notable effects against chronic inflammation, cardiovascular hypertension, cancer prevention or metabolic syndrome regulation [28].

Basic skeleton structure of anthocyanins is shown in Fig. 2. Structurally, anthocyanins are heterosides of an aglycone unit (anthocyanidin) which is a derivative of the flavylium ion. The main differences among the different anthocyanins are the number of hydroxylated groups in the anthocyanidin, the nature and the number of bonded sugars in their structure, the aliphatic or aromatic carboxylates bonded to the sugar in the molecule, and the position of these bonds [28]. The structure of anthocyanins influences their chemical properties, which have important implications for their stability, their aqueous equilibrium, their colour, their copigmentation effects, their reactivity and their antioxidant properties. As these aspects are beyond the purpose of this article, we recommend recent reviews by Castaneda-Ovando et al. [29] and Rivas-Gonzalo [30].

The search for anthocyanins with potential health benefits, together with the latest advances in separation techniques and identification methods, has lead to a considerable increase in the number of structures characterized. New research for finding bioactive anthocyanins and their beneficial effects has been initiated, and it has not been restricted to traditional sources. For example, in recent years Chirinos et al. have investigated the properties of anthocyanins from purified mashua extracts [31], Thompson et al. have shown the function of some potato cultivars [32] against breast cancers, Mezadri et al. have investigated the anthocyanin fraction of acerola [33], Prata and Oliveira have proposed fresh coffee husks as potential sources of anthocyanins [34], Longo et al. have identified the presence of malvidin-3,5-O-diglucoside as the only anthocyanin present in *Eugenia myrtifolia* Sims fruits [35], and Escribano-Bailon et al. have published an interesting review [36] on anthocyanins in cereals focused on maize, rice, wheat and sorghum.

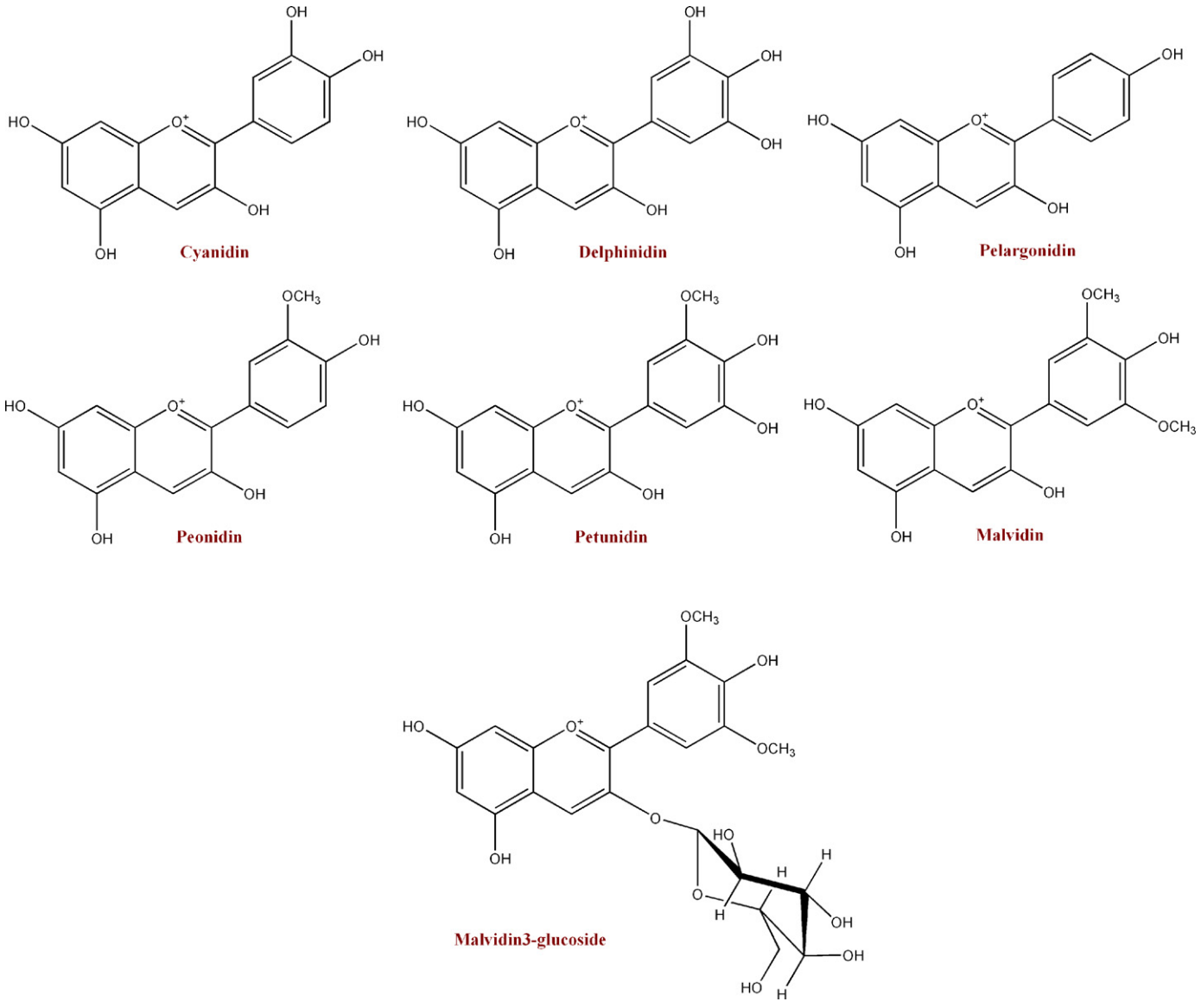


Fig. 2. Structure of common anthocyanidins present in nature and example of a glucose attached to the aglycon in 3-position.

A recent review by Andersen et al. [27] of the different natural anthocyanins isolated from plants summarized a total of 539 anthocyanins. But despite this increasingly large number, structures were derived from only 27 different anthocyanidins (including newly found desoxyanthocyanidins and pyranoanthocyanidins), and in fact almost 94% of the new anthocyanins discovered are based on only the six common anthocyanidins: pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin.

2.2. Flavan-3-ols, proanthocyanidins and tannins

Flavan-3-ols are a subclass of flavonoids that contain varying degrees of hydroxylation on the 5 and 7 positions of the A-ring and 3′, 4′ and 5′ positions on the B-ring (Fig. 3). The 3-position on the C-ring is commonly a hydroxyl group or is esterified with gallic acid. Catechin, epicatechin, catechin gallate, epicatechin gallate, gallocatechin, epigallocatechin, gallocatechin gallate and epigallocatechin gallate are the most common flavan-3-ol monomers.

Proanthocyanidins are oligomers of flavan-3-ol monomers units, most frequently linked by C–C bonds either at 4 → 6 or 4 → 8 (B-type proanthocyanidins). They are classified as procyanidins (derived from catechin, epicatechin and their gallic esters) and prodelphinidins (derived from gallocatechin, epigallocatechin and their galloylated derivatives). A-type proanthocyanidins occur with the formation of a second interflavonoid bond by C–O oxidative coupling. Condensed tannins are highly polymerized proanthocyanidins.

Proanthocyanidins are found in such commonly consumed fruits as blueberries, cranberries, grapes, apples, kiwis and pears, and are also found in teas, the skin of peanuts, the seed coat of almonds and in certain cocoas. Processed foods made from these raw materials, such as red wine, grape juice, beer or chocolate, can also contain proanthocyanidins, although the concentrations found in the final products are affected by processing techniques [37].

Proanthocyanidins have a wide range of biological activities [38]. They function as powerful antioxidants and can have anti-inflammatory activities. Flavanols products have been widely been exploited in the field of nutraceuticals and dietary supplements, whereas tea extracts are becoming extremely popular for producing functional drinks. On the other hand, tannins and flavan-3-ols determine the astringency and bitterness of wines, which depend heavily on the degree of polymerization and on particular structures. The development of new isolation techniques and separation methods may contribute to a better understanding of these effects, and so the analysis of proanthocyanidins in foods has been the subject of constant research.

2.3. Isoflavones

Isoflavones are a subclass of flavonoids that are also described as phytoestrogen compounds, since they exhibit estrogenic activity. However, it would be advisable not to mix terms, because other polyphenolic structures such as lignans and coumestans, among others, have similar estrogenic effects [39].

The basic characteristic isoflavone structure is a flavone nucleus, composed of two benzene rings (A and B) linked to a heterocyclic ring C (Fig. 4). The benzene ring B position is the basis for the categorization of the flavonoid class (position 2) and the isoflavonoid class (position 3). Isoflavonoids from soybeans include the isoflavones genistein (4′,5,7-trihidroxyisoflavone) and daidzein (4′,7-dihidroxyisoflavone), which occur mainly as the glycosides genistin and daidzin. Other sources such as red clover, alfalfa or puerarria are rich in other aglycones, like biochanin A, formonetin, or glycitein. Respective glycosides, acetyl or malonyl forms can also be found. In total, more than 1600 isoflavonoids were described up to 2004, with soybean being still the most studied source [27].

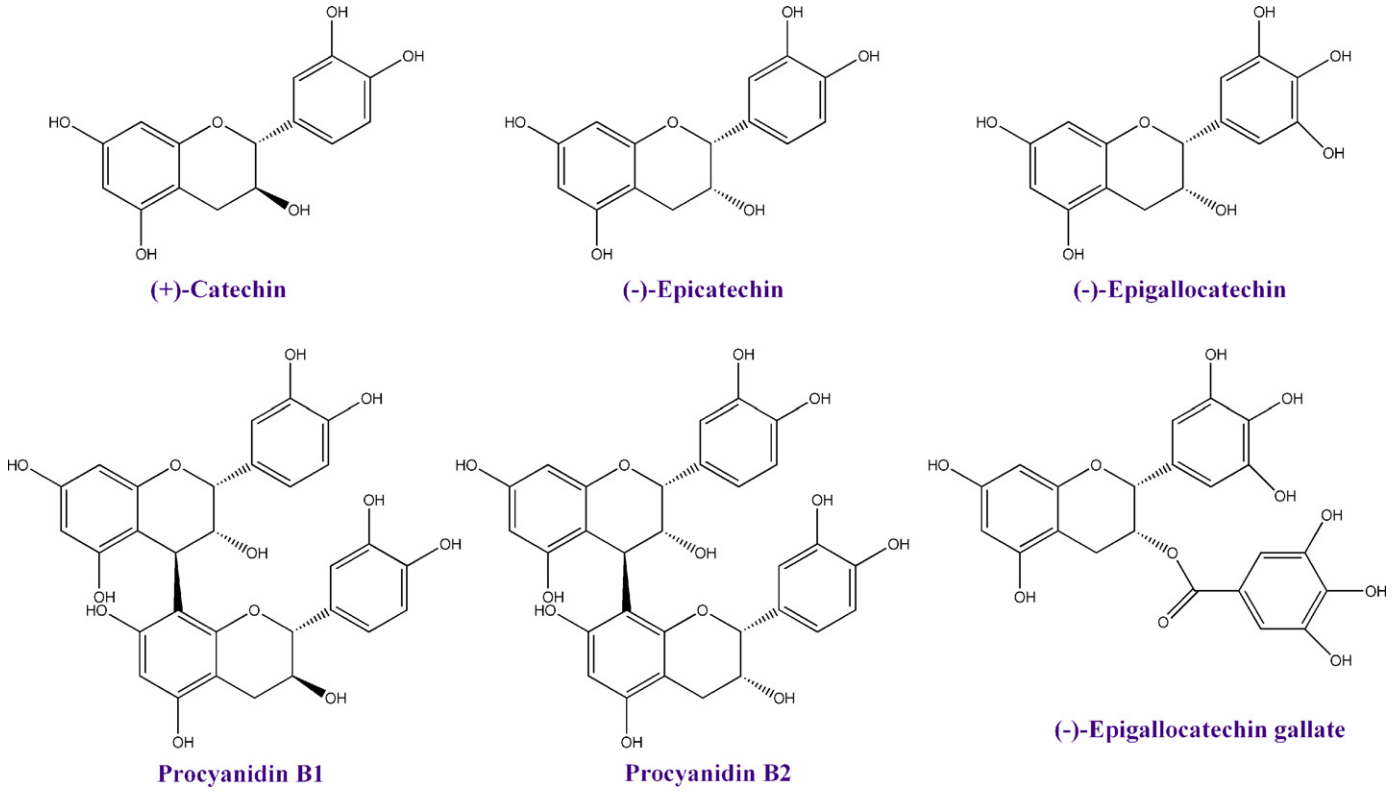


Fig. 3. Structures of some common flavan-3-ol monomers and proanthocyanidins, including 4 → 6 dimers.

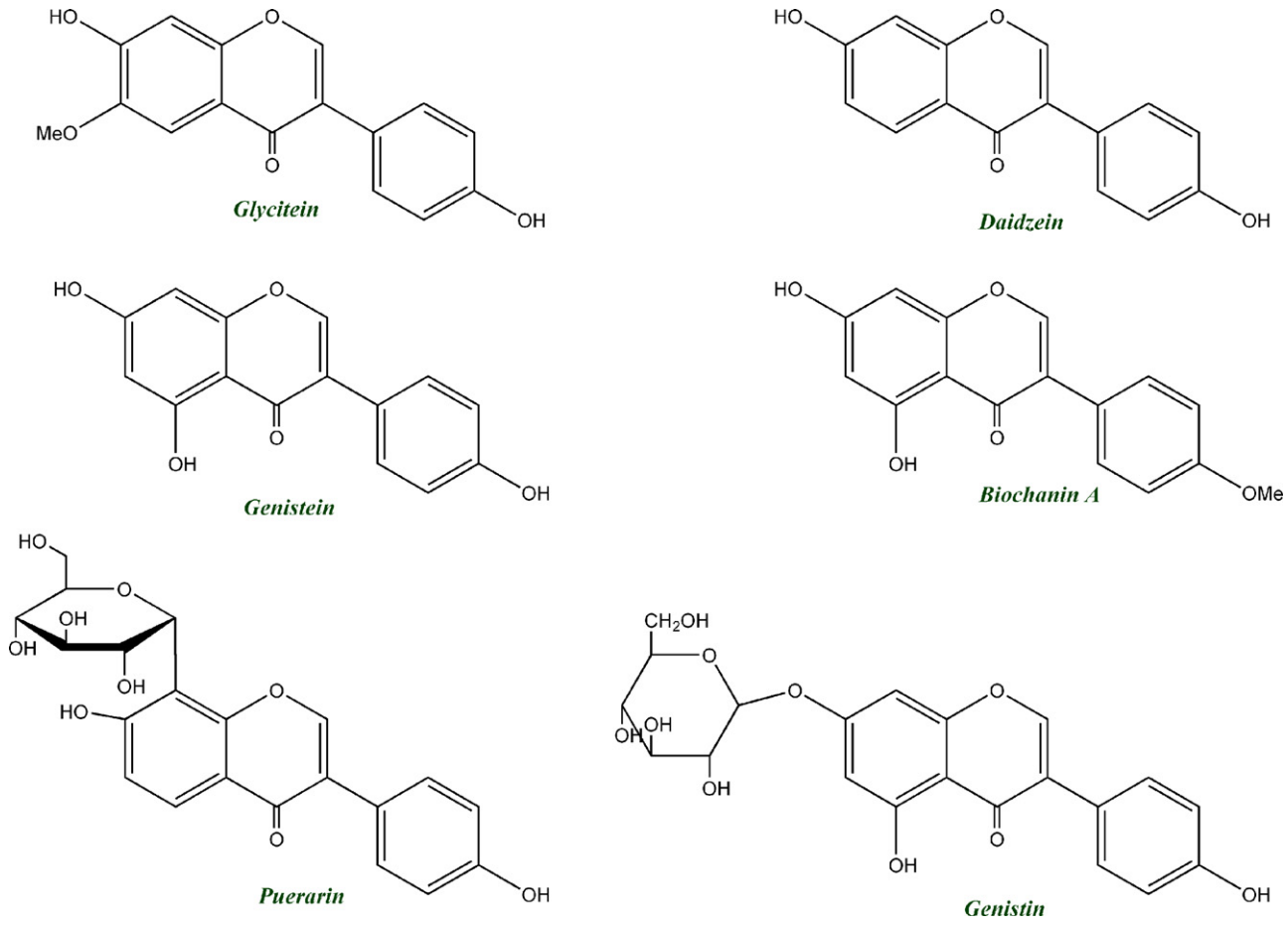


Fig. 4. Structure of common isoflavones.

The potential of soybeans as healthy ingredients for the formulation of functional foods is currently being explored by the food industry. The protective effect of isoflavones against osteoporosis and menopausal symptoms has led to the launch of several soy-enriched products (for example soymilks, dairy products and cheeses).

3. Fractionation and purification methods by counter-current chromatography

Generally, conventional methods such as low pressure chromatography (with silica gel, polyamide, Sephadex LH-20) and preparative reversed-phase liquid chromatography are used to fractionate or isolate pure products from plants, but they are tedious, time and solvent consuming, and require multiple chromatographic steps [40]. Counter-current chromatography (CCC) can be an excellent alternative.

CCC is a liquid–liquid partition chromatography process in which both the mobile and the stationary phase are liquids. The "column" is simply a long length of tubing wound on a drum (the bobbin) which is geared to the main rotor in such a way that it simultaneously rotates at twice the speed of the rotor (planetary motion). This double motion sets up a fluctuating force field which forces one phase (the less dense or lighter phase) to move by definition to the "head" end of the coil/column and the other (the more dense, heavier phase) to go by displacement to the opposite end of the coil/column called the "tail". Changing the direction of motion simply changes the head/tail notation around. Hence the name "counter-current" chromatography [41].

In practice however, the counter-current nature of the process is rarely used. Instead, the column is filled with the phase intended to be the "stationary" phase and the other phase is pumped in the end of the column, which allows the other phase to be retained. So if the heavier phase is the mobile phase (often the aqueous phase) then it is pumped from head-to-tail (descending mode) in the opposite direction to the way the lighter phase wants to go. If the lighter phase is the mobile phase, then it is pumped tail-to-head (ascending mode).

By measuring the displacement of the stationary phase from the column during the equilibration process, it is possible to calculate the amount of stationary phase left in the column and predict exactly when compounds will elute on the basis of their distribution ratio (sometimes referred to as the partition coefficient).

Modern commercial counter-current chromatographs have been applied to polyphenols separation. They can be classified into hydrodynamic systems, such as high-speed counter-current chromatographs (HSCCC), or multilayer coil counter-current chromatographs (MLCCC), and hydrostatic systems, such as droplet counter-current chromatographs (DCCC), or centrifugal partition chromatographs (CPC). For a detailed explanation of both families and their main characteristics, a comprehensive review by Pauli et al. was published in 2008 [41]. Although the principle of separation is very similar for all equipments, there are some differences in efficiency, mainly related to the number of coils in the system.

Counter-current chromatography operates under gentle conditions and enables non-destructive isolation even of labile natural compounds. Due to the absence of any solid stationary phase,

adsorption losses are minimized and 100% sample recovery is guaranteed. The liquid nature of the stationary phase means that the compounds retained in this phase can be easily recovered by simply pumping this phase out. This is an important feature for samples containing polyphenols, which tend to get irreversibly absorbed in HPLC columns [42].

The undeniable applicability of this separation technique has led to an enormous number of papers on the fractionation and isolation of various compounds from plant sources, including alkaloids, terpenes and phenolic compounds (mainly flavonoids). In 2009 Sutherland and Fisher published an excellent comprehensive review of the application of these techniques to the study of Chinese herbal medicines [43]. They report the isolation of 354 relatively pure different molecules (almost 50% of which were polyphenols) across a wide range of polarities, chemical classes and molecular weights.

Like other preparative and semipreparative-scale techniques, counter-current chromatography is a powerful tool for phytochemists, as it allows the isolation of molecules in large enough quantities for identification techniques and bioactivity assays to be applied. So it is by no means a coincidence that many of the studies that have used counter-current chromatography have led to the discovery of known compounds previously unreferenced in a particular plant and of previously unknown structures.

However, most studies are very source-dependent, with mixtures of different specific compounds, so general conclusions for concrete families are hard to infer. The aim of our review is to compile general procedures for selected families of compounds rather than for selected plants, so that common methods can be established.

3.1. Anthocyanins separation by CCC

Counter-current chromatography has been successfully applied for the fractionation and isolation of pure anthocyanins from a number of sources. Table 2 compiles the most relevant research in this area.

The solvents mixtures used for anthocyanidin separation by CCC can be considered as very polar. They often contain n-butanol, which can be difficult to handle when evaporations or concentrations must be performed, but allows both phases to have high polarity while maintaining the biphasic system. They also include acidification with trifluoroacetic acid (TFA) to displace the anthocyanidin equilibrium to the flavylium ion, which improves separations.

The normal mode of operation is head-to-tail: the lesser dense phase is the stationary phase, and the more dense phase is the mobile phase. In consequence, it is a descending elution mode. However, ascending mode methods can also be found [44,45].

A variety of solvent systems can be used to improve the fractionation and purification of anthocyanins. It is advisable to optimize solvent systems every time a new source is tested, since anthocyanin composition may differ considerably from one source to another. The utilization of gradients can be a solution [44,45], always taking into account not to break the biphasic system maintained in the rotor. Iterative separations, which constitute a procedure where co-eluting compounds are again chromatographed under different conditions in order to improve their separation, are also useful. For example, Renault et al. [44] obtained pure peonidin-3-glucoside and malvidin-3-glucoside from grape in one step, while in a second step the pure cyanidin-3-glucoside was isolated.

The more hydrophobic solvent systems enable the elution of polymeric anthocyanins and diglucosides, while monomeric anthocyanins and some vitisins are retained in the stationary phase. By using less hydrophobic systems, the monomeric anthocyanidins can be eluted with the mobile phase. Anthocyanins are separated according to the degree of substitution of their B-ring and to the nature of the anthocyanidin substituent (glucoside, acetylglucoside, or coumaroylglucoside). Normally, acylated anthocyanins will precede non-acylated anthocyanins in head-to-tail separations. Coelution patterns suggested to Renault et al. [44] and Du et al. [46] that the polarity of the sugars dominated the selectivity of the CPC method, which is in contrast with TLC (thin layer chromatography) behaviors, where the aglycon is the element that plays a major role in separation. But Salas et al. [47] observed that the B-ring had also a considerable influence on separations: the anthocyanins that are trisubstituted in the B-ring (delphinidin, petunidin and malvidin) tend to elute first than the disubstituted anthocyanins (peonidin, cyanidin) when working in head-to-tail mode. That seems to happen also for the corresponding acylated anthocyanins, and even for some carboxyl-pyranoanthocyanins.

Other factors that influence separation are the number of coils in the equipment, and the flow rate applied. For example, Degenhardt et al. [48] obtained no separation when they used a single-coil system, but separated anthocyanins from black currant with a three-coil CCC. Schwarz et al. [49] improved the resolution for coeluting anthocyanidin glucosides by reducing the flow rate.

CCC can be applied directly to raw sources dissolved in one of the system phases. However, better yields are provided when prepurification steps (i.e., Amberlite XAD polymeric resin, clean up of samples) are done, since in that situation greater amounts can be injected into the column.

To sum up, with the use of CCC anthocyanins from various sources can be isolated, in amounts up to several hundred milligrams of pure compounds. CCC can have a crucial role for identifying new structures, because it provides large enough quantities for NMR studies of unknown anthocyanidins present at very low concentrations in natural sources, such as pyranoanthocyanidins from wines [47,50,51] or other beverages or in plants like *Tradescantia pallida* [49]. In these cases, the HPLC–MS of eluted fractions will give valuable information so that these compounds can be identified.

3.2. Flavan-3-ols and procyanidins separation by CCC

Table 3 shows the different solvent systems used to date to isolate proanthocyanidins monomers and oligomers by CCC.

Most studies on procyanidins separation by CCC have focused on two of the richest sources of these compounds: green tea and grape seeds. However, the results can be translated to other derived foods such as wines or musts, although in these cases a greater complexity must be expected.

Since they are normally more hydrophobic than anthocyanins, the solvent systems used for the separation of procyanidins are usually less polar.

As the hydrophobicity properties of procyanidins differ considerably because of their degree of polymerization or their galloylation, different solvent systems and strategies must be taken into account. Yanagida et al. [54] showed that the elution peaks are perfectly reversed if the system is changed from the head-to-tail to the tail-to-head mode. So, in order to maximize efficiency solvent systems should be selected very carefully.

For example, an ascending mode with an apolar system is suitable for isolating monomers epicatechin (EC), catechin (C), epigallocatechin (EGC), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG), which would appear too late in the descending mode (with broadening peaks) or even remain retained on the stationary organic phase. In contrast, the descending mode is more suitable for proanthocyanidins dimers and trimers. Yanagida's results also demonstrate that the hydrophobicity of EC oligomers considerably decreases when their degree of polymer-

Table 2
Anthocyanins separation by CCC.

Column capacity, flow, rpm Solvent system	*Source* Anthocyanins isolated (time)	Ref.
Wines		
850 ml, 3.5 ml/min, 800 rpm *MTBE:BuOH:ACN:W 0.1% TFA* (*2:2:1:5*) *Head-to-tail* (*descending*)	*Wine pigments* Polymeric pigments: carboxyl-pyranoanthocyanins, anthocyanidin-flavanol adducts, anthocyanidin-diglucosides, anthocyanidin dimers (170 min) Dp3gluc, Pt3gluc, Mv3gluc (220 min) Pn3gluc, Cy3gluc (270 min) Acylated anthocyanins, anthocyanidin dimers, coumaryl carboxypyranoanthocyanins (330 min)	Salas et al., 2005 [47]
850 ml, 3.7 ml/min, 1000 rpm *TBME:BuOH:ACN:W* (*2:2:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Red wine* Polymeric pigments (120 min); diglucosides (130 min); Mv-3-gluc (190 min)	Schwarz et al., 2003 [49]
850 ml, three coils, 2 ml/min, 1000 rpm *TBME:BuOH:ACN:W* (*1:3:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Red wine* Polymeric pigments (180 min); Mv-3,5-digluc (240 min); Pn-3,5-digluc (280 min)	Schwarz et al., 2003 [49]
850 ml, 5 ml/min, 1000 rpm *TBME:BuOH:ACN:W* (*2:2:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Californian red wine* Mixture Pn-3,5-digluc + Mv-3,5-digluc (80 min), Mv-3-gluc (120 min), Mv-3-cu-gluc-5-gluc (150 min), Pn-3-cu-gluc-5-gluc (200 min), Mv-3-ac-gluc (250 min)	Degenhardt et al., 2000 [52]
850 ml, 5 ml/min, 1000 rpm *AcEt:BuOH:W* (*2:3:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Pre-purified mixture of wine anthocyanins* Mv-3,5-digluc (90 min); Pn-3,5-digluc (105 min); Mv-3-ac-glu-6-gluc (120 min) *German red wine*: Vitisin A (90 min), acetylvitisin A (120 min)	Degenhardt et al., 2000 [50]
850 ml, 5 ml/min, 1000 rpm *AcEt:W* (*1:1*), *0.1% TFA* *Head-to-tail* (*descending*)	*German red wine*: Mv-3-cou-glu, Mv-3-cg-glu	Degenhardt et al., 2000 [50]
Berries		
200 ml, 2 ml/min, 800 rpm *System gradient* *TBME:BuOH:ACN:W*, *0.02% TFA* (*2:2:0.1:5*) *TBME:BuOH:ACN:W*, *0.02% TFA* (*2:2:2.5:5*) *Tail-to-head* (*ascending*)	*Grape skins and rosé wine* Five anthocyanin coumarates (40 min) Anthocyanidin-caffeoates (45 min) Anthocyanidin-acetates (55–80 min) Anthocyanidin-monoglucosides (95 min, 120 min)	Vidal et al., 2004 [45]
850 ml, 5 ml/min, 1000 rpm *AcEt:BuOH:W* (*4:1:5*), *0.1% TFA*	*Grape skin extract*: Mv-3-ac-glu, Dp-3-ac-glu, Pt-3-ac-glu	Degenhardt et al., 2000 [50]
(a) 230 ml, 3 ml/min, 1400 rpm (b) 5470 ml, 60 ml/min, 1400 rpm *AcEt:BuOH:W*, *0.2% TFA* (*77:15:8*) *initial mobile phase* (*40:46:14*) *final mobile phase* (*5:5:90*) *stationary phase* *Tail-to-head* (*ascending*)	*Black currant anthocyanins* Cy-3-gluc (100 min), Dp-3-gluc (150 min), Cy-3-rut (200 min), Dp-3-rut (230 min) *Blue grapes anthocyanins* Impure acylated anthocyanins (100 min), Pn-3-gluc (160 min), Mv-3-gluc (230 min), Cy-3-gluc (260 min), Pt-3-gluc (270 min), Dp-3-gluc (270 min)	Renault et al., 1997 [44]
850 ml, 5 ml/min, 1000 rpm *TBME:BuOH:ACN:W* (*2:2:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Black currant anthocyanins*: Dp-3-rut (40 min), Cy-3-rut (50 min), Dp-3-gluc (80 min), Cy-3-gluc (120 min) *Black chokeberry*: Cy-3-gal (130 min), Cy-3-ara (240 min) *Roselle*: Dp-3-sam (30 min), Cy-3-sam (50 min) *Red cabbage*: Cy-3-digluc-sin, 6-gluc (20 min); Cy-3-digluc-disin, 6-gluc (30 min); Cy-3-digluc-sin-fer, 6-gluc (50 min); Cy-3-digluc-sin-fer, 6-gluc (70 min)	Degenhardt et al., 2000 [48]
850 ml, 5 ml/min, 1000 rpm *TBME:BuOH:ACN:W* (*1:3:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Elderberry* Mixture Cy-3-sambubioside-5-gluc + Cy-3,5-digluc (120 min) Cy-3-sambubioside (180 min); Cy-3-gluc (270 min)	Schwarz et al., 2003 [49]
850 ml, 2.5 ml/min, 1000 rpm *TBME:BuOH:ACN:W* (*1:3:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Elderberry* Cy-3-sambubioside-5-gluc (150 min); Cy-3,5-digluc (190 min)	Schwarz et al., 2003 [49]
850 ml, 3.7 ml/min, 1000 rpm *TBME:BuOH:ACN:W* (*2:2:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Blackberries* Cy-3-rut (170 min); Cy-3-gluc (190 min)	Schwarz et al., 2003 [49]
850 ml, 5 ml/min, 1000 rpm *TBME:BuOH:ACN:W* (*2:2:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Purple corn*: Cy-3-gluc (170 min); Cy-3-mal-gluc (200 min) non pure; Pn-3-mal-gluc (240 min) non pure *Purple heart*: Cy-3-ara-gluc-gluc-7′,3′-di-fer-gluc (60 min)	Schwarz et al., 2003 [49]
380 ml, 1.5 ml/min, 650 rpm *MTBE:BuOH:ACN:W:TFA* (*1:4:1:5:0.01*) *Head-to-tail* (*descending*)	*Bilberry fruit* Dp-3-sambubioside (200 min); Cy-3-sambubioside (280 min) Others unidentified	Du et al., 2004 [53]

Table 2 (*Continued*)

Column capacity, flow, rpm Solvent system	*Source* Anthocyanins isolated (time)	Ref.
1200 ml, 3 ml/min, 700 rpm *MTBE:BuOH:ACN:W:TFA* (*1:3:1:5:0.001*) *Head-to-tail* (*descending*)	*Mulberry* Unknown mixture (200 min), Cy-3-rha (300 min), Cy-3-rha-gal (350 min), Cy-3-gluc (460 min), Cy-3-gal (600 min), Cy-7-gluc (800 min)	Du et al., 2008 [46]
850 ml, 3.5 ml/min, 800 rpm *TBME:BuOH:ACN:W* (*1:3:1:5*), *0.1% TFA* *Head-to-tail* (*descending*)	*Cherry juice and fermented cherry beverages* Cy-3-gluc-rut; Cy-3-sophoroside; 5-carboxypyranocyanidin-3-gluc-rut; Cy-3-rut; Cy-3-gluc; pn-3-rut; Pn-3-gluc	Rentszsch et al., 2007 [51]

EtAc: ethyl acetate; EtOH: ethanol; BuOH: n-butanol; W: water; Hex: hexane; Ac: acetic acid; ACN: acetonitrile; MTBE: methyl ter-butyl ether. Cy: cyaniding; Dp: delphinidin; Pt: Petunidin; Pn: peonidin; Mv: malvidin; gluc: glucoside; rut: rutinoside; gal: galactoside; ara: arbinoside; sam: sambioside; sin: sinapoyl; fer: feruloyl; cu: coumaroyl; and cff: caffeoyl.

ization increases [54]. So, the more polymerized the sample is, the greater the polarity the solvent systems should have.

Sample clean-up before the CCC separation can provide a lower baseline level for UV on-line detection. Pentane precipitation methods or polyamide columns can be applied for this purpose, but it should be pointed out that they both eliminate higher oligomeric compounds, so yields for compounds above tetramers will be reduced [40].

Excluding monomers, it is hard to obtain other pure compounds from grape seed extracts, since in this source there is a complex

Table 3
Flavan-3-ols and proanthocyanidins separation by CCC.

Column capacity, flow, rpm Solvent system	Source, proanthocyanidins isolated (time)	Ref.
Teas		
260 ml, 2 ml/min, 800 rpm *Hex:EtAc:W* (*1:20:30*) *Descending mode* (*head-to-tail*)	*Green tea* EGCG (360 min)	Cao et al., 2000 [57]
260 ml, 2 ml/min, 800 rpm *Hex:EtAc:W* (*1:3:4*) *Descending mode* (*head-to-tail*)	*Green tea* ECG (200 min)	Cao et al., 2000 [57]
230 ml, 1.5 ml/min, 800 rpm *Hex:EtAc:W* (*10:1:12*) *Descending mode* (*head-to-tail*)	*Cultured tea cells* EC (100 min); C (150 min) Gallate-type catechins retained in the stationary phase	Du et al., 2001 [58]
300 ml, 800 rpm, 1.5 ml/min *Hex:EtAc:MeOH:W* (*1:7:1:7*) *Descending mode*, *ascending mode*	*Tea* Head-to-tail elution mode (3.5 h): C, EGC, EC, EGCG, ECG Tail-to-head elution mode (1 h): CG, EGCDG	Kumar et al., 2005 [59]
40 ml, 1000 rpm, 2 ml/min *MTBE:ACN:W* (*2:2:3*), *0.1% TFA Ascending mode* (*tail-to-head*)	*Tea* Theflavins (6 min), ECg (8 min), EGCg (10 min), EC (15 min), Gallic acid (16 min), EGC (20 min), Caffein (30 min), theophylline (40 min), theobromine (70 min)	Yanagida et al., 2006 [54]
300 ml, 800 rpm, 2 ml/min *Hex:EtAc:MeOH:W* (*1:5:1:5*) *Descending mode* (*head-to-tail*)	*Tea leaves* EGCG-(4 → 6)-EGCG (30 min); ECG-(4 → 6)-EGCG (70 min) EGCG-(4 → 6)-ECG (100 min); EGCDG (140 min) EAG-(4 → 6)-EGCG (190 min); ECG-(4 → 6)-ECG (300 min) ECDG (420 min)	Kumar et al., 2009 [55]
Grape Seed		
850 ml, 1000 rpm, 2.5 ml/min *EtAc:IprOH:W* (*40:1:40*) *Descending mode* (*head-to-tail*)	*Grape seed extracts* Dimer B1 and trimer C1 (180 min); dimer B2 and dimer B3 (270 min); dimer B4 (52.6%) (300 min)	Kohler et al., 2008 [40]
215 ml, 3–4 ml/min, 1000 rpm *Hex:EtAc:EtOH:W* (*1:8:2:7*) *Ascending mode* (*tail-to-head*)	*Vitis vinifera grape seeds* Monomers: C, E (30 min); gallic acid (45 min) Procyanidin dimers (80–180 min)	Delaunay et al., 2002 [60]
850 ml, 1000 rpm, 2.9 ml/min *EtAc:IprOH:W* (*20:1:20*) *Descending mode* (*head-to-tail*)	*Grape seed extract* Tetrameric cinnamtannin A2 (160 min), dimer B1 (200 min), trimer C1 (210 min), dimer B2 and dimer B3 (250 min); dimer B4 (300 min)	Kohler et al., 2008 [40]
850 ml, 1000 rpm, 2.7 ml/min *EtAc:BuOH:W* (*14:1:15*) *Descending mode* (*head-to-tail*	*Grape seed extract* Tetrameric A2 and dimer B1 (240 min), trimer C1 (300 min), dimer B2 and dimer B3 (360 min), dimer B4 (410 min)	Kohler et al., 2008 [40]
850 ml, 1000 rpm, 3 ml/min *Hex:EtAc:MeOH:W* (*1:10:1:10*) *Descending mode* (*head-to-tail*)	*Grape seed extract* Dimer ECG-C (180 min), dimer B5 (270 min), dimer B7 (290 min) EC (300 min); C (370 min)	Kohler et al., 2008 [40]

EtAc: ethyl acetate; EtOH: ethanol; BuOH: n-butanol; W: water; Hex: hexane; Ac: acetic acid; ACN: acetonitrile; MTBE: methyl ter-butyl ether; EC: epicatechin; C: catechin; EGC: epigallocatechin; EGCG: epigallocatechin gallate; ECG: epicatechin gallate; ECDG: epicatechin digallate; and IprOH: isopropanol.

mixture of different proanthocyanidins isomers which are difficult to resolve chromatographically. The successive application of different separation and sample clean-up systems can result in better separations, as shown by Kohler et al. [40], but a last step of purification by preparative or semipreparative HPLC may be needed. Controlling the *K*-value of distribution between the two phases can be very useful, as resolution of two peaks can require differences in *K*-values of over 0.1 (the exact value can depend on the system) [55].

Overall, CCC is a suitable technique for fractionating proanthocyanidins and catechins with aims to produce extracts or purified compounds that can be applied as HPLC standards, for biological studies, or to evaluate their organoleptic properties [56].

3.3. Isoflavonoids separation by CCC

Separation methods of Isoflavones from *Pueraria lobata* [42], soybeans [61], *Astragalus* [62] and red clover [63] by high-speed counter-current chromatography have been described (Table 4). Due to the difficulties that arise from the low solubility of some isoflavones, CCC can be a suitable technique for the isolation of isoflavones at large scales [63].

The lack of active surfaces in CCC ensures an efficient preparative isolation even of labile isoflavones. Reported methods have allowed separation of isoflavone free aglycones (daidzein, biochanin A, etc.), glycosides (daidzin and genistin), diglycosides, and acetyl and malonyl glycosides. Sometimes it is necessary to perform the separation in a two-steps procedure, since it is difficult to find a system of solvents able to provide partition coefficients different enough for all the isoflavones. And sometimes it is only possible to obtain pure isoflavones after a semipreparative or preparative HPLC isolation. But even in that case, the previous CCC fractionation is very useful, since application of preparative or semipreparative HPLC will then be easier [63]. In overall, CCC methods constitute a fast and reliable technique to obtain great amounts of rare isoflavones in order to examine their physiological effects, metabolism and bioavailability [63].

For all the methods found, the stationary phase was the upper phase, so the system was in the descending mode (head-to-tail). The most common solvent systems used are based on ethyl acetate:water with an alcohol that distributes between both phases. Ethanol, methanol and n-butanol have been the most widely used alcohols in the various separations, although several methods report the use of acetonitrile instead. The polarity of the stationary phase can be adjusted by partially or totally replacing the ethyl acetate by more apolar solvents such as hexane or tert-methyl butyl ether.

The work of Yang et al. [64] is a clear example of manipulating the selectivity of the separation by changing the system mixture. In a first run, only the less polar isoflavones (the aglycones) were well separated, while the polar glucosides remained retained on the column, due to their large partition coefficient values in this solvent system. However, when a little n-butanol was added to the system, the mobile phase was a little more polar, the aglycones eluted faster and the glucosyl derivatives were finally eluted. By applying another system of solvents where methyl ter-butyl ether and tetrahydrofurane replaced chloroform and methanol, selectivity changed enormously, and peaks that remained poorly resolved

Table 4
Isoflavonoids separation by CCC.

Column capacity, flow, rpm Solvent system	Source, isoflavonoids isolated (time)	Ref.
230 ml, 2 ml/min, 800 rpm *EtAc:EtOH:BuOH:W* (*30:10:6:50*)	*Astragalus membranaceus* Calycosin-7-O-β-D-glycoside + formononetin-7-O-β-D-glycoside (180 min)	Ma et al., 2003 [62]
230 ml, 2 ml/min, 800 rpm *EtAc:EtOH:W* (*5:1:5*)	*Astragalus membranaceus* Calycosin-7-O-β-D-glycoside (120 min), Formononetin-7-O-β-D-glycoside (180 min)	Ma et al., 2003 [62]
260 ml, 2 ml/min, 700 rpm *Hex:EtAc:BuOH:MeOH:Ac:W* (*1:2:1:1:5:1*)	*Soybeans* Daidzin (100 min), Genistin (150 min), 6″-O-malonyldaidzin (180 min), 6″-O-malonylgenistin (280 min)	Du et al. 2001 [61]
850 ml, 3.2 ml/min, 600 rpm *MTBE:ACN:W* (*2/2/3*)	*Soy* Daidzin + glycitin (160 min), genistin (260 min), 6″-O-acetyldaidzin (320 min)	Stuertz et al., 2006 [63]
850 ml, 3 ml/min, 800 rpm *Hex:EtAc:MeOH:W:*(*6/5/6/5*)	*Red clover* Formononetin (200 min), Irilone (260 min), prunetin (360 min), biochanin A (480 min)	Stuertz et al., 2006 [63]
175 ml, 1 ml/min, 1000 rpm 1st separation. *$CHCl_3$–MeOH–W* (*4:3:2*) 2nd separation. *$CHCl_3$–MeOH–BuOH–W* (*4:3:0.5:2*) 3rd separation. *MtBE–THF–0.5% TFA* (2:2:4)	*Crude soybean extract* 1st: Glycitein (65 min), daidzein (100 min), acetylgenistein (190 min), acetyldqaidzin (340 min) 2nd: Glycitein (70 min); daidzein (80 min), acetylgenistein (100 min), acetyldaidzin (160 min), genistin (320 min), glycitin (380 min), daidzin (540 min) 3rd separation: Glycitin (60 min), gensitin (420 min)	Yang et al., 2001 [64]
850 ml, 2.8 ml/min, 600 rpm *MTBE:ACN:W* (*6/3/8*)	*Red clover* Ononin (270 min), Sissotrin (500 min)	Stuertz et al., 2006 [63]
260 ml, 2 ml/min, 800 rpm *EtAc:BuOH:W* (*2:1:3*)	*Pueraria lobata* Puerarin xyloside (70 min), Puerarin xyloside (90 min), 3′-hydroxylpuerarin (120 min), 3′-methoxy-puerarin (150 min), puerarin (200 min), daidzin (400 min)	Cao et al., 1999 [42]

EtAc: ethyl acetate; EtOH: ethanol, BuOH: n-butanol, W: water, Hex: hexane, Ac: acetic acid, ACN: acetonitril, MTBE: methyl ter-butyl ether; $CHCl_3$: chloroform; MeOH: methanol; TFA: trifluoroacetic acid; and THF: tetrahydrofuran.

(genistin and glycitin) in the previous system were perfectly separated.

In conclusion, the one-step separation of all isoflavones with a single solvent system is not practical, while better and fast results can be obtained by combining different runs and applying different kinds of solvents that provide differentiated partition coefficients for the compounds of interest.

3.4. Conclusions

The main advantage of CCC when compared to equivalent techniques such as low pressure liquid chromatography is that there are no losses by adsorption in the stationary phase. The range of selectivities offered by chromatographic resins can find its equivalent on the range of selectivities offered by different systems solvents, although one main limitation is that system solvents must keep two immiscible phases. However, iterative strategies can easily lead to great yields of pure polyphenols, even if a final purification by more resolutive techniques such as semipreparative HPLC can be envisaged.

4. High performance liquid chromatography

High performance liquid chromatography (HPLC) is the analytical technique most used for the separation and characterization of phenolic compounds [65–67]. Columns chosen for the determination of phenolic compounds are almost exclusively reverse phase columns, composed of a C18 stationary phase with an internal diameter ranging from 2.1 to 5 mm and a particle size ranging from 3 to 5 μm, although narrow-bore columns (internal diameter ranging from 2.1 to 1.1 mm) packed with very small particles (1.7 μm) have been reported for mass spectrometry coupled to liquid chromatography [68]. The solvents system consists on an aqueous phase and an organic phase (mainly methanol or acetonitrile). Typically, an acid is added to the solvents, being acetic acid or formic acid the most used. This combination of columns and system of solvents has been successfully applied to a great number of phenolic compounds families, and this review will report some examples for anthocyanins, flavanols and isoflavones, but other polyphenol families including phenolic acids [69], flavonols (quercetin, kaempferol), flavones (apigenin, luteolin) and flavanones (naringenin, neohesperidin) [70,71], and also stilbenes [72–74] are also easily analyzed by HPLC.

In overall, HPLC methods combined with electrochemical, UV and fluorescent detectors have been widely used in food polyphenols research, and have proven to be a highly effective analytical tool. The weakness of these detection methods is their lack of structural information and some non-specificity leading to the possibility of sample matrix interference and misattribution of peaks. Mass spectrometry is currently the most selective analytical technique for the identification and quantification of unknown compounds from crude and partially purified samples of foods and natural supplements [75–79]. Over the last few years, the coupling of mass spectrometry detectors to liquid chromatography systems has notably improved the identification and structural characterization of phenolic compounds [80].

The most important aspects for the optimal performance of LC–MS analysis are the choice of the interface and the kind of detection provided by the equipment.

Electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) interfaces are highly sensitive, show great ionization stability, and they have become the methods of choice for polyphenols analysis [81]. The view is generally held that APCI can be used for relatively non-polar compounds that can undergo acid–base reactions in the gas phase, whereas ESI is more suitable for compounds that can be ionized in solution [82]. According to most studies, for both APCI and ESI the negative mode provides best sensitivity [83].

The application of tandem mass spectrometry (MS-MS) is useful in the identification and quantification of polyphenolic compounds [81]. For quantitative analysis, triple-quadrupole (QqQ) mass spectrometers, which are capable to perform multiple reaction ion monitoring (MRM) are extensively used. However, MS-MS fragmentations and low mass resolution may be limited and insufficient to infer the molecular formula of an unknown compound [84].

Ion-trap mass spectrometers (ITMS), with their high sensitivity in the scanning mode and the ability to perform MS^n experiments, are well suited for many identification purposes. That means that they can carry out sequential fragmentations first of the parent molecular ion and second of the daughter ions [81]. This can be a particular advantage for the analysis of glycosides of isomeric flavonoids [81], which are not distinguishable in the MS^2 spectra. On the limitations side, mass resolution obtained by ion-trap spectrometers is not the best, and in fact it is comparable to the quadrupoles [84].

When qualitative analysis has to be performed, the highest resolution for identification purposes is provided by time-of-flight (TOF) mass spectrometers. Their resolving power may be enough to provide a molecular formula and to confirm or deny a suggested structure [84]. Even more useful is a quadrupole-TOF combination (qTOF) as it allows MS-MS experiments to be performed, providing more structural information, and the selection of a parent ion to be analyzed by TOF-MS, which adds selectivity [84].

Considering all these aspects, the choice of the instrument will depend on the study. The analysis of polyphenols in biological samples is one of the most interesting fields in which HPLC–MS can be applied. Mass spectrometry has proved to be one of the most effective techniques for the analysis of diet polyphenols in biological samples, since the ESI-MS/MS allows very low limits of detection (LOD), at the range of ng/ml. With MS in the selected ion monitoring (SIM) or in the product ion analysis mode, LOD are even better than for fluorescence or electrochemical detection [83]. A complete review of the determination of representative flavonoids and their metabolites in biofluids by LC–MS can be found [20,24].

Hyphenation with mass detectors has caused modifications in previously well established HPLC separation methods of polyphenols. The selectivity offered by MS scan mode, such as SRM, makes HPLC separation less crucial than before, since co-eluting flavonoids are scanned separately using the MS detector. Scan modes are usually applied for the fast detection of compounds in the sample, and determining the molecular weight allows to verify the identity of compounds. However, a good separation is anyway recommended, given that matrix effects can worsen the ionization of the analytes, and on the other hand, by separating the analytes chromatographically, a further element of selectivity is added [24].

It should also be noted that common mobile phase modifiers such as trifluoroacetic acid or sodium or potassium phosphate can quenche the ionization process, thereby requiring modifications of existing chromatographic methods. Alternative modifiers include formic acid in the place of trifluoroacetic acid and ammonium acetate instead of phosphate buffers [81].

The column effluent flow rate can influence the sensitivity of mass detectors. Though most mass spectrometers can nowadays operate at flow rates up to 2 ml/min for ESI and 4 ml/min for APCI, lower flow rates are advisable for the instruments maintenance. Similarly, acid concentration of mobile phases should be reduced for certain analysis, specially in the case of anthocyanins, since recommended percentages for ESI are 1% formic acid while classic HPLC-UV methods utilized 10% formic acid. Both reduction in mass flow and acid proportions can lead to worse resolution in the HPLC separation, but this limitation can be overcome by

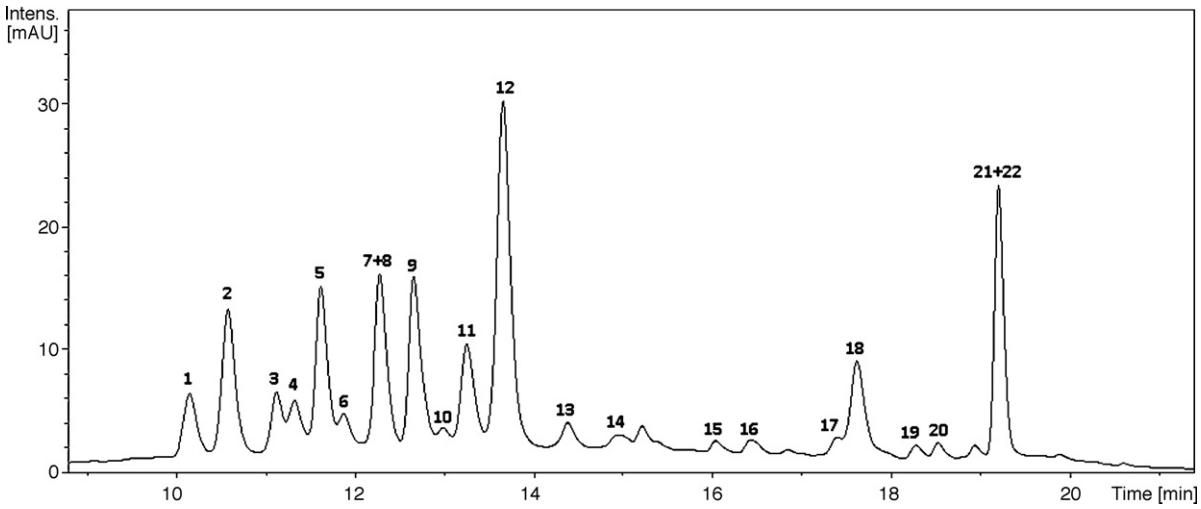


Fig. 5. HPLC separation of a mixture of anthocyanins from red fruits mix extract (535 nm) on a C18 150 × 2.1 mm, 3.5 μm column, with linear gradient of mobile phase (A) water, 0.2% trifluoroacetic acid and (B) MeOH, 0.2% trifluoroacetic acid. 1. Dp-galactoside, 2. Dp-glucoside, 3. Cy-galactoside, 4. Dp-arabinoside, 5. Cy-glucoside, 6. Pt-galactoside, 7. Cy-arabinoside, 8. Pt-glucoside, 9. Pg-glucoside, 10. Pt-arabinoside, 11. Pn-glucoside, 12. Mv-glucoside, 13. Mv-arabinoside, 14. Dp-acetylglucoside, 15. Cy-acetylglucoside, 16. Pt-acetylglucoside, 17. Pn-acetylglucoside, 18. Mv-acetylglucoside, 19. Cy-coumaroylglucoside, 20. Pt-coumaroylglucoside, 21. Pn-coumaroylglucoside, and 22. Mv-coumaroylglucoside (Dp: delphinidin, Cy: cyanidin, Pt: petunidin, Pn: peonidin, Pg: pelargonidin, Mv: malvidin).

columns with a smaller particle size. HPLC using 1.7 μm particles provides significantly more resolution while reducing run times and improving the sensitivity for the analysis of many compound types. In consequence, in recent years, interest in microcolumn LC has increased considerably. This is mainly due to the ability to work with small sample sizes, small volumetric flow rates, easy coupling to mass spectrometers and secondary chromatographic systems, and an enhanced detection performance with the use of concentration sensitive detectors due to the reduced chromatographic dilution. The use of microHPLC prior to ESI/MS detection can give an enhanced sensitivity that allows mass spectra to be obtained also for minor components present in the matrix [85–87]. An ultra-high pressure system (UPLC) is often used for small particle-packed columns with small diameter, which gives a positive effect on both system efficiency and analysis time. The technique is suitable for fingerprinting analysis, considering its speed, robustness and high sample-throughput. It also allows the detection of wide-range metabolites, including both hydrophilic and hydrophobic metabolites with a single chromatogram run.

4.1. HPLC of anthocyanins

Numerous methods have been developed for anthocyanins characterization. Usually, routine analyses of anthocyanins involve spectrophotometric and chromatographic techniques. Reversed-phase high performance liquid chromatography (RP-HPLC) with C18 columns is the usual method of choice for separating anthocyanins in different sources. With these columns the elution pattern of anthocyanins is mainly dependent on the partition coefficients between the mobile phase and the C18 stationary phase, and on the polarity of the analytes. The mobile phase consists normally on solvent A (water:carboxylic acid, 90:10) and solvent B (methanol or acetonitrile:carboxylic acid, 90:10). The acidic media allows the complete displacement of the equilibria to the flavylium cation, thus resulting in a better resolution and a great characteristic absorbance between 515 and 540 nm, which explains the universal application of DAD detection for anthocyanins quantification.

The elution order in C18 columns is normally a function of the number of hydroxyl groups and their degree of methoxylation (delphinidin (Dp) < cyanidin (Cy) < petunidin (Pt) < pelargonidin (Pg) < peonidin (Pn) < malvidin (Mv)), along with the number of glucosides and their acylation pattern (diglucosides (digluc) < monoglucosides (gluc) < acetylglucosides (ac-gluc) < caffeoyl glucosides (cff-gluc) < coumaroyl-glucosides (coum-gluc)) [88].

Fig. 5 gives an example of this pattern of elution, provided by a synthetic mixture of common anthocyanins that can be found in several berries.

However, although this classic method can work very well for the separation of anthocyanin monoglucosides, it has proven to have some limitations for the separation and determination of other compounds. Common problems with existing methods include fast moving anthocyanidin-diglucosides eluting together as mixed peaks or combined with anthocyanidin monoglucosides, along with considerable overlap between the acetic and coumaric ester series. The appearance of multiple p-coumaric peaks for a given aglycone, attributed to cis and trans isomers, also complicates anthocyanin studies. Caffeoyl esters, generally present only in trace amounts, are particularly difficult to detect because their polarities are very similar to their p-coumaroyl derivatives, and the molecular masses for the 3,5-diglucosides and the caffeoyl glucosides are identical, limiting absolute identification by HPLC–MS.

New columns have been tested in order to try to improve the separation of anthocyanins. McCallum et al. [89] obtained better separations for anthocyanins with mixed mode HPLC columns such as the Prime-Sep column based dual functional column, i.e., ion-exchange and reversed phase. Using the Prime-Sep column, a total of 37 anthocyanin peaks were detected in the Concord skin extract, better than many C18 methods (27–40 peaks). The unique elution pattern produced by the mixed column greatly simplifies peak identification, as the anthocyanins are separated into distinct, repetitive and uninterrupted series (Dp < Cy < Pt < Pn < Mv), with the relative amounts of each aglycone within a group conserved.

In the field of HPLC, monolithic or rod columns represent one of the most interesting innovations. Their common characteristic is that they are constituted by one single piece of a porous material and that this piece fills the entire column. Replacing conventional columns with monolithic columns can allow shorter assay times, faster column equilibration, and larger void volume between the

Table 5
Selection of LC–MS analysis of anthocyanins in different sources.

Columns	Solvents	Detection	Source/anthocyanins	Ref.
LiChrospher 100 RP-18 250 × 10 mm, 10 μm	A: water/formic acid 90:10 B: methanol/water/formic acid 50:40:10	HPLC–DAD–ITMS ESI(+) N_2: 40 T^a: 220 °C Capillary: 3000 V Scan: 150–1000 *m*/*z* DAD: 520 nm	Grape samples 22 compounds	Favretto et al. 2000 [97]
Novapack C18 250 × 4.6 mm, 5 μm T^a 50 °C	A: water/acetonitrile 95:5 B: water/acetonitrile 50:50 pH 1.3 con TFA (trifluoroacetic acid)	HPLC–PDA–QMS ESI(+) N_2: 12 l/min T^a: 325 °C Capillary: 4000 V Frag. volt: 90–120 V Scan: 50–2000 *m*/*z* DAD: 520 nm	Grape samples Wine 20 compounds	García et al. 2003 [91]
C18 250 × 4.6 mm, 5 μm T^a 25 °C	A: water/formic acid/AcN 87:10:3 B: water/formic acid/AcN 40:10:50	HPLC–DAD–ITMS ESI(+) N_2: 11 l/min T^a: 365 °C Neb: 65 psi Scan: 50–1000 *m*/*z* DAD: 520 nm	Pomace from red and white grapes 13 anthocyanins 12 phenolics acid 15 anthoxantines and stilbenes	Kammerer et al. 2004 [98]
Capcell Pak C18 150 × 4.6 mm, 5 μm T^a 40 °C	A: water/TFA 0.1% B: acetonitrile/water (50:50), 0.1% TFA	LC–PDA–QMS ESI(+) T^a: 320 °C Capillary: 5000 V Scan: 50–1000 *m*/*z* DAD: 250–600 nm	Bilberry Blueberry Black currant	Nakajima et al. 2004 [99]
Novapack C18 150 × 3.9 mm	A: water/formic acid 90:10 B: water/methanol/formic acid 45:45:10	HPLC–PDA–QMS ESI(+) N_2: 10 l/min T^a: 350 °C Spray volt: 4000 V Fragmentor: 100–120 V DAD: 530 nm	Grape skins 21 compounds	Núñez et al. 2004 [100]
Symmetry C18 75 × 4.6 mm, 3.5 μm	A: water/10%formic acid B: acn (acetonitrile)	HPLC–PDA–QqQ ESI(+) N_2: 17 l/min T^a: 420 °C Capillary: 3000 V Collision energy: 25 eV DAD: 200–600 nm	Black raspberries Red raspberries Blueberries 15 compounds	Tian et al. 2005 [92]
Novapack C18 150 × 3.9 mm, 4 μm Room T^a	A: water/formic acid, 90:10 B: water/methanol/formic acid, 45/45/10	HPLC–DAD–QMS ESI(+) N_2: 10 l/min T^a: 350 °C Neb.: 55 psi Capillary: 4000 V Fragmentor: 100–120 V Scan: 100–1500 *m*/*z* DAD: 530 nm	Commercial dietary ingredients (grape seeds, skin, pomace and leaves)	Monagas et al. 2006 [101]
Symmetry C18 250 × 4.6mm, 5 μm T^a 25 °C	A: water, 1% formic acid B: acetonitrile	HPLC–DAD–ITMS ESI(+) T^a: 275 °C Capillary: 4000 V Scan: 250–2000 *m*/*z* DAD: 250–600 nm	Blackberry, black raspberry, blueberry, cranberry, red raspberry, strawberry	Seeram et al. 2006 [102]
Luna C18 250 × 4.6 mm, 5 μm T^a 20 °C	A: water/formic acid, 90:10 B: acetonitrile/formic acid, 90:10	HPLC–DAD–QMS ESI(+) N_2:8 l/min T^a: 250 °C Capillary: 3500 V Scan: 100–1500 *m*/*z* DAD: 530 nm	Grape pomace	Ruberto et al. 2006 [67]

Table 5 (*Continued*)

Columns	Solvents	Detection	Source/anthocyanins	Ref.
Symmetry C18 150 × 3.0 mm, 5 μm	A: water/formic acid, 95:5 B: methanol/formic acid, 95:5	HPLC–DAD–QMS ESI(+) N_2: 15 l/min Neb.: 0.1 MPa Scan: 200–700 *m/z* DAD: 520 nm	Muscadine grapes	Huang et al. 2009 [103]

packed particles. Monolithic columns have also other advantages over conventional particle-columns such as higher flow rates at lower backpressures. The use of a monolithic column offered the possibility to obtain separation of 24 anthocyanins in a complicated red cabbage sample in a very short time (18 min) [90]. Moreover, the fast equilibration of the monolithic column also resulted in higher sample-throughput.

Successful methods of microLC with narrow-bore columns have been published in the works of Dugo et al. [85], Palikova et al. [87] or Nicoletti et al. [86]. The latter described the separation of different phenolic compounds together with anthocyanins.

4.1.1. Detection

The separation procedures described above, specially the reversed-phase HPLC, have proven to be very useful, and HPLC coupled to diode array detection (DAD) has become the method of choice for monitoring anthocyanic profiles. With this technique, compound identification is based primarily on the UV–vis spectrum or retention time as compared with standard compounds. However, DAD detection is not sufficient to discriminate between compounds with similar spectroscopic characteristics and the lack of reference compounds for comparison makes mass spectrometry a supporting technique in anthocyanin characterization.

Since UV–vis detectors cannot differentiate co-eluted compounds, the structure information and compound identification capacity provided by these methods are very limited. Therefore, confirmatory analysis using more advanced instrumentation is needed. HPLC coupled with electrospray ionization mass spectrometer (MS), especially the tandem mass spectrometer (MS/MS) can provide mass spectra of intact molecular ion and fragment ions. Peaks can be identified categorically by matching their mass spectrum and retention time with reference compounds. The structural information from HPLC–ESI-MS methods (Table 5) allows to verify the results of anthocyanins profile obtained by HPLC–DAD systems.

Table 5 shows some of the HPLC–DAD–MS methods used for the detection of anthocyanins in different matrixes published in the literature since the year 2000.

Electrospray ionization in the positive mode is the unanimous choice of detection for the analysis of anthocyanins. ESI is known to be a soft ionization technique producing, under positive ion mode, protonated molecules. The compounds under analysis are present in acidic conditions as flavylium ions. Thus, the production of molecular cations M^+ in their ESI mass spectra should occur ideally with the highest yields. As acid modifiers in the mobile phase, formic acid and trifluoroacetic acid are used. To analyze anthocyanins, pH values of the mobile phase must range from 1 to 2. When formic acid is used as acid modifier, high proportions (5–10%) of this acid were used to reach this pH in conventional HPLC–DAD methods, but in HPLC–MS it may decrease the sensibility of detection. A reduction in proportion of the carboxylic acid is recommended, but in this case a narrow-bore column should be used to compensate the loss of resolution. On the other hand, those methods which use trifluoroacetic acid at a very low proportion (0.2–0.6%), limit the formation of ionic pairs that may decrease the detection sensibility by MS [91].

Table 6 summarizes the data obtained by HPLC–DAD–ITMS with electrospray ionization in positive ion mode and anthocyanin composition in the berries mixture extract shown in Fig. 5.

Tandem mass spectrometry (MS/MS) using quadrupole mass analyzers or ion-trap analyzers facilitates the conduction of vari-

Table 6
Profiling of anthocyanins found in a berries mixture extract analyzed by HPLC–DAD–ITMS, related to their retention times (RT), and fragmentation patterns (M^+, M^+-X).

Peak	RT (min)	Anthocyanin	M^+ (*m/z*)	M^+-X (*m/z*)	Transition (MS_2) (*m/z*)
1	10.3	Dp-3-galactoside	465	303 (M^+-galac)	465 → 303
2	10.7	Dp-3-glucoside	465	303 (M^+-glu)	465 → 303
3	11.3	Cy-3-galactoside	449	287 (M^+-galac)	449 → 287
4	11.5	Dp-3-arabinoside	435	303 (M^+-arab)	435 → 303
5	11.8	Cy-3-glucoside	449	287 (M^+-glu)	449 → 287
6	12.1	Pt-3-galactoside	479	317 (M^+-galac)	479 → 317
7	12.4	Cy-3-arabinoside	419	287 (M^+-arab)	419 → 287
8	12.4	Pt-3-glucoside	479	317 (M^+-glu)	479 → 317
9	12.8	Pg-3-glucoside	433	271 (M^+-glu)	433 → 271
10	13.0	Pt-3-arabinoside	449	317 (M^+-arab)	449 → 317
11	13.3	Pn-3-glucoside	463	301 (M^+-glu)	463 → 301
12	13.7	Mv-3-glucoside	493	331 (M^+-glu)	493 → 331
13	14.6	Mv-3-arabinoside	463	331 (M^+-arab)	463 → 331
14	15.2	Dp-3-(6-acetyl)-glucoside	507	303 (M^+-gluAc)	507 → 303
15	16.2	Cy-3-(6-acetyl)-glucoside	491	287 (M^+-gluAc)	491 → 287
16	16.7	Pt-3-(6-acetyl)-glucoside	521	317 (M^+-gluAc)	521 → 317
17	17.5	Pn-3-(6-acetyl)-glucoside	505	301 (M^+-gluAc)	505 → 301
18	17.8	Mv-3-(6-acetyl)-glucoside	535	331 (M^+-gluAc)	535 → 331
19	18.4	Cy-3-(6-coumaroyl)-glucoside	595	287 (M^+-gluCou)	595 → 287
20	18.7	Pt-3-(6-coumaroyl)-glucoside	625	317 (M^+-gluCou)	625 → 317
21	19.2	Pn-3-(6-coumaroyl)-glucoside	609	301 (M^+-gluCou)	609 → 301
22	19.3	Mv-3-(6-coumaroyl)-glucoside	639	331 (M^+-gluCou)	639 → 331

Dp: delphinidin; Cy: cyanidin; Pt: petunidin; Pg: pelargonidin; Pn: peonidin; and Mv: malvidin.

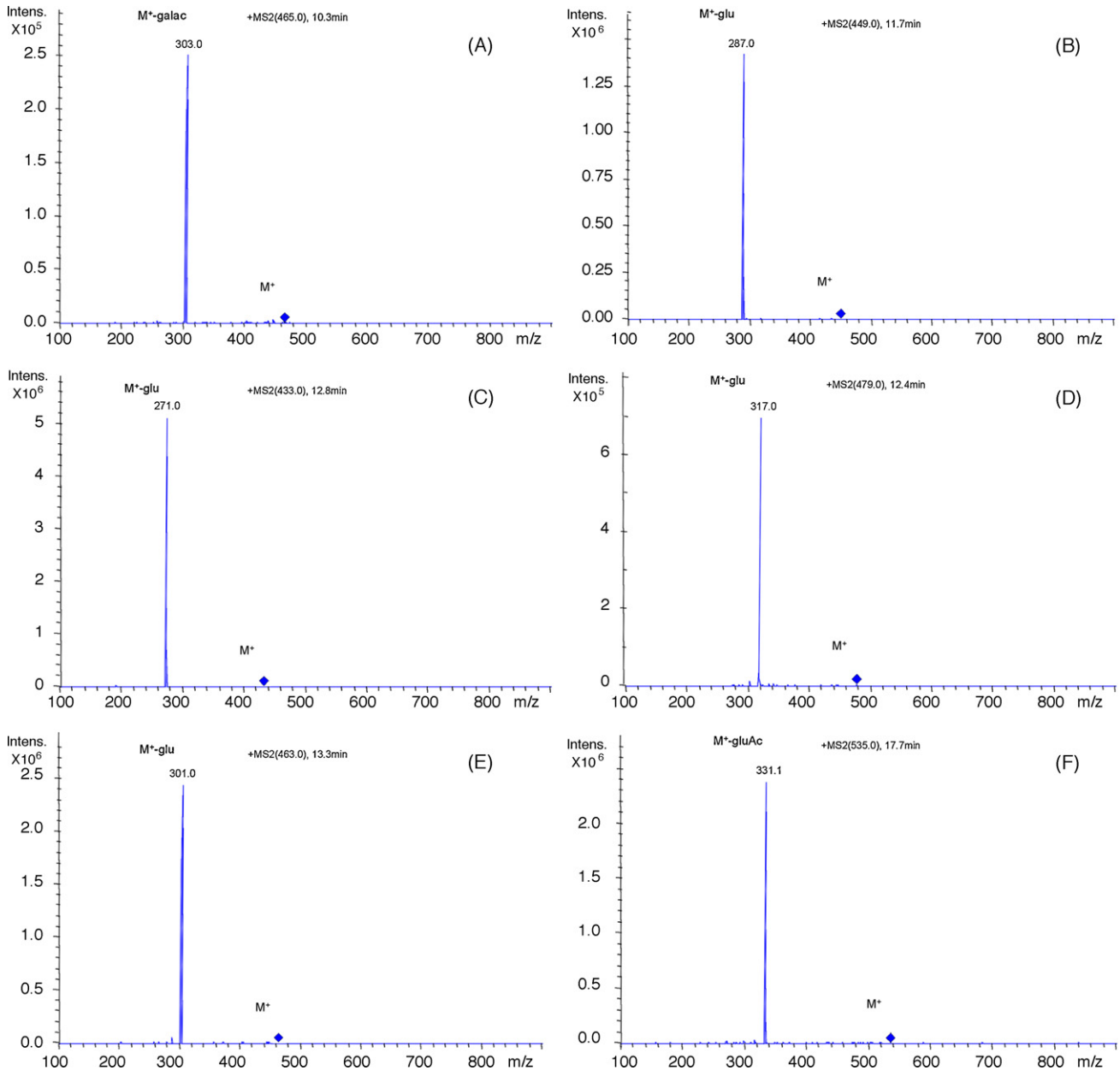


Fig. 6. MS/MS spectra of several anthocyanins obtained by ITMS. (A) Delphinidin-3-galactoside; (B) cyanidin-3-glucoside; (C) pelargonidin-3-glucoside; (D) petunidin-3-glucoside; (E) peonidin-3-glucoside; and (F) malvidin-3-(6-acetyl)-glucoside.

ous MS/MS experiments and significantly increases the sensitivity of detection [92]. Fig. 6 shows the MS/MS spectra of some anthocyanins obtained by ITMS. The mass spectra present the molecular ions M^+. The fragmentation patterns show the loss of a glucose, galactose, arabinose, acetylglucoside (gluAc) or coumaroylglucoside (gluCou) moiety, corresponding to the aglycon M^+-X ions. Hence, as it can be stated, for all cases the MS^2 spectra reflects the cleavage of the glycosidic bonds directly linked to the flavylium ring, thus giving the corresponding aglycone even in the case of malvidin–acetylglucoside. However, there can be exceptions to these general MS/MS spectra, since Wu and Prior [93] have observed that cleavages can also occur between the sugar and the other moieties, thus resulting in the detection of the anthocyanidin glucosides in the MS^2 spectra.

HPLC–MS analysis of anthocyanins in food has a wide range of applications. For example, the great sensitivity of LC–MS/MS allows the detection and quantitation of parent species and metabolites in biomatrices, which are hardly detected by HPLC–DAD methods. In a recent study, determination by specific MRM transitions has been used to detect and identify unchanged anthocyanins and their glucuronide metabolites in human plasma and urine [94].

Another example of application concerns the detection of new compounds. The information about molecular and fragment ions supplied by the MS^n analysis has provided a powerful tool to detect new anthocyanin-derived pigments that can play a crucial role in the colour of aged wines. A comprehensive study [95] has been published including more than 100 anthocyanin-derivated pigments, belonging to pyranoanthocyanins, direct anthocyanins–flavanols condensation products, and acetaldehyde-mediated condensation products. This spectrometric technique also yielded for the first time evidence confirm-

ing the existence of anthocyanin oligomers in the grape skin extract [96].

4.2. HPLC of flavan-3-ols and proanthocyanidins

4.2.1. Reversed-phase separations

The chromatographic separation of proanthocyanidins (PAs) is complicated because of the enormous variety of similar isomeric oligomers in plant or food sources. Reversed-phase HPLC by C18 columns has been the primary method of analysis for proanthocyanidins in food samples, and it allows the separation of small oligomers of the same degree of polymerization (DP), such as dimers and trimers. However, the elution order of these monomers and oligomers does not exactly correspond to their degree of polymerization [104]. A typical chromatogram showing separation for grape seed and green tea proanthocyanidins is presented in Figs. 7 and 8, respectively.

The common used columns for proanthocyanidins separations are analytical size columns (4.0–4.6 mm id). Recently, new columns have been proposed for this separation. The selection of a narrow-bore column (2.0 mm id), in combination with a microvolume detection cell, showed efficient separations of phenolic compounds in grapes, allowing the mobile phase low flow rates required for hyphenation with ESI interfaces [86].

Ultra-performance liquid chromatography with C18 columns has also been described. In this case, reduction in particle size and id. of the columns provides significant advances concerning selectivity, sensitivity and speed for the analysis of tea catechins [105]. Flow can be increased to 3 times because of smaller particles, and shortening of the column by one-third makes complete separations in 1/9 time while maintaining resolution. This kind of HPLC is very appropriated for the hyphenation with mass detectors required for the analysis of biological samples. The technique is suitable for fingerprinting analysis of commercial teas, considering its speed, robustness and high sample-throughput. [105].

4.2.2. Detection

Detectors commonly used for the detection of proanthocyanidins include DAD and fluorescence detectors, the latter providing the greatest sensitivity. But it is undoubtedly the hyphenation of HPLC with mass detectors like TOF, qTOF, QqQ, single quadrupole, or ion trap the most powerful technique nowadays, since it has provided a reliable tool to detect dimers up to decamers in food samples.

It is considered that the best response for proanthocyanidins is usually obtained in negative mode, despite the presence of acetic acid in the mobile phase. Acetic acid should improve the efficiency of the ionization in positive mode, whereas in the negative mode it may favor the formation of acetate/phenolate adducts, which sometimes makes the interpretation of the spectra more difficult [106]. Anyway, the negative mode ionization is still more effective, and however, most equipments can acquire both modes simultaneously.

Several recently reported studies of proanthocyanidin analysis using liquid chromatography with mass spectrometric detection are compiled in Table 7.

The main inconvenient in proanthocyanidins identification is that even exact mass peak ions reported by TOF detectors are exactly the same among isomers with the same degree of polymerization and galloylation, and fragmentations patterns obtained by MS/MS detectors do not differ either. In these cases, co-elution with pure standards is the usual method of choice for identifying proanthocyanidins. Table 8 shows ion peak and characteristic fragmentations of proanthocyanidins, while Fig. 9 shows the TOF mass spectra of some of them.

Besides the utilization for detecting and quantifying proanthocyanidins and catechins in food samples, liquid chromatography coupled with mass spectrometry (LC–MS/MS) has also emerged as the preferred technology for quantitative determination of flavanols and proanthocyanidins metabolites in different biomatrices, due to its sensitivity and selectivity through MS/MS experiments and the fact that it enables structural identification. Mata-Bilbao et al. [22] have detected green tea catechin metabolites (sul-

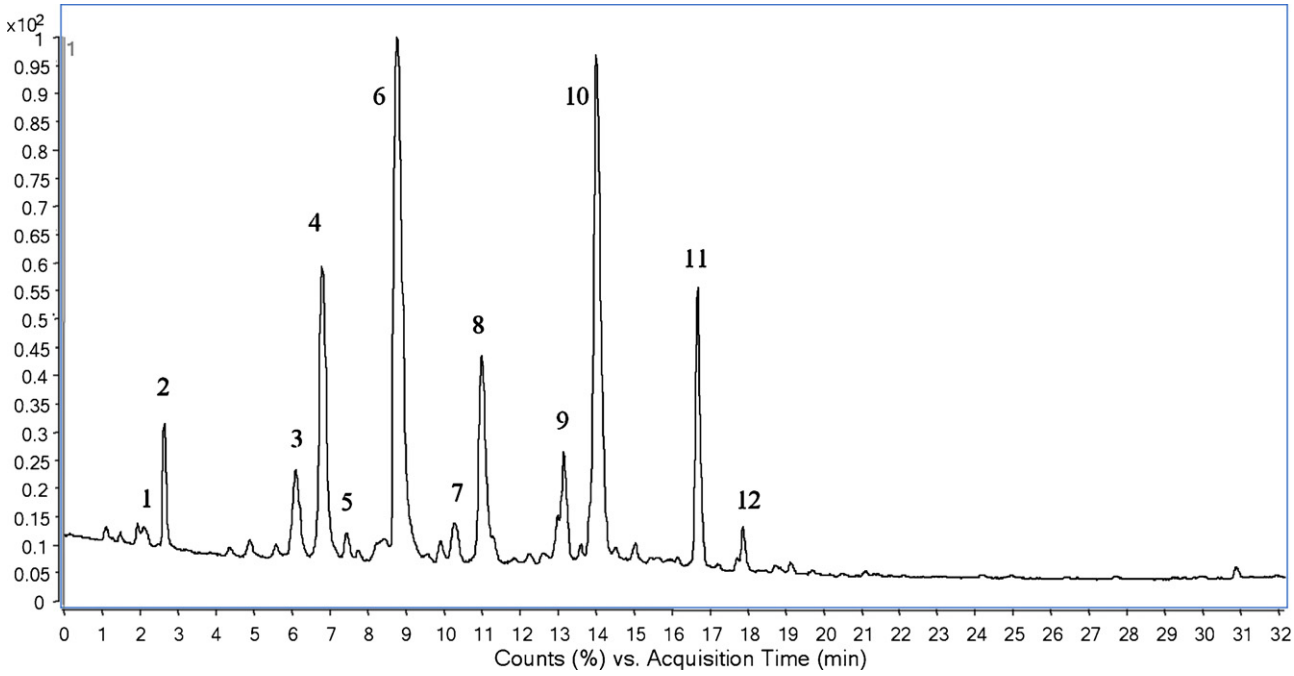


Fig. 7. Total Ion Chromatogram (TIC) of a grape seed extract obtained by a HPLC–TOF-MS method using a C18 150 × 2.1 mm, 3.5 μm column, with linear gradient of mobile phase (A) water, 0.1% formic acid and (B) MeOH, 0.1% formic acid. 1. Procyanidin T trimer, 2. gallic acid, 3. procyanidin B dimer, 4. procyanidin B dimer, 5. procyanidin T trimer, 6. catechin, 7. procyanidin B dimer, 8. procyanidin B dimer, 9. procyanidin dimer gallate, 10. epicatechin, 11. epicatechin gallate, and 12. procyanidin B dimer.

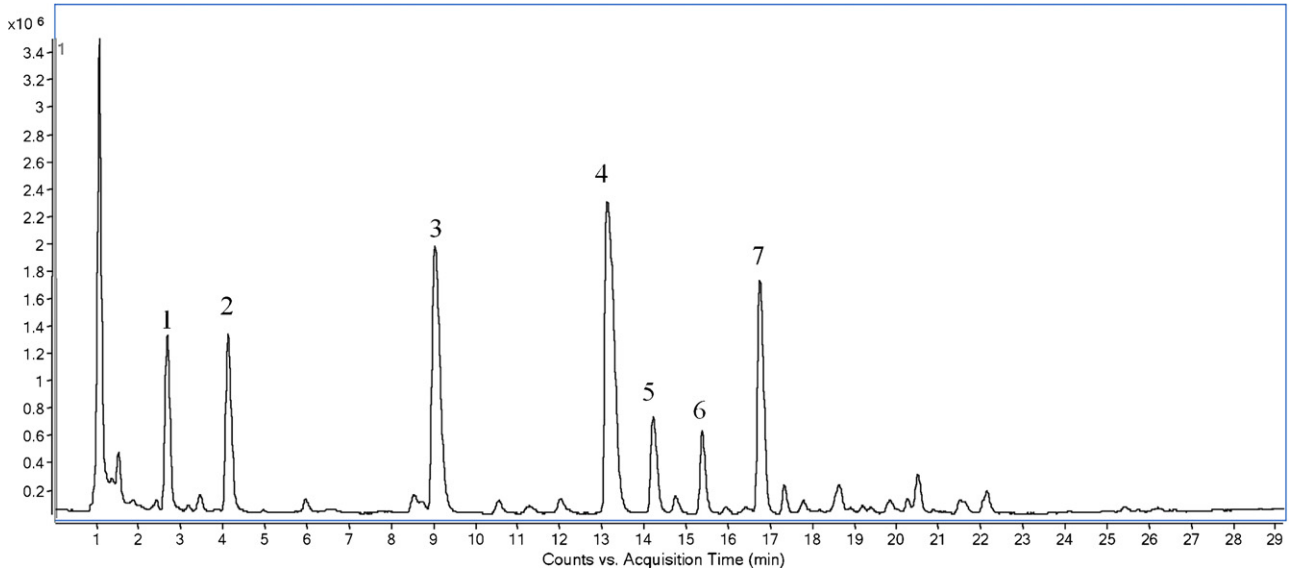


Fig. 8. Total Ion Chromatogram (TIC) of a green tea extract obtained by a HPLC–TOF-MS method using a C18 150 × 2.1 mm, 3.5 μm column, with linear gradient of mobile phase (A) water, 0.1% formic acid and (B) MeOH, 0.1% formic acid. 1. Galloyl quinic acid, 2. gallocatechin, 3. epigallocatechin, 4. epigallocatechin gallate, 5. epicatechin, 6. gallocatechin gallate, and 7. epicatechin gallate.

fates and glucuronides) in urine and plasma after oral intake of a green tea extract in dogs, and Roura et al. [107] have applied HPLC–MS to identify epicatechin metabolites in human plasma and urine. Serra et al. [25] have successfully determined grape seed procyanidins and their metabolites in rat plasma samples by UPLC–ESI-MS/MS.

4.2.3. Normal phase separations

Although reverse-phase HPLC correctly separates flavanol-3-monomers and dimers, and it is the most convenient method for their quantification in foods, severe limitations occur with higher oligomers (DP > 3). Since the number of isomers increases with increasing degrees of polymerization, the higher oligomers (DP > 3) co-elute in C18 columns as a large unresolved peak, that tends to overlap the separation profiles [104].

For more efficient separation of highly polymerized proanthocyanidins, several useful techniques have been established. They do not allow to isolate individual compounds, but at least they can separate proanthocyanidins with the same polymerization degree. Normal-phase HPLC using a bare silica column and elution with an organic mobile phase shows an efficient separation performance. Proanthocyanidins from apple [108], pine bark powder [108], lingonberry [108], grape [108] and cocoa [109] were clearly separated up to decamers with a gradient elution of dichloromethane–methanol solvent mixture (containing a small volume of acidic water), and polymeric proanthocyanidins with DP > 10 appeared as a single peak at the end of the chromatogram [110]. Gradient elutions of acetone–hexane have proven to elute up to pentamers, but the higher polymerized procyanidins remain retained in the silica phase. Cacao samples were the easiest to identify, since cacao contains procyanidins mainly consisting of epicatechin as the monomeric units. When A-type and B-type procyanidins coexist, or when the source contains galloylated oligomers, it is difficult to completely separate the higher oligomers into discrete peaks.

Although this normal phase silica procedures are efficient for separating proanthocyanidins, there are several inconveniences that complicate the routine use of these methods. One limitation is that the mobile phase contains chlorinated solvents, such as methylene chloride, raising concerns with respect to laboratory exposure, environmental protection, and disposal costs. Moreover, when scaled up for proanthocyanidins isolation, methylene chloride can be problematic if further in vitro, in vivo or clinical studies are targeted. Kelm et al. [19] have successfully avoided these solvents by using a diol stationary phase and a mobile phase that consisted of a gradient elution of acetonitrile–methanol slightly acidified. This column exhibited stronger retention characteristics than silica, which turned into larger separation times, but at the same time also allowed a better speciation for monomers epicatechin and catechin, usually unresolved in normal phase columns.

More recently, Yanagida et al. [111] have described a novel separation method for natural PAs by hydrophilic interaction chromatography (HILIC) using a silica-based stationary phase bonded with acrylamide. By this method, a mixture of proanthocyanidins from apple was separated according to their degree of polymerization up to decamers. The main advantage is that the elution was performed with an aqueous acetonitrile mobile phase, which would have been unacceptable for common normal phase columns.

Isolation of oligomer peaks by preparative or semipreparative HPLC and their depolymerization reactions in presence of nucleophiles like phloroglucinol can offer also information about the exact monomers present in terminal units and extension units. However, it cannot give a clue of the nature of the linkage between monomers, and the correct order of monomers when DP > 2 cannot be established. In those cases, hyphenation with NMR techniques can help to assign the correct structure.

4.3. HPLC of isoflavones

Due to their biomedical importance and wide applicability, numerous studies of isoflavones identification and analysis in foods have appeared in the last few years, together with some reviews (Table 9). Rostagno et al. [118] have published a comprehensive review summarizing the most recent advances in sample preparation and analysis methods of isoflavones from soybean and soy foods, while Vacek et al. [119] have written an accurate review on methods of separation and identification of isoflavones, including liquid chromatographic methods.

Table 7
Selection of LC–MS analysis of proanthocyanidins in different sources.

Columns	Solvents	Detection	Source/proanthocyanidins	Ref.
Symmetry C18 (250 × 4.6 mm), 5 μm	A: water/formic acid 99:1 B: acetonitrile/formic acid 99:1	HPLC–DAD–ESI(±)-MSD N_2: 13 l/m T^a: 350 °C Capillary: 4000 V Scan: 100–2000 *m/z* DAD: 350, 310, 270 nm 1 ml/min	*Green and fermented teas* 96 phenolic compounds, 18 flavanols, including gallates, gallocatechins and trimers	Lin et al., 2008 [112]
Acquity T3 column (100 × 2.1 mm), 1.8 μm	A: water/acetic acid 98:2 B: acetonitrile	UPLC–ESI(±)-TQD N_2: 800 l/h T^a: 400 °C Capillary: 4000 V Flow rate: 0.4 ml/min	*Different cocoa sources* SRM (selected Reaction monitoring) Monomers up to nonamers	Ortega et al., 2008 [113]
Acquity UPLC BEH C18 column (150 × 2.1 mm), 1.7 μm	A: water/formic acid 99:1 B: acetonitrile/formic acid 99:1	UPLC–ESI(±)-TOF N_2: 500 l/h T^a: 350 °C Capillary: 2000 V Flow rate: 0.3 ml/min	*Green tea* Metabolic fingerprinting approach, key compounds: EGC, ECGC, ECG	Ponsuwan et al., 2008 [105]
Acquity UPLC BEH C18 column (100 × 1 mm), 1.7 μm	A: water/formic acid 99.9:0.1 B: methanol	UPLC–ESI(−)-TQD N_2:600 l/h T^a: 210 °C Capillary: 3000 V Scan: 100–1900 *m/z* Flow rate: 0.2 ml/min	*Berries, chokecherries and seabuckthorn* Monomers, dimers, oligomers	Hosseinian et al., 2007 [114]
Supelcosil C18 column (250 × 4.6 mm), 5 μm	A: water/formic acid 99.7:0.3 B: acetonitrile	HPLC-APCI(±)-TQD N_2: 118 l/h T^a: 550 °C Scan: 100–1700 *m/z* Flow rate: 0.7 ml/min	*Grape seed and pine bark* B dimers, monogallate dimers, digallate dimers	Weber et al., 2007 [115]
Spherisorb S3 ODS-2 C8 (4.6 × 150 mm), 3 μm	A: water/acetic acid 97.5:2.5 B: acetonitrile:acetic acid 90:10	HPLC-APCI(±)-ITMS N_2:360 l/h T^a: 270 °C Capillary: 4500 V Scan: 150–2000 *m/z*	*Red wines procyanidins* Monomers, galloyled monomers, dimers and trimers	González-Manzano et al., 2006 [106]
LichroCart Purospher RP-18e column (125 × 3 mm), 5 μm	A: water/formic acid 99:1 B: acetonitrile/formic acid 99:1	HPLC–ESI(±)-ITMS Flow rate: 0.5 ml/min	*Lingonberry, cranberry, bilbery, bog whortleberry* B-series dimers and trimers A-series dimers and trimers	Maatta-Riihinen et al., 2005 [116]
Lichrospher 100 RP-18 (250 × 4 mm), 5 μm	A: water/acetic acid 99:1 B: water/acetic acid 90:10	HPLC–ESI-MS N_2: 6 l/h T^a: 340 °C Capillary: 4500 V Scan: 200–3000 *m/z* Flow rate: 1 ml/min	*Lentils* Dimers, trimers, tetramers	Dueñas et al., 2003 [117]

The most used analysis technique for the quantification of isoflavones in solid samples is, without doubt, reverse-phase HPLC using C18 based columns with water and methanol or acetonitrile containing small amounts of acid as the mobile phase [118].

Table 8
Fragmentation of some proanthocyanidins identified in natural sources. Mass spectra obtained by ITMS.

Proanthocyanidin	M^- (*m/z*)	Fragments (*m/z*)
Catechin	289	245, 205
Epicatechin	289	245, 205
Gallocatechin	305	287, 261, 219, 179, 125
Epigallocatechin	305	287, 261, 219, 179, 125
Catechin gallate	441	331, 289, 245, 193, 169
Epicatechin gallate	441	331, 289, 245, 193, 169
Gallocatechin gallate	457	331, 305, 287, 193, 169
Epigallocatechin gallate	457	331, 305, 287, 193, 169
Procyanidin B dimer	577	559, 451, 425, 407, 289
Procyanidin T trimer	865	847, 739, 695, 577, 451, 425, 407, 289
Procyanidin dimer gallate	729	711, 603, 577, 559, 441, 407, 289
Procyanidin trimer gallate	1017	891, 847, 729, 695, 603, 559, 451, 407, 289

Separation of isoflavones on reversed-phase sorbents is based on hydrophobic interactions of individual isoflavones with the stationary phase, and retention times of separated substances depend primarily on their solubility in water. In most cases aglycones, glucosides and derivatives are separated in a single run on a reversed-phase column, from the less hydrophobic to the most hydrophobic. The expected order of elution on a C18 stationary phase would be as follows: Glucosides of isoflavones are eluted in the order puerarin < daidzin < glycitin < genistin. Corresponding aglycones would follow, in the same order: daidzein < glycitein < genistein. Aglycones as formononetin (4-methylated form of daidzein) and biochanin A (4-methylated from of genistein) would be the most retained [120–122]. In fact, aglycones are eluted from the stationary phase only at high contents of organic modifier. For example, biochanin A glucosides needed 85–100% of methanol in the mobile phase to be eluted. Derivatives like 2′-methoxy-flavone and 6-methoxyflavone, which some authors have considered suitable internal standards, are still more retained [121]. By substituting octadecyl C18 stationary phases by more polar phenyl- or cyanopropyl-groups, aglycones

can elute at lower methanol contents [123]. Other isoflavone forms (acetyl- or malonylglucosides) also follow the same pattern of elution, and they elute between the glucosides and the aglycones.

To summarize, the hydrophobicity of individual isoflavones increases in the order β-glucosides < malonylglucosides < acetylglucosides < free aglycones. As expected, when separation is performed in hydrophilic interaction chromatography, elution orders of soybean isoflavones are exactly the opposite [124].

Because of the occurrence of different isoflavone forms, a common practice for their analysis is a previous acidic hydrolysis to give the aglycones (although puerarin is not hydrolyzed because the glucose is bonded with a C–C bond to the isoflavone ring [121]). The hydrolysis produces very simple chromatograms where only the aglycones are present, which can be resolved in fast times under 2 min [79].

In recent years the number of assays developed for isoflavones using LC–MS has increased. Both ESI and APCI have been used to ionize analytes in both positive and negative ion modes and various types of mass spectrometers have been employed, including ion trap, single quadrupole, triple quadrupole and time-of-flight instruments. By comparing ionization sources, Rybak et al. [122] found that electrospray ionization did improve measurement precision, sensitivity and limit of detection over atmospheric-pressure chemical ionization in certain cases, specially for equol (LOD_{ESI} = 0.3 ng/ml; LOD_{APCI} = 2.7 ng/ml), whose detection frequency has been a challenge on the past.

The described LC–QqQ-MS/MS methodologies operating in the selected reaction monitoring (SRM) allow the analysis of sub-ppb concentrations of isoflavones in matrices like urine and serum with a very high degree of reproducibility [125,126]. In fact, HPLC–MS/MS with triple quadrupole even showed better sensitivity than estradiol enzyme-linked immunoabsorbent assay (ELISA)

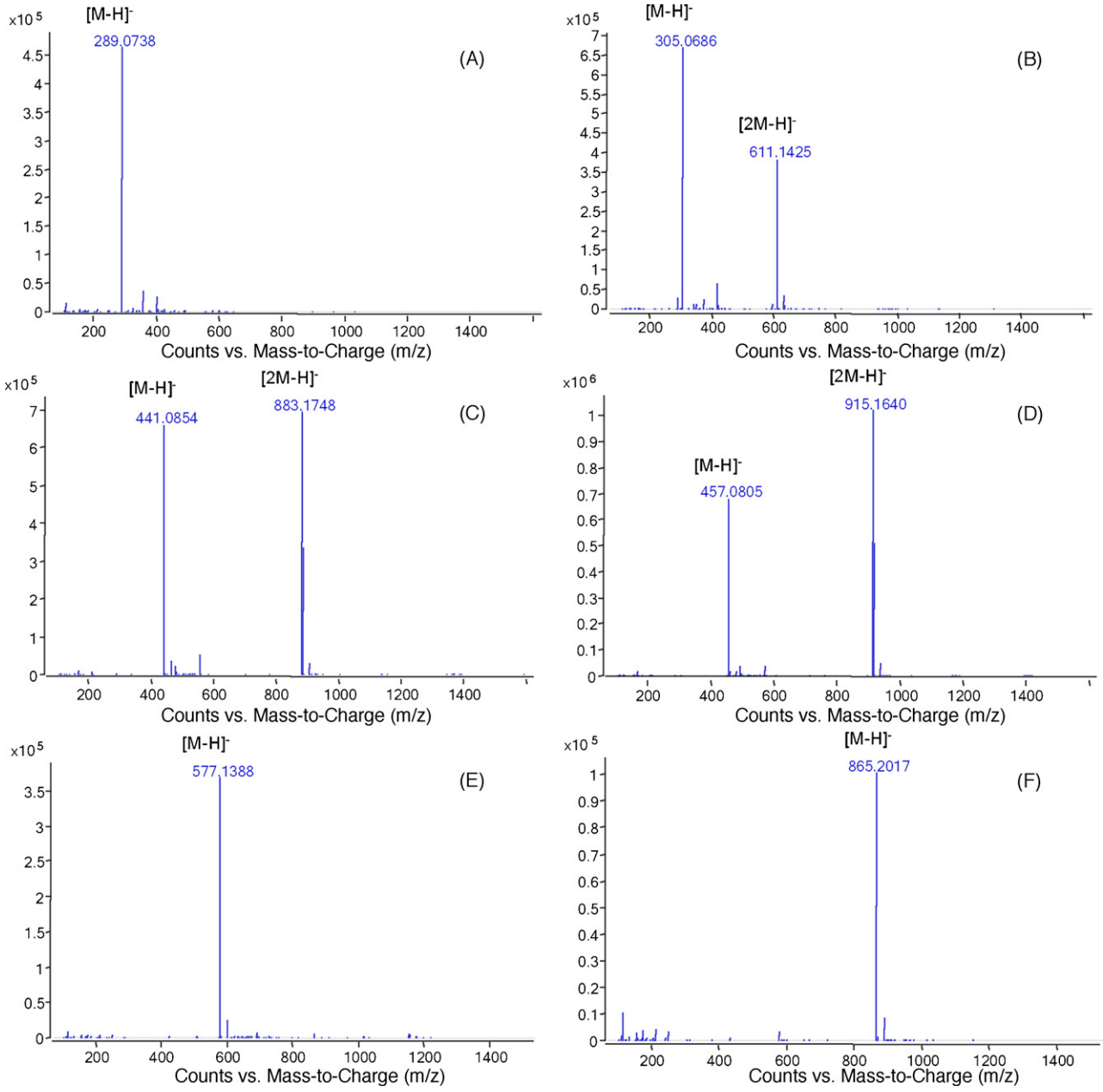


Fig. 9. Mass spectra of proanthocyanidins obtained by TOF-MS. (A) catechin/epicatechin; (B) gallocatechin/epigallocatechin; (C) catechin gallate/epicatechin gallate; (D) gallocatechin gallate/epigallocatechin gallate; (E) procyanidin B dimer; and (F) procyanidin T trimer.

Table 9
Selection of HPLC methods to separate isoflavones.

Columns	Solvents	Detection	Source/isoflavones (time)	Ref.
50 × 3 mm, 5 μm C18 column	10 mM ammonium acetate buffer, pH 6.5/methanol:acetonitrile	HPLC–MS/MS (QqQ) Negative mode	Urine phytoestrogens Daidzein, equol, genistein (6 min)	Rybak et al., 2008 [122]
50 × 2.1 mm, 1.7 μm C18, CNP, Phenyl columns	Aqueous acetic acid (0.3%)/methanol	UPLC-PDAD	10 isoflavones (1.9 min)	Kledjus et al., 2008 [123]
150 × 2.1 mm, 3 μm C18 column	Ammonium acetate 0.1%, pH 4.8/methanol	QqQ, SRM mode QTrap Negative	Urine and serum phytoestrogens Daidzein, equol, genistein, glycitein (8 min)	Grace et al., 2007 [125]
250 × 2.1 mm, 5 μm ODS-3 column	Acetonitrile/water	ESI-QqQ, SRM mode Positive ionization	*Astragalus mongholicus* in rabbit plasma 3 isoflavonoid glycosides (15 min)	Zhang et al., 2007 [126]
150 × 4.6 mm, 5 μm C18 column	Ammonium formate 50 mM, pH 4/acetonitrile	LC/APCI/Q Negative ionization	Soy acetylglucosides and glucosides in human urine (40 min)	Chen et al., 2007 [131]
(a) 125 × 2 mm, 5 μm RP-18 e columns (b) 50 × 2.1 mm, 1.7 μm C18 column	Water/acetonitrile 400 μl/min	(a) HPLC–QqQ (b) UPLC–qTOF-MS UPLC–qTOF-MS/MS	Water samples Daidzein, genistein, biochanin A (16 min)	Farré et al., 2007 [127]
25 mm × 2.0 mm, 5 μm C18 column	Methanol–aqueous formic acid 0.01% Isocratic 0.5 ml/min	ESI-QqQ	Isoflavonoid aglycones in soybeans Daidzein, genistein, formononetin Biochanin A (2 min)	Careri et al., 2007 [134]
150 × 3.9 mm, 4 μm C18 column	Water–acetonitrile, acidified with acetic acid (0.1%) 1 ml/min	HPLC-PDA (260 nm)	Soy, red clover and kudzu isoflavones 19 isoflavones (90 min)	Delmonte et al., 2006 [121]
150 × 4.6 mm, 5 μm C18 column	Water:acetonitrile	HPLC–ion trap Negative mode APCI	Nutritional supplements isoflavones 12 isoflavones (aglycones, glucosides, acetylglucosides) (50 min)	Chen et al., 2005 [120]
150 × 2.1 mm, 3.5 μm dC18 column	Acetate buffer 0.15 M pH 5.5:acetonitrile, 0.4 ml/min	HPLC-ED HPLC–ESI-Quad Positive mode	Soybean foods and human urine 6 isoflavones (28 min)	Klejdus et al., 2004 [128]
150 × 3.2 mm, 5 μm ODS-3 column	Formic acid (0.1%) in water/acetonitrile 1 ml/min	HPLC–ESI-MSD trap Positive mode	Red clover isoflavones (25 isoflavones) (40 min)	Wu et al., 2003 [133]
150 mm × 2 mm, 3 μm C18	0.2% Acetic acid:acetonitrile, 0.3ml/min	ESI-quadrupole Positive mode	Red clover isoflavones (49 isoflavones) (60 min)	Kjeldus et al., 2001 [132]

for the analysis of estrogens in water samples [127]. However, optimized electrochemical detection can still offer a more sensitive detection [128].

Application of columns of smaller inner diameter and with a smaller stationary phase particle size are very suitable for separation of isoflavones. They allow reduction of retention times to less than 60 s for complete separation of 10 isoflavones. Methods based on UPLC–qTOF showed some complementary advantages to the HPLC–MS/MS methods, such as shorter analysis times and improved selectivity. Exceptionally, Farre et al. showed better detection limits for daidzein, genistein and biochanin A in water samples by using UPLC–qTOF (8, 5 and 30 ng/l, respectively) than by using HPLC–MS/MS (QqQ), where these isoflavones could not be detected [127]. But this is not the usual response, since a loss in sensitivity up to one order of magnitude can be expected in comparison with QqQ analysis. However, this can be compensated by using more concentrated samples, since qTOF is less influenced by ion suppression than QqQ. On-line in-tube solid-phase microextraction with poly(divinylbenzene) resins coupled to HPLC is an easy way to preconcentrate the analytes, and has the advantage that it reduces the usual sample preparation required by other methods [129,130].

Table 10
Fragmentation of some isoflavones identified in natural sources.

Isoflavones	M^- (m/z)	Fragments MS^2 (m/z)
Daidzein	253.2	224.3
Genistein	269.2	225.2
Glycitein	283.2	268.2
Daidzin	415.0	253.4, 252.3
Genistin	431.2	268.3, 269.3
Glycitin	445.4	283.2

Table 10 shows major precursor and fragmentation ions of selected isoflavonoids in negative mode. For a more comprehensive list, MS^3 fragments or more details on fragmentation pathways, see Refs. [120,128,131–133].

5. Capillary electrophoresis

5.1. Introduction

HPLC is the most popular and widely used technique for the analysis of dietary polyphenols. But in recent years capillary elec-

trophoresis (CE) is becoming an attractive alternative, mainly due to its high separation efficiency, small sample and solvents consumption, and speed, as the separation time can be reduced to only several minutes. In addition, while HPLC columns have short lifetime because of numerous co-existing interferences and column contaminations, capillaries used in CE are much easier to wash [135]. Moreover, CE is relatively well suited for the analysis of samples with complex matrices, as it allows in-capillary concentration through electrokinetic stacking [136]. On-line sample preconcentration in capillary electrophoresis has been recently reviewed by Simpson et al. [137], including reported methods for phenolic acids and flavonoids. The drawbacks of CE are generally lower sensitivity and worse reproducibility compared to HPLC. Relative properties of CE and HPLC are often discussed and compared [138]. For example, in 2000 da Costa et al. [139] reviewed and compared the analysis of anthocyanins in foods by different techniques, including CE. Comparative analysis of tea catechins by HPLC and CE has also been widely discussed [140–142].

The term "capillary electromigration methods" refers collectively to several separation techniques having different operational characteristics and separation principles. These modes of capillary electrophoresis include capillary zone electrophoresis, micellar electrokinetic chromatography (MEKC), capillary electrochromatography (CEC), capillary gel electrophoresis (CGE), capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). Although these modes differ in their fundamentals and in the background electrolyte used, all of them have in common that the separation is based on differences in electromigration between analytes in a given electric field.

Some recent reviews have been published concerning the application of electromigration methods to the separation and analysis of phenolic compounds. Sadecka and Polonsky reviewed in 2000 the application of electrophoretic methods in the analysis of beverages, including phenolic compounds in beers, hard drinks, juices and wines [143]. In 2005 Herrero et al. reviewed the analysis of natural antioxidants by electromigration methods [144]. The most recent reviews we have found concerning these methodologies come from 2006 by Jac et al. [145], that described recent trends in polyphenols electrophoretic analysis, and by Cifuentes [146], who offered a detailed reviewed of the application of capillary electromigration methods for food analysis, including the analysis of natural antioxidants like polyphenols. For our current review, we have picked up where these authors left and have added some new references.

Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography are the two "classical" modes of electromigration methods chosen for polyphenols separation.

CZE separation is based on different migration of charged solutes (caused by the differences in their charge to mass ratio) in a conductive liquid placed in a capillary under the influence of a high-voltage electric field. The movement of solutes in a silica capillary is also affected by the electroosmotic flow (EOF) that originates thanks to negatively charged silanoate groups of the capillary walls. Cations of the electrolyte are attracted by the negatively charged wall to form a fixed layer; other cations form a mobile layer which migrates toward the cathode while the bulk of the buffer solution co-migrates with it and gives rise to the EOF. Since the EOF is greater than the electrophoretic mobility of the negatively charged solutes, both negatively and positively charged solutes can be analyzed within one run.

Micellar electrokinetic chromatography is another electromigration technique widely used. MEKC is capable of separating molecules with similar electrophoretic mobility, such as neutral analytes, as well as charged solutes. MEKC is based in the incorporation of ionic surfactant micelles to the separation solution. Conventional micelles are formed in the running buffer by adding the surfactants at a higher concentration than their critical micellar concentration (CMC) [147]. The micelles formed serve as a "pseudostationary phase" and the analytes undergo partitioning between the micelles (hydrophobic phase) and the buffer (hydrophilic phase). Here the mechanism of separation is also based on the differences in the lipophilicity of analytes.

Microemulsion electrokinetic chromatography (MEEKC) is similar to MEKC. The main difference between both techniques is that, while in MEKC the pseudostationary phase is the micelle, in MEEKC, surfactant-coated oil droplets in a microemulsion serve as the pseudostationary phase. The oils in water microemulsions are similar to micelles for their ability of solubilizing hydrophobic compounds, but display a much larger capacity due to their larger droplet size. The separation in MEEKC for neutral compounds is based on the analyte partioning between the moving charged oil droplets and the aqueous buffer phase. The microemulsion droplets are generally obtained by mixing an oil such as n-heptane with water and by adding a surfactant such as sodium dodecyl sulphate (SDS) to reduce the surface tension between the immiscible liquids. A cosurfactant (e.g., 1-butanol) is also added to further stabilize the microemulsion as part of the interfacial film with the primary surfactant. Under alkaline conditions, the negatively charged oil droplets attempt to oppose the EOF. Hydrophobic analytes favor partitioning in the oil phase, and thus will migrate later than the water-soluble analytes. When an acidic medium buffer is used, the EOF is greatly suppressed, and using reverse polarity hydrophobic analytes in the oil droplets will exhibit lower migration times than the hydrophilic ones [148].

5.1.1. Isotachophoresis and on-line combination of electrophoretic techniques

On-line combination of electrophoretic techniques can also be achieved. Isotachophoresis has been successfully applied as a pre-separation stage before CE. In isotachophoresis (ITP) a zone containing a mixture of analytes (cations or anions) is introduced between two different buffers. When an electric field is applied to the capillary, the analytes are stacked into zones according to their mobilities, and in equilibrium state these distinct zones migrate at the same velocity. The analyte zones closely follow one another (with sharp boundaries, no gap between them). In one run either cations or anions can be separated but not both. In comparison with CZE and MEKC the zone dispersion in ITP is significantly decreased. The on-line combination of ITP–CZE significantly increases the separation capability and sensitivity of capillary electrophoresis. This technique has been used for separation and quantification of flavonoids (kaempferol, quercetin, myricetin, quercitin, rutin, epicatechin, catechin) and phenolic acids (ie gallic acid, p-coumaric acid, caffeic acid) in red wines [149].

Capillary electrochromatography is a hybrid technique of capillary zone electrophoresis and microHPLC. It employs a capillary column containing the stationary phase, which has fixed charges at the interface, and electroosmotic flow of the mobile phase generated by high electric fields. The separation of ionized sample components is determined by differences in both their retention on the stationary phase and their electrophoretic mobility [150]. However, columns fragility and long conditioning times are recurrent problems that seriously overshadow its potential. Hence, few electrochromatographic separations of polyphenols have been reported until now. To give a recent example, Fonseca et al. [151] developed a fast (7.5 min) methodology to determine 11 bioactive phenolic compounds (coumarins, phenolic acids, flavones and flavonols) in chamomile extracts in a Hypersil SCX/C18 column with pH 2.8 phosphate buffer at 50 mM containing 50% acetonitrile.

5.2. Optimization of variables influencing electrophoretic separation of polyphenols

Electromigration techniques being still relatively new, it is normal that most studies undergo optimization procedures in order to establish reliable efficient methods. Univariate design is the most common and simplest approach for achieving the best resolution and analysis time. Strategies for the optimization of CE for the analysis of phytochemical bioactive compounds have recently been reviewed by Li et al. [152].

Electrophoretic separation is influenced by experimental variables such as voltage, temperature, injection time and mode, capillary characteristics and buffer composition (including pH, addition of organic solvents and modifiers) [153]. In MEKC, factors like the micelle concentration, pH (especially if the mixture contains charged and not charged solutes), modifier concentrations or the addition of an organic solvent, can modify the distribution constants of the micellar phase and therefore can improve the analysis resolution.

The acidity and concentration of the running buffer play an important role in CE. Borate buffers with pH 8–11 and a concentration of 25–200 mM are commonly used, as borate can form complexes with o-dihydroxyl groups on the flavonoid nucleus and with vicinal cis-hydroxyl groups of sugars and therefore ease the separation [154]. Buffer concentration influences the viscosity of the solution, which affects not only the resolution and migration time of analytes, but also the peak current. The migration time and the resolution normally increase with increasing buffer concentrations, unless they become too high, because if the ionic strength exceeds a certain value, increased sample dispersion occurs due to excessive Joule heating. It can also cause a negative effect on the detection limits when electrochemical detection is used [135].

The buffer pH is one of the most important parameters for CE separation. Acidity affects the overall charge of polyphenols, which affects their migration time and separation. On the other hand, the migration time is also dependent on EOF, which increases with pH [152].

The separation voltage affects the electric field strength, which in turn affects the EOF and the migration velocity of charged particles. Increasing the voltage gives shorter migration times but can also produce more Joule heat and increase the background noise. Decreasing the voltage can improve resolution, but it can also increase the analytical time considerably, which leads to peak broadening. Experimentally, the optimal voltage is determined by performing runs at increasing voltages until deterioration in resolution is noticed [152].

Temperature influences the buffer viscosity, which is related to both the electrophoretic mobility and the EOF. As the temperature increases, the viscosity decreases and thus the electrophoretic mobility increases as well, leading to a decrease on migration times of analytes [152].

Organic solvents can be employed in electrokinetic separations to improve the selectivity and resolution of CE by improving both the separation and the peak shape, increasing the solubility, changing the migration time, and/or decreasing the adsorption and diffusion of analytes [152,155]. The separation of standard polyphenols mixtures both in aqueous buffer and in non-aqueous solutions has been often investigated, and changes in the separation selectivity and the mobility of ions is clearly influenciated by the amount of the organic solvent.

Surfactants are also used for controlling EOF and electrophoretic migration in MECK. The most common employed surfactants are anionic, especially SDS, but cationic (CTAB), neutral (Triton X-100) or zwitterionic surfactants have also been used. For example, Tween 20 (polyoxyethylene sorbitan monolaurate) is a non-ionic surfactant that improves the separation of caffeic and chlorogenic acids in Chinese herbal samples [156].

Ionic liquids (IL) are a broad class of low-melting semi-organic salts or salt mixtures that have appreciable liquid range. Interest in IL for their potential uses in different chemical processes is increasing, because they are environmentally benign and they act as good solvents for both organic and inorganic materials [152].

5.3. Addition of cyclodextrins and chiral capillary electrophoresis

A recent field of interest has been the inclusion of chiral selectors in buffers, mainly cyclodextrins, as media modifiers to achieve the separation of isomeric compounds [157]. This is based on the so-called "host–guest" principle, which represents a simple model of weak and non-covalent interaction. The host molecule cyclodextrin (CD) is well known to form inclusion complexes with guest molecules (the analytes) that possess suitable polarities and dimensions. This possible stereoselective inclusion-complexation of the chiral analytes into CD enables their successful use for analytical purposes, such as the chiral separation in liquid chromatography and capillary electrophoresis [158]. In MEKC, the CDs then play also a role as "secondary pseudostationary phase" in competition with micelles.

As chiral selectors having universal properties do not exist, the optimization of chiral selectors used for enantioseparations is an important field of research. The optimized chiral modifier depends largely on the analytes to be separated, so it is advisable to do some research before making the final choice. So far, native cyclodextrins (α-, β-, γ-cyclodextrin), neutral cyclodextrin derivatives (dimethyl-β-cyclodextrin, hydroxypropyl-β-cyclodextrin), or charged cyclodextrin derivatives (carboxymethyl-β-cyclodextrin, carboxyethyl-β-cyclodextrin) have been used as buffer additives. Sulfated cyclodextrins like sulphated-substituted β-cyclodextrin or β-cyclodextrin sulfobutyl ether have also been employed. Beyond cyclodextrins, the use of neutral cyclosophoraoses and highly sulfated cyclosophoraoses has been as well described. Bile salts like sodium cholate, sodium taurocholate and sodium deoxycholate, are also chiral surfactants used in MEKC.

Several studies have analyzed the resolution improvement for flavanones mixtures by the addition of chiral modifiers [159–162]. Normally, cyclodextrins are the chiral modifiers used, but bile salts [162] and novel microbial cyclic β-(1 → 3),(1 → 6)-glucans [159] have also been employed. The use of chiral modifiers has been applied for separating flavanones, flavanone glucosides and hydroxyflavanones.

Chiral modifiers have also been employed to develop new methods of separation for complex matrices. For example, t-resveratrol, astilbin, taxifolin and some phenolic acids were separated in *Smilacis glabrae* rhizomes thanks to the addition of β-CD [163]. β-Cyclodextrin also improves separation of flavones (apigenin, luteolin), flavonols (kaempferol, quercetin) and flavanols (catechin, epicatechin) in *Chrysanthemum* [164], and it also improves separation of flavonols (rutin, quercetin) and phenolic acids in *Prunella vulgaris* [165]. The analysis of red wine polyphenols (phenolic acids, catechins, flavanones) can also be simplified by the presence of different cyclodextrins [149]. In another example, HP-β-CD allows the enantioselective separation of (+)-catechin and (−)-catechin in *Theobroma cacao* [166].

5.4. Hyphenation of CE with mass detectors

UV and DAD have been the most widely applied detectors in capillary electrophoresis for the analysis of polyphenols. Two main limitations concern these detections. On the one hand, the sensitivity obtained with UV-absorption detection is still, in many cases, unsatisfactory, although electrochemical detection, the use of

high-sensitivity cells [167], and preconcentration by large-volume sample stacking [168] or anion-selective exhausting injection-sweeping [169] can improve the sensitivity for the detection of polyphenols. On the other hand, the lack of a powerful identification tool is a major disadvantage. Both limitations can be overcome by the hyphenation with mass detectors.

In general, if a separation technique is coupled with MS the interpretation of the analytical results can be more straightforward. Additionally, MS/MS detection provides high sensitivity. So, the hyphenation of CE as the analytical separation technique coupled to mass spectrometry as the detection system can provide important advantages in food analysis thanks to the combination of the high separation capabilities of CE and the power of MS for identification and confirmation.

The weakest point in coupling on-line CE to MS has been the interface between both. First of all, voltages applied for CE separations are not suitable for mass detectors. Buffer solutions for CE usually include borate, phosphate or SDS, that are not suitable for ionization in the mass detector. And appropriate buffers for MS like acetic acid, ammonium carbonate or ammonium acetate, may not allow satisfactory separations in electromigration methods.

An alternative strategy could be the off-line fraction collection in capillary electrophoresis, as proposed in recent works by Helmja et al. [170], where the cathode end of the capillary is placed inside a stainless steel needle using a coaxial liquid-seath-flow configuration. The fractions can then be analyzed by multiple techniques like MALDI-TOF, HPLC–ESI-Q-TOF and ICP-MS. The main inconvenient for this approach is that CE sample injection volumes and solvent consumption are very low, and then concentrations of separated analytes are also very low and volumes can be in the nanoliters range. Anyway, by combining CE-fraction collection with ESI-Q-TOF, an extract of *Sophora japonica* gave 20 fractions in 22.3 min, and mass analysis identified several flavonoids (genistein, kaempferol, cyanidin, rutin or quercetin derivatives).

On-line coupling between CE and mass spectrometers is also possible. For the connection between the CE system and the electrospray ion source of the mass spectrometer, the outlet of the separation is fitted into the electrospray needle of the ion source and a flow of conductive sheath liquid establishes electrical contact between the capillary effluent and water for the electrospray needle. Volatile buffers are used for the electrophoretic separation, mainly with a high percentage of an organic solvent.

5.5. Electrophoretic separations of anthocyanins

Selected representative methods of anthocyanins separation by electrophoretic techniques can be found in Table 11.

Although most flavonoids analysis by CE is generally achieved with basic buffers, when it concerns to anthocyanins, both acidic and basic buffers can be used.

For some authors, acidic buffers are preferred, as they stabilize the highly absorbent flavylium cation, which improves the sensitivity of the detection method by UV–vis at 520–560 nm. da Costa et al. [171] observed broadening of peaks with increasing pH between ranges of 1.5–2.8 used in their studies, that were attributed to flavylium cation equilibria. So, the more acidic the buffer, the better resolution. But pH lower than 1.5 provide an excessive current (over 100 μA). The addition of an organic modifier (methanol or acetonitrile) greatly improved the separation of blackcurrant anthocyanidins and strawberry anthocyanins [171].

Surfactants also have influence on separation. Bicard et al. [172] suggested that in acidic buffers the flavylium cation could be retained on the silanol group of the coated capillary, and so the use of a flow modifier like cetyltrimethylammoniumbromide (CTAB), a cationic surfactant, under its critical micellar concentration, was recommended for the migration of the anthocyanins to the detector. Experiences with a short length quaternary ammonium salt or with an alkyl long chain sulphate (SDS) did not produce a quantifiable peak [172]. However, this does not mean that MEKC cannot be applied for the separation of anthocyanins. On the contrary, the electrolyte system without the micellar agent SDS did not provide a good separation of a model mixture of anthocyanins with different sugar moieties [173]. The accurate migration time determination of MECK mobilities has allowed Muller et al. [174] to evaluate the estimation partition coefficients of seven anthocyanins by an iterative method, which is related to their hydrophobicity value, an important parameter for investigation of the distribution of pharmaceuticals and bioactive compounds in the human body.

As well as surfactants, the use of cyclodextrins in acidic buffers has proven beneficial in sharpening the cyanidin and peonidin peaks [175], as well as preserving the compounds. Because β-cyclodextrin forms hydrogen bounds between its inner hydroxyl groups and the anthocyanin, the more β-cyclodextrin added to the buffer, the longer the run time. However, applicability of chiral electrophoresis to anthocyanins is still relatively unexplored.

While acidic buffers provided a lower limit of detection, basic borate buffers can increase selectivity. That is attributed to the interaction of vicinal hydroxyls with borate, which influences the migration times. The anthocyanins migrate as anions, and therefore anthocyanins with a lower molecular mass have a higher charge/size and show longer migration times. But orthohydroxylated anthocyanins cyanidin, delphinidin and petunidin, can form complexes with borate, and therefore will show the longest migration time [176]. On the other hand, anthocyanins are primarily separated by the type of conjugated sugar. That is, glucosides move faster than galactosides, which are followed by arabinosides [177].

This behavior of anthocyanins in basic buffers is very interesting for the analysis of wines. Saenz-Lopez et al. [176] found that when working at pH 8.4, the basic medium allowed a faster separation than the acidic medium. The lower sensitivity for this basic medium could be improved by adding SO_2 to the samples, as an increase on the absorbance at 599 nm could be obtained. Using these methodologies, they found good correlations between the CZE methodology and the standard HPLC method [176], but the electrophoretic method was much shorter (only 13 min, for 50 min by HPLC). Since the CZE method used positive polarity and positive electroosmotic flow, anthocyanins (negatively charged at basic pH) with higher charge/size ratios displayed longer migration times, except for the ones that could interact with borate buffer (cyanidin, delphinidin and petunidin).

When applied to the analysis of red wines of different ages, the electropherogram showed not only peaks for anthocyanidin monomers, but also for dimers of malvidin-3-glucoside with catechin, and pyruvic acid derivatives characteristic for aged red wines [178]. So, CZE is a suitable technique to study these hydroxycinnamic acids and malvidin-3-glucoside derivatives. Moreover, the appearance of a group of seven peaks that increased with wine age suggested the detection of polymeric pigments, consisting of an anthocyanin molecule and one or more flavanol molecules, usually unvisualized by HPLC methods with C18 columns. In consequence, CE may be the technique of choice to analyze pigmented polymers, because they can be properly separated in different peaks, whereas with other techniques these compounds elute as diffuse humps or a single peak [179]. An example of the applicability of electrophoretic techniques to the analysis of the stabilization of coloured forms in aged red wines has been recently shown. Saenz-Navajas et al. [180] have studied by CE-UV the occurrence of these derivatives in model wine solutions containing malvidin-3-glucoside and ferulic, caffeic and p-coumaric acids, and several pyranoanthocyanins derivatives like malvidin-3-glucoside 4-vinylcatechol (pinotin A), malvidin-3-glucoside 4-vinylguaiacol, malvidin-3-glucoside 4-

Table 11
Selected examples of methods of separation of anthocyanins in foods by capillary electrophoresis.

Separation conditions:	Sample/detection Anthocyanins (time)	Ref.
Acidic Buffers		
CZE 25 kV, 20 °C Fused-silica capillary: 70.4 cm × 50 μm 25 mM NaH_2HPO_4–25 mM Na_2HPO_4 (pH 1.5) 30% AcN	*Blackcurrant anthocyanins*/UV–vis (DAD) 520 nm Cy-3-gluc (28 min); Cy-3-rut (29 min); Dp-3-gluc (31 min); Dp-3-rut (32 min)	daCosta et al., 1998 [171]
MEKC −25 kV, 25 °C Fused-silica capillary 72 cm, 50 μm 160 mM phosphate buffer (pH 2.1) 0.25 mM CTAB (cationic surfactant)	*Black glutinous rice and commercial natural anthocyanins*/UV–vis (DAD) 520 nm Cy-3-gluc; Cy-3-galactoside; Cy-3,5-digluc; Mv-3,5-digluc	Bicard et al., 1999 [172]
CZE 20 kV fused-silica capillary 60 cm, 50 μm id 200 mM Chloroacetate ammonium (pH 2)	*Wine*/ESI-MS-IT (positive mode) Vitisin B (6.90 min), Mv-3-gluc (7.34 min), Pn-caff-gluc (8.30), Mv-caff-digluc (8.65), vitisin A (9.75 min)	Bednar et al., 2005 [182]
CZE 20 kV, 27 °C Fused-silica capillary 48 cm, 75 μm 150 mM phosphoric acid, 3 M urea (pH 2.11) 50 mM β-Cyclodextrin	*Cranberries anthocyanidins*/UV–vis (DAD) 525 nm Peonidin (14.74 min) Cyanidin (18.01 min)	Watson et al., 2004 [175]
CZE 23 kV, 25 °C Polyimide-coated 45 cm, id 50 μm 250 mM sodium phosphate, 30% AcN (pH 1.4)	*Strawberry*/UV–vis (DAD) 510 nm Pg-glu (18.5 min), Cy-glu (19.5 min), Pg-rut (21 min)	Comandini et al., 2008 [184]
CZE −12 kV Poly-LA 313-coated fused-silica 57 cm, id 50 μm 15 mM Formic acid (pH 1.9)	*Red onion*/ESI-MS-TOF (positive mode) Cy-3-laminaribioside (13.5 min), Cy-3-malonoylglucoside (13.9 min), Cy-3-acetoylglucoside (14.3 min), Cy-3-gluc (14.3 min), Pn-3-malonoylglucoside (13.8), Pn-3-glu (14.3 min), Cy (16 min), Mv-3-glu (16.4 min)	Petersson et al., 2008 [181]
Basic Buffers		
CZE 25 kV, 25 °C Fused-silica capillary 50 cm, 75 μm id 150 mM sodium borate (pH 8)	*Strawberry*/UV–vis (DAD) 560 nm Pg-3-rut (4.2 min), Pg-3-gluc (4.3 min), Cy-3-gluc (5.4 min), Pg-3-succinylgluc (5.7 min) *Elderberry*: Cy-3-sambubioside-5-gluc (4 min), Cy-3,5-digluc (4.8 min), Cy-3-sambubioside (5 min), Cy-3-gluc (5.4 min)	Bridle, 1997 [185]
MECK 15 kV Fused-silica capillary (50 cm × 50 μm) 30 mM phosphate 400 mM borate–Tris (pH 7.0) 50 mM SDS	*Red grape skins*/UV–vis (DAD) 280 nm Mv-3,5-digluc (12 min), Pg-3-gluc (13 min), Mv-3-gluc (13.5 min), Mv-3-gal (13.8 min), Cy-3,5-digluc (14.2 min), Cy-3-gal (15.5 min)	Bednar et al., 2003 [173]
CZE 20 kV Fused-silica capillary 60 cm, 50 μm id 200 mM borate–ammonium (pH 9)	*Synthetic mixture* (*musts*)/ESI-MS-ion trap (positive mode) Mv-3,5-digluc (8.5 min), Cy-3,5-digluc (10 min), Mv-3-gluc (11 min), Mv-3-gal (11.5 min), Cy-3-gal (12 min), pelargonidin (15 min), Dp (16 min)	Bednar et al., 2005 [182]
CZE 25 kV, 10 °C Fused-silica capillary 46 cm, 75 μm 50 mM sodium tetraborate, 15% Methanol (pH 8.4)	*Wine*/UV–vis (DAD) 599 nm Mv-3-(6-p-coum)gluc (9.5 min), Mv-3-(6-ac)gluc (9.8 min), Pn-3-(6-ac)gluc (9.95 min), Mv-3-gluc (10.3 min), Pn-3-gluc (10.6 min), Mv-3-gluc + catechin dimer (10.9 and 11.25 min), Pt-3-(6-ac)gluc (11.35 min), Mv-3-gluc + pyruvic acid deriv. (11.5 min), Pt-3-gluc + pyruvic acid deriv. (11.6 min), Pt-3-gluc (12.2 min), Dp-3-gluc (12.4 min), Cy-3-gluc (12.6 min)	Calvo et al., 2004 [186]
CZE 25 kV Fused-silica capillary 72.5 cm × 50 μm 30 mM Na–borate (pH 8.78) containing 7.5 mM CyDTA	*Bilberry anthocyanins*/UV–vis (DAD) 580 nm Mv-3-gluc (8.5 min), Pn-3-gluc (8.6), Mv-3-gal (8.9), Pt-3-gluc (9), Pn-3-gal + Mv-3-ara (9.1), Cy-3-gluc (9.2), Dp-3-gluc + Pn-3-ara (9.25), Pt-3-gal (9.4), Cy-3-gal + Pt-3-ara (9.5), Dp-3-gal (9.6), Cy-3-ara (9.9), Dp-3-ara (10)	Ichiyanagi et al., 2004 [177]
CZE 25 kV Fused-silica capillary 80 cm × 50 μm 200 mM Boric acid (pH 9.0)	*Dried calyces of karkade*/ESI-ion trap ESI-TOF Cy-3-rut (10.4 min), Cy-3,5-digluc (11.2 min), Cy-3-sam (11.8 min), Dp-3-sam (11.9 min), Dp-3-gluc (12.6 min)	Segura Carretero et al., 2008 [183]

vinylphenol, or malvidin-3-glucoside 4-vinylsyringol have been detected [180].

As stated above, since anthocyanins absorb at visible wavelengths, UV–vis is the detector most commonly used for their determination in electrophoretic procedures. However, these methods lack for powerful identification tools, so the identification of anthocyanins has to be done by co-elution with pure expensive standards or by isolating peaks to further characterize them by MS or NMR, which is time-consuming. For example, in the work of Guadalupe et al. [179], condensation derivatives had to be previously analyzed by UPLC–TOF in order to verify their chemical structure. But recently, new instruments that combine CE with MS have been used for the analysis of anthocyanins. The main limitation is that not all the buffers used for CE are suitable for the ionization source. Petersson et al. reported for the first time a CE–TOF-MS analysis method for detecting anthocyanins in red onion [181], using poly-LA 313-coated capillaries and a buffer consisting of formic acid with ionic strength 15 mM and pH 1.9 for the electrophoretic separation, and a sheath-flow interface that provided 2 μl/min of an acidified methanol/water make-up liquid to the electrospray ionization source.

Hyphenation of capillary electrophoresis/ion-trap mass spectrometry has also been described in the work of Bednar et al. [182]. Ion trap provides the sequential fragmentation of the anthocyanidin skeleton. Both acidic and basic buffers were used for separating the common glucosidic anthocyanins, and the sheath liquid methanol:water:acetic acid allowed the ionization of anthocyanins. Optimized methods for both buffers were applied to monitor the changes in anthocyanin profile in red wines as well as the process of release of anthocyanins to wine must. CE/MS/MS of a Dornfelder wine sample confirmed the presence and identity of caffeoyl derivatives, vitisins and diglucosides. When applied to musts, only glucosylated derivatives (malvidin-3-glucoside, malvidin-(6-acetyl)-glucoside, malvidin-(6-coumaryl)-glucoside, and petunidin-3-glucoside) were found.

A recent work by Segura-Carretero et al. [183] has analyzed the anthocyanins from roselle, an annual herb that has gained an important position in the soft drinks market, by CZE–ESI-TOF and CZE–ESI-IT. Both methodologies gave almost identical product ion mass spectra for the anthocyanins examined, and the identity of anthocyanins was confirmed by using the accurate mass data obtained by the TOF detector and the fragmentation ions (MS^2) information obtained by the IT detector.

The results obtained show the high potential of electrophoretic applications for the analysis of anthocyanins, although further studies are required in order to improve its quantification repeatability, and make CZE an effective alternative to HPLC. The optimized electrophoretic methods present the traditional benefits of CE analysis, such as high separation efficiency with a low consumption of solvents and samples; and they also result in considerable reduction in analysis time of anthocyanins. However, the major volumes injected in HPLC and the different detectors employed enable to reach normally lower LOD and LOQ in HPLC.

5.6. Electrophoretic separations of flavanols and procyanidins

CE being still a relatively new technique in food analysis, most of the research has not been focused on a complete separation of different flavanols and proanthocyanidins, but in trying to establish new methods for food analysis. In consequence, we can find lots of methods for quantifying catechin and epicatechin, together with other important polyphenols, in several sources. In this sense, wine has been the most studied source to separate and quantify polyphenols from different families. For example, Herrero et al. and Sun et al. have provided micellar electrokinetic methods to determine different flavonoid aglycones (including flavanols, flavanones, flavonols, flavones) in wine [187,188]. Pazourek et al. [189] have applied a CZE method to detect catechin and epicatechin, together with phenolic acids in Canary Island wines, while Arce et al. [190] have determined the same phenolics and resveratrol.

Together with catechin and epicatechin, monomeric galloylated catechins from tea have also been widely analyzed by electrophoretic methods. The more complex study of CE separations for oligomer procyanidins has rarely been undertaken, with some exceptions like their separation in cocoa [166] or the separation of several B-type dimers by Cifuentes et al. [191]. A possible explanation is that the greatest limiting factor of proanthocyanidin analysis is the characterization of the polymerization degree, which can be provided by mass detectors that only recently have been successfully hyphenated with CE. Until now, HPLC–MS was the most common technique used for analyzing these oligomers. But since a good combination of electrophoretic moieties and electroosmotic flows should allow the migration of all oligomeric proanthocyanidins, without the irreversible retentions in the stationary that take place in HPLC columns, CE–MS can in the future provide helpful methods of analysis of oligomeric and polymeric proanthocyanidins, analogue to what has been observed for polymeric anthocyanins [178].

The most representative examples of flavanols and proanthocyanidins analysis by CE are shown in Table 12.

Although the first electrophoretic methods for the separation of catechins were CZE methods [192], the separation between several peaks was unsatisfactory and hence MECK was soon preferred [193,194]. SDS concentration is particularly important for separating similar structures [193], and can lead to changes in the migration order [191].

As usual, the type of buffer and pH are also important. Borate basic buffers are the most usual ones. Vicinal hydroxyl groups on catechins can undergo complexation with borate buffers, resulting in negatively charged catechin-borate ions [195] that experiment electromigration.

Despite that the use of acidic buffers has been scarcely applied for MECK separations, mainly due to the reduction of the EOF that could avoid the migration to the detection point, Cifuentes et al. [191] obtained a much better separation of procyanidin dimers and monomers at pH 5.0 than at basic pH. All procyanidins tested in this buffer were carried to the detection point in less than 5 min. The optimized method was successfully applied for the separations of dimers and monomers in lentils, white beans, almond peels and black beans, and the most clean electropherograms were obtained (i.e., less interfering compounds detected) at pH 5.0 [191].

Opposite to what has been observed for polymeric anthocyanins, little information can be found about the separation of proanthocyanidin oligomers. There is the possibility that some of the interfering compounds that appear as large broaden peaks at the end of electrophoretic runs include some higher polymerized oligomers, but it has not been verified. However, capillary electrophoresis can still be a tool to characterize polymerized procyanidin mixtures, given that it can serve for the fast separation of the major depolymerized components after thiolysis with cysteine. The quantification of monomer-cysteine adducts after thiolysis is a way to determine the size and composition of procyanidins. MECK can be effectively used to separate the catechins and their cysteinyl derivatives in less than 14 min, which is faster (only 14 min) and less solvent consuming than the classic RP-HPLC method [196].

Gotti et al. [166] have developed an optimized enantioselective CD–MECK method with hydroxypropyl-β-cyclodextrin, that not only improved separation of the most important phenolic compounds in *T. cacao*, but also allowed the enantioseparation of racemic catechin. This enantioselective method can evidence the epimerization of (−)-epicatechin to (−)-catechin caused by the heat development during the manufacture of chocolate and cocoa. Sim-

Table 12
Selected examples of methods of separation of catechins and proanthocyanidins in foods and natural sources by capillary electrophoresis.

Separation conditions	Sample/detection Proanthocyanidins (time)	Ref.
CZE		
CZE, 30 kV, 23 °C Fused-silica capillary, 77 cm × 50 μm 20 mM borax (pH 8.0)	*Green tea infusions*: Uv (200 nm) EGC (7. 1 min), E (7.2 min), C (7.3 min), EGCG (9.2 min), EG (9.4 min)	Horie et al., 1997 [192]
MEKC		
MEKC, −27 kV, 30 °C Fused-silica capillary, 48.5 cm, 50 μm^2 5 mM phosphate, 2% MeOH (pH 2.0) 2.89% SDS	*Tea and grapes*: UV (200 nm) EG (4 min); E (4.5 min); EGCG (5 min); C (5.25 min); EGC (6 min); GC (13 min)	Huang et al., 2005 [197]
MEKC, 25 °C Fused-silica capillary 37 cm × 50 μm 50 mM acetate buffer (pH 5.0) 100 mM SDS	*Synthetic mixture*: UV (200, 280 nm) B3 (2.1 min), B1 (2.6 min), C (2.9 min), B2 (3.3 min), E (4.5 min)	Cifuentes et al., 2001 [191]
MECK, 30 kV, 21 °C Fused-silica capillary, 85 cm × 50 μm 4 mM Tetraborate, 12 mM hydrogenphosphate, 40 mM SDS (pH 7.0)	*Green and black tea lyophilized extracts*: UV (200 nm) C (7.9 min), EGC (9 min), EGCG (10 min), E (11.75 min), ECG (13.5 min)	Barroso, 1999 [194]
Chiral		
MEKC, 10 kV, 20 °C Fused-silica capillary 56 cm × 50 μm 200 mM Borate–20 mM phosphate (pH 6.4) 240 mM SDS; 25 mM 6G-β-CD	*Green, oolong and black teas*: UV (210 nm) (−)-C (9.6 min); (+)-C (10 min); (−)-GC (10.4 min); (−)-EGCG (11 min); (−)-ECG (11.4 min); (−)-EGC (15.2 min); (+)-EC (19.5 min); (−)-EC (20 min)	Kodama et al., 2004 [195]
MEKC, 15 kV, 30 °C Fused-silica capillary, 38.5 cm, 50 μm 50 mM Britton–Robinson buffer (pH 2.5) 90 mM SDS; 12 mM HP-β-CD	*Theobroma cacao*: UV (220 nm) EC (1 min), B2 (2 min), B1 (4 min), (+)-C (4.25 min), (−)-C (4.5 min)	Gotti et al., 2006 [166]
CZE, 30 kV, 25 °C Fused-silica capillary, 56 cm, 50 μm 50 mM borate buffer (pH 8.5) 1 mM β-CD	*Human plasma after green tea ingestion*: UV (210 nm) C (5.5 min) E (5.7 min)	Abd El-Hady et al., 2008 [167]
MEKC, 20 kV Fused-silica capillary, 47 cm, 50 μm 50 mM phosphate (pH 7.0) 25 mM SDS; 25 mM sodium cholate 10% methanol	*Flavonoid aglycones in foods* (*wine*): UV (214 nm) C (5.41 min) E (5.80 min)	Herrero-Martínez et al., 2007 [187]
MEEKC		
MEEKC, −27 kV, 30 °C Fused-silica capillary, 48.5 cm, 50 μm 25 mM phosphate (pH 2.0); 2% MeOH 2.89% SDS; 1.36% heptane; 7.66% cyclohexanol	*Tea and grapes*: UV (200 nm) EG (6.5 min); C (7.75 min); EGCG (8 min); EC (10 min); EGC (13 min); GC (15 min)	Huang et al., 2005 [197]
MEEKC, −10 kV, 40 °C Fused-silica capillary, 24 cm × 50 μm 88.09% Phosphate buffer 50 mM (pH 2.5) 2.89% SDS; 1.36% n-heptane; 7.66% cyclohexanol	*Chinese and Indian teas*: UV (230 nm) ECG (3 min); EGCG (3.8 min); EC (4.5 min); C (5 min); EGC (6 min); GC (8 min)	Pomponio et al., 2003 [148]

EG: (−)-epicatechin gallate; C: (+)-catechin; EGCG: (−)-epigallocatechin gallate; E: epicatechin; EGC: (−)-epigallocatechin; and GC: (−)-gallocatechin.

ilarly, Kodama et al. [195] have tested the effect of several different cyclodextrins on enantiomeric separations of catechins from commercial teas. When the negatively charged catechin-borate ions are included in the cyclodextrins cavity, the inclusion complexes that are formed have charges identical with those of the free negatively charged C-borate ions but increased molecular masses, and hence lower electrophoretic mobilities. In consequence, they will be more easily migrated by the EOF to the cathode. When the complexes are incorporated into the micelles, they migrate with a micellar velocity that is lower than the velocities of the negatively charged C-borate ions and those complexes with CD. The portioning of the solute between the CD and the micelle depends on CD and surfactant (CD) concentrations, and thus has an influence on migration times. An optimized method with 6-α-glucosyl-β-cyclodextrin for the enantioseparation of catechin and epicatechin racemic isomers in commercial teas has been applied to study the epimerization during thermal sterilization, distribution and storage [195].

Microemulsion electrokinetic chromatography has also been applied to the separation of catechins. Huang et al. [197] developed and compared two optimized methods of MEKC and MEEKC for the analysis of phenolic compounds in grape and tea, mainly flavanols. Selectivity for both methods was completely different. As expected, a higher SDS level reduced the total separation time of all analytes, but it also produced a relatively high current. SDS levels greatly altered the migration order in MEEKC, but not in MEKC. Other parameters, like organic modifiers, voltage applied and temperature, were also optimized for both techniques.

Pomponio et al. [148] analyzed the influence on MEEKC separation of nine different cosurfactants for the analysis of green tea catechins. In this case, and opposite to Huang et al. [197], SDS levels did not alter significantly the migration order of analytes. More influence could be credited to the cosurfactant, since four different selectivities were obtained by changing the cosurfactant. However, this change in selectivity was only obtained when the concentration of cosurfactant was much higher than that of the oil (n-heptane).

Table 13
Selected examples of methods of separation of isoflavones in foods and natural sources by capillary electrophoresis.

Separation conditions	Sample/detection Isoflavones (time)	Ref.
CE-ED, 14 kV 50 mM borate (pH 9.5) Fused-silica capillary: 75 cm, 25 μm id	*Red clover isoflavones*/ED (0.85 V) Biochanin A (20 min), daidzein (23 min), genistein (24 min)	Peng and Ye, 2006 [199]
CE-ED, 9 kV 50 mM borate (pH 9.0) Fused-silica capillary: 40 cm, 25 μm id	*Pueraria radix* (*root of Pueraria lobata*)/ED (0.90 V) Puerarin (9.5 min), daidzein (10.5 min), rutin (11.5 min)	Chen et al., 2001 [207]
CE-ED, 9 kV 50 mM borate (pH 9.0) Fused-silica capillary: 75 cm, 25 μm id	Pericarps-seeds of *Sophora japonica*/ED (0.95 V) Genistin (9 min), genistein (13 min), rutin (13.5 min), kaempferol (15 min), quercetin (17 min)	Chu et al., 2005 [201]
CZE 15 kV, 25 °C 30 mM borax buffer (pH 9.29) Fused-silica capillary: 40 cm, 75 μm id	*Kudzu* (*plant of Pueraria*)/UV–vis (192 nm) Daidzin (8.5 min), 3-methoxipuerarin (10.4 min), puerarin (10.8 min), daidzein (12.6 min),	Fang et al., 2006 [202]
CZE 15 kV, 25 °C 50 mM Ammonium acetate (pH 10.5) 20% Methanol Fused-silica capillary: 30 cm, 75 μm id	*Common bean and soybean seedlings*/UV–vis (214 nm) Glycitein (9.5 min), daidzein (10.5 min), genistein (11 min), kaempferol (15.7 min)	Dinelli et al., 2007 [203]
CZE 20 kV, 16 °C 25 mM ammonium acetate (pH 9.0) 20% AcN Fused-silica capillary: 40 cm, 75 μm id	*Traditional medicinal preparations* (*Pueriae radix and Scutellarie radix*)/UV–vis (273 nm) Puerarin (9.5 min), daidzein (10.5 min), wogonin (15 min)	Li et al., 2005 [204]
MEKC, 25 kV, 20 °C 10 mM STB, 40 mM SDS (pH 9.3) 1% Methanol Uncoated fused-silica capillary: 58.5 cm, 75 μm	*Soy germ* (*pharmaceutical capsules*)/UV–vis (269 nm) Glycitein (5.5 min), daidzein (5.7 min), genistein (5.9 min), Daidzin (6.2 min), glycitin (6.9 min), genistin (7.2 min)	Micke et al., 2006 [205]
MEKC, 25 kV, 25 °C 30 mM Borate, 20 mM SDS, 4 mg/ml HP-β-CD (pH 10.1) 5% Ethanol Uncoated fused-silica capillary: 60 cm, 75 μm id	*Red clover*/UV–vis (254 nm) Biochanin A (8 min), formononetin (8.5 min), genistein (9.5 min), daidzein (11 min)	Zhang et al., 2007 [206]

ED: electrochemical detection.

In this sense, two methods with cyclohexanol and 2-hexanol were successfully applied to the analysis of catechins in green tea samples. In conclusion, MEEKC selectivity can be favorably changed by an appropriate choice of cosurfactant.

Limits of detection with normal detection cells are normally far from those obtained by HPLC–MS/MS [167,187]. The use of high-sensitivity cells has led to an improvement of 10-fold for the quantification of catechins in biological samples [167]. However, advances in hyphenation with mass detectors should allow the improvement on the detection limits for catechins, and they can promote their applicability for the analysis of biological samples, in an analogue way to what has occurred with LC–MS/MS methods. Additionally, the identification of peaks by mass detectors will enormously simplify the analysis, since it will allow the extraction of unambiguously identified masses, and in consequence interferences will be reduced.

5.7. Electrophoretic separations of Isoflavones

CE with different detection methods has been successfully applied for the determination of isoflavones in food products. Table 13 compiles some representative methods of these separations.

Daidzein and genistein were determined in soy products in the work of Peng et al. [198] in a method that lasted 20 min. The optimum conditions consisted in 100 mM borate buffer (pH 11.0) and 12 kV separation voltage. Increasing the separation voltage gives shorter migration times, but it is not beneficial for the resolution of both isoflavones. Too low separation voltage increases the analysis time considerably, which in turn causes peak broadening. The same team have also applied a borate buffer (50 mM, pH 9.0) at 14 kV to separate three isoflavonoids (biochanin A, genistein and daidzein) from red clover within 25 min [199]. Same buffer and pH, but lower separation voltage (9 kV) was applied by Chen et al. for separating puerarin and daidzein (together with rutin) [200]. Chu et al. [201] separated genistein and genistin, together with rutin, kaempferol and quercitin in medicinal parts of *S. japonica* within 18 min at the separation voltage of 16 kV in a 50 mM borax running buffer (pH 9.0). A very similar method with borax 30 mM (pH 9.29) was used for Fang et al. [202] to determine isoflavonoids (puerarin, 3′-methoxypuerarin, daidzin and daidzein) in Kudzu samples.

Improvements for these methodologies included the use of organic modifiers. Dinelli et al. [203] determined daidzein, glycitein and genistein (together with kaempferol) in common beans and soybean seedlings in 16 min by using 50 mM ammonium acetate buffer at pH 10.5 containing 20% of methanol. Li et al. [204] separated three bioactive isoflavones (puerarin, daidzein and wogonin) in traditional Chinese medicinal preparations using a non-aqueous buffer system of 20% acetonitrile, 25 mM ammonium acetate and pH 9.00, with applied voltage and capillary temperature of 20 kV and 16 °C, respectively. Migration time was 16 min, but puerarin and daidzein were already resolved in less than 11 min.

The potential application of MEKC for isoflavones determination has also been studied. In these cases, SDS has been added to the buffer in order to generate the micelles. Dinelli et al. [203] found that SDS led to a remarkable increase in migration times without improving resolution, so the use of micelles was discarded. But Micke et al. [205] achieved the separation of six isoflavones (glycitein, daidzein, genistein, daidzin and genistin) in less than 7.5 min in an optimum electrolyte composition of 10 mM sodium tetraborate buffer (pH 9.3) containing 40 mM SDS and 1% MeOH. Also Zhang et al. [206] have established an optimized micellar electrokinetic method for the separation and determination of four isoflavones (biochanin A, formononetin, genistein and daidzein) in

red clover. In this case, since SDS alone improved the resolution but was still not satisfactory, hydroxypropyl-β-cyclodextrin was added to the buffer. The final electrolyte consisted of 30 mM borate, 20 mM SDS, 4 mg/ml hydroxypropyl-β-cyclodextrin containing 5% ethanol at pH 10.1, and migration time was less of 12 min.

5.8. Conclusions

The use of capillary electromigration methods to analyze antioxidants and phenolic compounds is nowadays increasing, although HPLC is still the technique of choice for the study of this type of compounds. Until now, CE methods do not offer the range of separation of complex samples that can be achieved with HPLC, and they do not offer the same sensitivity either. However, recent hyphenations with mass detectors are set to improve both limitations, and the fact that electrophoretic separations offer different selectivities than chromatographic separations, the easy incorporation of chiral separations by adding cyclodextrins to the samples, together with the non existence of irreversible adsorptions, make CE a very attractive technique to study. Moreover, for routine analysis it may be the technique of choice, since it is less solvents consuming and it can be faster than HPLC.

6. General conclusions

As summarized in this review, research in new methods of separation of food polyphenols is experimenting a large increase in recent years, since the development of new instruments allows a better characterization both qualitative and quantitative of complex natural sources.

We have shown that methods of CCC are very well suited for the isolation of different polyphenols, and thus in certain cases they can replace traditional techniques like low pressure columns or semipreparative and preparative HPLC, since CCC can provide great resolution and greater yields at lower costs.

We have also analyzed the current trends in hyphenated procedures, specially HPLC–MS, that have boosted the identification of new polyphenolic compounds and that have provided the greatest sensitivities for the analysis of polyphenols. HPLC remains the most advisable choice for investigating polyphenols, since the separation methods are already well established, and hyphenation with mass detectors is easy to achieve. Recent development of new techniques of UPLC has proven to minimize times without compromising the resolution.

However, the development of hyphenated CE–MS instruments should also promote the utilization of these methodologies for analyzing samples that may need a different selectivity, and with some advantages such as faster analysis and less consume of solvents.

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References

[1] J.A.M. Kyle, G.G. Duthie, in: O. Andersen, K. Markham (Eds.), Flavonoids. Chemistry, Biochemistry and Applications, CRC Press, Boca Ratón, 2006, p. 219.
[2] L. Rolle, S. Guidoni, J. Int. Sci. Vigne Vin 41 (2007) 193.
[3] C. Llaudy Maria, R. Canals, J.-M. Canals, N. Rozes, L. Arola, F. Zamora, J. Agric. Food Chem. 52 (2004) 742.
[4] T. Stark, S. Bareuther, T. Hofmann, J. Agric. Food Chem. 53 (2005) 5407.
[5] A. Scalbert, C. Manach, C. Morand, C. Remesy, L. Jimenez, Crit. Rev. Food Sci. Nutr. 45 (2005) 287.
[6] G. Tucker, K. Robards, Crit. Rev. Food Sci. Nutr. 48 (2008) 929.
[7] S.N. Nichenametla, T.G. Taruscio, D.L. Barney, J.H. Exon, Crit. Rev. Food Sci. Nutr. 46 (2006) 161.
[8] V. Juturu, Curr. Nutr. Food Sci. 4 (2008) 227.
[9] H.-J. Jung, J. Choi, J.-H. Nam, H.-J. Park, J. Med. Food 10 (2007) 702.
[10] I. Erlund, R. Koli, G. Alfthan, J. Marniemi, P. Puukka, P. Mustonen, P. Mattila, A. Jula, Am. J. Clin. Nutr. 87 (2008) 323.
[11] X. Terra, J. Valls, X. Vitrac, J.-M. Merrillon, L. Arola, A. Ardevol, C. Blade, J. Fernandez-Larrea, G. Pujadas, J. Salvado, M. Blay, J. Agric. Food Chem. 55 (2007) 4357.
[12] C. Riviere, T. Richard, X. Vitrac, J.-M. Merillon, J. Valls, J.-P. Monti, Bioorg. Med. Chem. Lett. 18 (2008) 828.
[13] R. Mayer, G. Stecher, R. Wuerzner, R.C. Silva, T. Sultana, L. Trojer, I. Feuerstein, C. Krieg, G. Abel, M. Popp, O. Bobleter, G.K. Bonn, J. Agric. Food Chem. 56 (2008) 6959.
[14] J.-A. Kim, Endocr. Metab. Immune Disord.: Drug Targets 8 (2008) 82.
[15] P. McCue, K. Shetty, Crit. Rev. Food Sci. Nutr. 44 (2004) 361.
[16] T.M. Spormann, F.W. Albert, T. Rath, H. Dietrich, F. Will, J.-P. Stockis, G. Eisenbrand, C. Janzowski, Cancer Epidemiol. Biomarkers Prev. 17 (2008) 3372.
[17] S. Dudonne, X. Vitrac, P. Coutiere, M. Woillez, J.-M. Merillon, J. Agric. Food Chem. 57 (2009) 1768.
[18] M.B. Roberfroid, V. Coxam, N. Delzenne, Aliments Fonctionnels, Éditions Tec & Doc, Paris, 2008.
[19] M.A. Kelm, J.C. Johnson, R.J. Robbins, J.F. Hammerstone, H.H. Schmitz, J. Agric. Food Chem. 54 (2006) 1571.
[20] C.D. Stalikas, J. Sep. Sci. 30 (2007) 3268.
[21] W. Hummer, P. Schreier, Nutr. Mol. Food Res. 52 (2008) 1381.
[22] M.d.L. Mata-Bilbao, C. Andres-Lacueva, E. Roura, O. Jauregui, C. Torre, R.M. Lamuela-Raventos, J. Agric. Food Chem. 55 (2007) 8857.
[23] M. Spanakis, S. Kasmas, I. Niopas, Biomed. Chromatogr. 23 (2009) 124.
[24] J. Xing, C. Xie, H. Lou, J. Pharm. Biomed. Anal. 44 (2007) 368.
[25] A. Serra, A. Macia, M.-P. Romero, M.-J. Salvado, M. Bustos, J. Fernandez-Larrea, M.-J. Motilva, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 1169.
[26] L. Bravo, Nutr. Rev 56 (1998) 317.
[27] O.M. Andersen, M. Jordheim, in: O. Andersen, K. Markham (Eds.), Flavonoids. Chemistry, Biochemistry and Applications, CRC Press, Boca Ratón, 2006, p. 471.
[28] J.-M. Kong, L.-S. Chia, N.-K. Goh, T.-F. Chia, R. Brouillard, Phytochemistry 64 (2003) 923.
[29] A.M. Castaneda-Ovando, d.L. Pacheco-Hernandez, M.E. Paez-Hernandez, J.A. Rodriguez, C.A. Galan-Vidal, Food Chem. 113 (2009) 859.
[30] J.C. Rivas-Gonzalo, in: C. Santos-Buelga, G. Williamson (Eds.), Methods in Polyphenols Analysis, The Royal Society of Chemistry, Cambridge, 2003, p. 338.
[31] R. Chirinos, D. Campos, M. Warnier, R. Pedreschi, J.-F. Rees, Y. Larondelle, Food Chem. 111 (2008) 98.
[32] M.D. Thompson, H.J. Thompson, J.N. McGinley, E.S. Neil, D.K. Rush, D.G. Holm, C. Stushnoff, J. Food Compos. Anal. (2009), doi:10.1016/j.jfca.2008.09.002.
[33] T. Mezadri, D. Villano, M.S. Fernandez-Pachon, M.C. Garcia-Parrilla, A.M. Troncoso, J. Food Compos. Anal. 21 (2008) 282.
[34] E.R.B.A. Prata, L.S. Oliveira, LWT – Food Sci. Technol 40 (2007) 1555.
[35] L. Longo, A. Scardino, G. Vasapollo, F. Blando, Innovative Food Sci. Emerg. Technol. 8 (2007) 329.
[36] M.T. Escribano-Bailon, C. Santos-Buelga, J.C. Rivas-Gonzalo, J. Chromatogr. A 1054 (2004) 129.
[37] S.A. Lazarus, M.A. Kelm, G.A. Wächter, J.F. Hammerstone, H.H. Schmitz, in: C. Santos-Buelga, G. Williamson (Eds.), Methods in Polyphenols Analysis, The Royal Society of Chemistry, Cambridge, 2003, p. 267.
[38] E. Rasmussen Salka, H. Frederiksen, K. Struntze Krogholm, L. Poulsen, Mol. Nutr. Food Res. 49 (2005) 159.
[39] B.D. Oomah, F.S. Hosseinian, in: W.J. Hurst (Ed.), Methods of Analysis for Functional Foods and Nutraceuticals, CRC Press, Taylor & Francis Group, 2008, p. 1.
[40] N. Koehler, V. Wray, P. Winterhalter, J. Chromatogr. A 1177 (2008) 114.
[41] G.F. Pauli, S.M. Pro, J.B. Friesen, J. Nat. Prod. 71 (2008) 1489.
[42] X. Cao, Y. Tian, T. Zhang, X. Li, Y. Ito, J. Chromatogr. A 855 (1999) 709.
[43] I.A. Sutherland, D. Fisher, J. Chromatogr. A 1216 (2009) 740.
[44] J.-H. Renault, P. Thepenier, M. Zeches-Hanrot, L. Le Men-Olivier, A. Durand, A. Foucault, R. Margraff, J. Chromatogr. A 763 (1997) 345.
[45] S. Vidal, Y. Hayasaka, E. Meudec, V. Cheynier, G. Skouroumounis, J. Agric. Food Chem. 52 (2004) 713.
[46] Q. Du, J. Zheng, Y. Xu, J. Food Compos. Anal. 21 (2008) 390.
[47] E. Salas, M. Duenas, M. Schwarz, P. Winterhalter, V. Cheynier, H. Fulcrand, J. Agric. Food Chem. 53 (2005) 4536.
[48] A. Degenhardt, H. Knapp, P. Winterhalter, J. Agric. Food Chem. 48 (2000) 338.
[49] M. Schwarz, S. Hillebrand, S. Habben, A. Degenhardt, P. Winterhalter, Biochem. Eng. J. 14 (2003) 179.
[50] A. Degenhardt, S. Hofmann, H. Knapp, P. Winterhalter, J. Agric. Food Chem. 48 (2000) 5812.
[51] M. Rentzsch, P. Quast, S. Hillebrand, J. Mehnert, P. Winterhalter, Innovative Food Sci. Emerg. Technol. 8 (2007) 333.
[52] A. Degenhardt, H. Knapp, P. Winterhalter, Vitis 39 (2000) 43.
[53] Q. Du, G. Jerz, P. Winterhalter, J. Chromatogr. A 1045 (2004) 59.
[54] A. Yanagida, A. Shoji, Y. Shibusawa, H. Shindo, M. Tagashira, M. Ikeda, Y. Ito, J. Chromatogr. A 1112 (2006) 195.

[55] N. Kumar, W.M.A. Maduwantha, B. Wijekoon, V. Kumar, P.A.N. Punyasiri, I.S.B. Abeysinghe, J. Chromatogr. A 1216 (2009) 4295.
[56] S. Vidal, L. Francis, A. Noble, M. Kwiatkowski, V. Cheynier, E. Waters, Anal. Chim. Acta 513 (2004) 57.
[57] X.L. Cao, Y. Tian, T.Y. Zhang, Y. Ito, J. Chromatogr. A 898 (2000) 75.
[58] Q. Du, H. Cheng, Y. Ito, J. Chromatogr. A 921 (2001) 331.
[59] N.S. Kumar, M. Rajapaksha, J. Chromatogr. A 1083 (2005) 223.
[60] J.-C. Delaunay, C. Castagnino, C. Cheze, J. Vercauteren, J. Chromatogr. A 964 (2002) 123.
[61] Q. Du, Z. Li, Y. Ito, J. Chromatogr. A 923 (2001) 271.
[62] X. Ma, P. Tu, Y. Chen, T. Zhang, Y. Wei, Y. Ito, J. Chromatogr. A 992 (2003) 193.
[63] M. Stuertz, V. Lander, W. Schmid, P. Winterhalter, Mol. Nutr. Food Res. 50 (2006) 356.
[64] F. Yang, Y. Ma, Y. Ito, J. Chromatogr. A 928 (2001) 163.
[65] A. Carrasco-Pancorbo, C. Neususs, M. Pelzing, A. Segura-Carretero, A. Fernandez-Gutierrez, Electrophoresis 28 (2007) 806.
[66] M. Naczk, F. Shahidi, J. Pharm. Biomed. Anal. 41 (2006) 1523.
[67] G. Ruberto, A. Renda, C. Daquino, V. Amico, C. Spatafora, C. Tringali, N. De Tommasi, Food Chem. 100 (2006) 203.
[68] J. Gruz, O. Novak, M. Strnad, Food Chem. 111 (2008) 789.
[69] R.J. Robbins, J. Agric. Food Chem. 51 (2003) 2866.
[70] N. Fabre, I. Rustan, E. de Hoffmann, J. Quetin-Leclercq, J. Am. Soc. Mass Spectrom. 12 (2001) 707.
[71] U. Justesen, P. Knuthsen, T. Leth, J. Chromatogr. A 799 (1998) 101.
[72] C.R. Horvath, P.A. Martos, P.K. Saxena, J. Chromatogr. A 1062 (2005) 199.
[73] W. Wu, C. Yan, L. Li, Z. Liu, S. Liu, J. Chromatogr. A 1047 (2004) 213.
[74] M. Boue Stephen, H. Carter-Wientjes Carol, Y. Shih Betty, E. Cleveland Thomas, J. Chromatogr. A 991 (2003) 61.
[75] I. Nicoletti, A. De Rossi, G. Giovinazzo, D. Corradini, J. Agric. Food Chem. 55 (2007) 3304.
[76] F. Buiarelli, F. Coccioli, R. Jasionowska, M. Merolle, A. Terracciano, Rapid Commun. Mass Spectrom. 21 (2007) 2955.
[77] J.B. Jean-Denis, R. Pezet, R. Tabacchi, J. Chromatogr. A 1112 (2006) 263.
[78] V. Jerkovic, D. Callemien, S. Collin, J. Agric. Food Chem. 53 (2005) 4202.
[79] M. Careri, C. Corradini, L. Elviri, I. Nicoletti, I. Zagnoni, J. Agric. Food Chem. 52 (2004) 6868.
[80] R. Flamini, Mass Spectrom. Rev. 22 (2003) 218.
[81] J.K. Prasain, C.-C. Wang, S. Barnes, Free Radical Biol. Med. 37 (2004) 1324.
[82] M.C. McMaster, LC/MS: A Practical's User Guide, John Wiley & Sons, NJ, USA, 2005.
[83] E. de Rijke, P. Out, M.A. Niessen Wilfried, F. Ariese, C. Gooijer, U. A.Th. Brinkman, J. Chromatogr. A 1112 (2006) 31.
[84] T. Reemtsma, J. Chromatogr. A 1000 (2003) 477.
[85] P. Dugo, O. Favoino, M. Lo Presti, R. Luppino, G. Dugo, L. Mondello, J. Sep. Sci. 27 (2004) 1458.
[86] I. Nicoletti, C. Bello, A. De Rossi, D. Corradini, J. Agric. Food Chem. 56 (2008) 8801.
[87] I. Palikova, J. Heinrich, P. Bednar, P. Marhol, V. Kren, L. Cvak, K. Valentova, F. Ruzicka, V. Hola, M. Kolar, V. Simanek, J. Ulrichova, J. Agric. Food Chem. 56 (2008) 11883.
[88] G. Takeoka, L. Dao, in: W.J. Hurst (Ed.), Methods of Analysis for Functional Foods and Nutraceuticals, CRC Press, Taylor and Francis Group, 2008, p. 247.
[89] J.L. McCallum, R. Yang, J.C. Young, J.N. Strommer, R. Tsao, J. Chromatogr. A 1148 (2007) 38.
[90] P. Arapitsas, C. Turner, Talanta 74 (2008) 1218.
[91] E. Garcia-Beneytez, F. Cabello, E. Revilla, J. Agric. Food Chem. 51 (2003) 5622.
[92] Q. Tian, M.M. Giusti, G.D. Stoner, S.J. Schwartz, J. Chromatogr. A 1091 (2005) 72.
[93] X. Wu, R.L. Prior, J. Agric. Food Chem. 53 (2005) 2589.
[94] D.N. Cooke, S. Thomasset, D.J. Boocock, M. Schwarz, P. Winterhalter, W.P. Steward, A.J. Gescher, T.H. Marczylo, J. Agric. Food Chem. 54 (2006) 7009.
[95] C. Alcalde-Eon, M.T. Escribano-Bailon, C. Santos-Buelga, J.C. Rivas-Gonzalo, Anal. Chim. Acta 563 (2006) 238.
[96] S. Vidal, E. Meudec, V. Cheynier, G. Skouroumounis, Y. Hayasaka, J. Agric. Food Chem. 52 (2004) 7144.
[97] D. Favretto, R. Flamini, Am. J. Enol. Vitic 51 (2000) 55.
[98] D. Kammerer, A. Claus, R. Carle, A. Schieber, J. Agric. Food Chem 52 (2004) 4360.
[99] J.-i. Nakajima, I. Tanaka, S. Seo, M. Yamazaki, K. Saito, J. Biomed. Biotechnol 5 (2004) 241.
[100] V. Nunez, M. Monagas, M.C. Gomez-Cordoves, B. Bartolome, Postharvest Biol. Technol 31 (2004) 69.
[101] M. Monagas, I. Garrido, B. Bartolome, C. Gomez-Cordoves, Anal. Chim. Acta 563 (2006) 401.
[102] N.P. Seeram, L.S. Adams, Y. Zhang, R. Lee, D. Sand, H.S. Scheuller, D. Heber, J. Agric. Food Chem 54 (2006) 9329.
[103] Z. Huang, B. Wang, P. Williams, R.D. Pace, LWT-Food Sci. Technol 42 (2009) 819.
[104] S.A. Lazarus, G.E. Adamson, J.F. Hammerstone, H.H. Schmitz, J. Agric. Food Chem. 47 (1999) 3693.
[105] W. Pongsuwan, T. Bamba, K. Harada, T. Yonetani, A. Kobayashi, E. Fukusaki, J. Agric. Food Chem. 56 (2008) 10705.
[106] S. Gonzalez-Manzano, C. Santos-Buelga, J.J. Perez-Alonso, J.C. Rivas-Gonzalo, M.T. Escribano-Bailon, J. Agric. Food Chem. 54 (2006) 4326.
[107] E. Roura, C. Andres-Lacueva, O. Jauregui, E. Badia, R. Estruch, M. Izquierdo-Pulido, R.M. Lamuela-Raventos, J. Agric. Food Chem. 53 (2005) 6190.
[108] J.K. Hellstrom, P.H. Mattila, J. Agric. Food Chem. 56 (2008) 7617.
[109] C. Counet, C. Ouwerx, D. Rosoux, S. Collin, J. Agric. Food Chem. 52 (2004) 6243.
[110] L. Gu, M. Kelm, J.F. Hammerstone, G. Beecher, D. Cunningham, S. Vannozzi, R.L. Prior, J. Agric. Food Chem. 50 (2002) 4852.
[111] A. Yanagida, H. Murao, M. Ohnishi-Kameyama, Y. Yamakawa, A. Shoji, M. Tagashira, T. Kanda, H. Shindo, Y. Shibusawa, J. Chromatogr. A 1143 (2007) 153.
[112] L.-Z. Lin, P. Chen, J.M. Harnly, J. Agric. Food Chem 56 (2008) 8130.
[113] N. Ortega, M.-P. Romero, A. Macia, J. Reguant, N. Angles, J.-R. Morello, M.-J. Motilva, J. Agric. Food Chem 56 (2008) 9621.
[114] F.S. Hosseinian, W. Li, A.W. Hydamaka, A. Tsopmo, L. Lowry, J. Friel, T. Beta, J. Agric. Food Chem 55 (2007) 6970.
[115] H.A. Weber, A.E. Hodges, J.R. Guthrie, B.M. O'Brien, D. Robaugh, A.P. Clark, R.K. Harris, J.W. Algaier, C.S. Smith, J. Agric. Food Chem 55 (2007) 148.
[116] K.R. Maeaettae-Riihinen, M.P. Kaehkoenen, A.R. Toerroenen, I.M. Heinonen, J. Agric. Food Chem 53 (2005) 8485.
[117] M. Duenas, B. Sun, T. Hernandez, I. Estrella, M.I. Spranger, J. Agric. Food Chem 51 (2003) 7999.
[118] M.A. Rostagno, A. Villares, E. Guillamon, A. Garcia-Lafuente, J.A. Martinez, J. Chromatogr. A 1216 (2009) 2.
[119] J. Vacek, B. Klejdus, L. Lojkova, V. Kuban, J. Sep. Sci. 31 (2008) 2054.
[120] L.J. Chen, X. Zhao, S. Plummer, J. Tang, D.E. Games, J. Chromatogr. A 1082 (2005) 60.
[121] P. Delmonte, J. Perry, J.I. Rader, J. Chromatogr. A 1107 (2006) 59.
[122] M.E. Rybak, D.L. Parker, C.M. Pfeiffer, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 861 (2008) 145.
[123] B. Klejdus, J. Vacek, L. Lojkova, L. Benesova, V. Kuban, J. Chromatogr. A 1195 (2008) 52.
[124] Q. Ma, M. Chen, H.-R. Yin, Z.-G. Shi, Y.-Q. Feng, J. Chromatogr. A 1212 (2008) 61.
[125] P.B. Grace, N.S. Mistry, M.H. Carter, A.J.C. Leathem, P. Teale, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 853 (2007) 138.
[126] X. Zhang, Y.G. Sun, M.C. Cheng, Y.Q. Wang, H.B. Xiao, X.M. Liang, Anal. Chim. Acta 602 (2007) 252.
[127] M. Farre, M. Kuster, R. Brix, F. Rubio, M.-J. Lopez de Alda, D. Barcelo, J. Chromatogr. A 1160 (2007) 166.
[128] B. Klejdus, J. Vacek, V. Adam, J. Zehnalek, R. Kizek, L. Trnkova, V. Kuban, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 806 (2004) 101.
[129] K. Mitani, S. Narimatsu, H. Kataoka, J. Chromatogr. A 986 (2003) 169.
[130] M. Satterfield, D.M. Black, J.S. Brodbelt, J. Chromatogr. B: Biomed. Sci. Appl. 759 (2001) 33.
[131] L. Chen, X. Zhao, L. Fang, D.E. Games, J. Chromatogr. A 1154 (2007) 103.
[132] B. Klejdus, D. Vitamvasova-Sterbova, V. Kuban, Anal. Chim. Acta 450 (2001) 81.
[133] Q. Wu, M. Wang, J.E. Simon, J. Chromatogr. A 1016 (2003) 195.
[134] M. Careri, C. Corradini, L. Elviri, A. Mangia, J. Chromatogr. A 1152 (2007) 274.
[135] Y. Peng, F. Liu, Y. Peng, J. Ye, Food Chem. 92 (2005) 169.
[136] P. Kuban, P. Kuban, V. Kuban, Electrophoresis 23 (2002) 3725.
[137] S.L. Simpson, J.P. Quirino, S. Terabe, J. Chromatogr. A 1184 (2008) 504.
[138] I. Molnar-Perl, Z. Fuzfai, J. Chromatogr. A 1073 (2005) 201.
[139] C.T. da Costa, D. Horton, S.A. Margolis, J. Chromatogr. A 881 (2000) 403.
[140] J.J. Dalluge, B.C. Nelson, J. Chromatogr. A 881 (2000) 411.
[141] H. Horie, K. Kohata, J. Chromatogr. A 881 (2000) 425.
[142] B.L. Lee, C.N. Ong, J. Chromatogr. A 881 (2000) 439.
[143] J. Sadecka, J. Polonsky, J. Chromatogr. A 880 (2000) 243.
[144] M. Herrero, E. Ibanez, A. Cifuentes, J. Sep. Sci. 28 (2005) 883.
[145] P. Jac, M. Polasek, M. Pospisilova, J. Pharm. Biomed. Anal. 40 (2006) 805.
[146] A. Cifuentes, Electrophoresis 27 (2006) 283.
[147] A.V. Shpak, A.V. Pirogov, O.A. Shpigun, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 800 (2004) 91.
[148] R. Pomponio, R. Gotti, B. Luppi, V. Cavrini, Electrophoresis 24 (2003) 1658.
[149] R. Hamoudova, M. Urbanek, M. Pospisilova, M. Polasek, J. Chromatogr. A 1032 (2004) 281.
[150] E. Wen, R. Asiaie, C. Horvath, J. Chromatogr. A 855 (1999) 349.
[151] F.N. Fonseca, M.F.M. Tavares, C. Horvath, J. Chromatogr. A 1154 (2007) 390.
[152] P. Li, P. Li Shao, T. Wang Yi, Electrophoresis 27 (2006) 4808.
[153] S. Sentellas, J. Saurina, J. Sep. Sci. 26 (2003) 875.
[154] M. Vaher, M. Koel, J. Chromatogr. A 990 (2003) 225.
[155] A. Varenne, S. Descroix, Anal. Chim. Acta 628 (2008) 9.
[156] X.-K. Wang, Y.-Z. He, L.-L. Qian, Talanta 74 (2007) 1.
[157] B. Steinbock, P.P. Vichaikul, O. Steinbock, J. Chromatogr. A 943 (2002) 139.
[158] L. Fan, W. Yan, C. Cao, W. Zhang, Q. Chen, Anal. Chim. Acta (2009), doi:10.1016/j.aca.2009.02.025.
[159] C. Kwon, H. Park, S. Jung, Carbohydr. Res. 342 (2007) 762.
[160] C.-H. Lin, W.-R. Fang, C.-M. Kuo, W.-Y. Chang, Y.-C. Liu, W.-Y. Lin, J.-C. Wu, C.-E. Lin, J. Chromatogr. A 1188 (2008) 301.
[161] J. Pan, S. Zhang, L. Yan, J. Tai, Q. Xiao, K. Zou, Y. Zhou, J. Wu, J. Chromatogr. A 1185 (2008) 117.
[162] D. Wistuba, O. Trapp, N. Gel-Moreto, R. Galensa, V. Schurig, Anal. Chem. 78 (2006) 3424.
[163] Q.-F. Zhang, S.-C. Li, W.-P. Lai, H.-Y. Cheung, Food Chem. 113 (2009) 684.
[164] S. Zhang, S. Dong, L. Chi, P. He, Q. Wang, Y. Fang, Talanta 76 (2008) 780.
[165] H.-Y. Cheung, Q.-F. Zhang, J. Chromatogr. A 1213 (2008) 231.
[166] R. Gotti, S. Furlanetto, S. Pinzauti, V. Cavrini, J. Chromatogr. A 1112 (2006) 345.
[167] D. Abd El-Hady, N. Abo El-Maali, Talanta 76 (2008) 138.

[168] H. Zhang, L. Zhou, X. Chen, Electrophoresis 29 (2008) 1556.
[169] H.-Y. Huang, W.-C. Lien, I.Y. Huang, Electrophoresis 27 (2006) 3202.
[170] K. Helmja, M. Borissova, T. Knjazeva, M. Jaanus, U. Muinasmaa, M. Kaljurand, M. Vaher, J. Chromatogr. A 1216 (2009) 3666.
[171] C.T. da Costa, B.C. Nelson, S.A. Margolis, D. Horton, J. Chromatogr. A 799 (1998) 321.
[172] V. Bicard, A. Fougerousse, R. Brouillard, J. Liq. Chromatogr. Related Technol. 22 (1999) 541.
[173] P. Bednar, A.V. Tomassi, C. Presutti, M. Pavlikova, K. Lemr, S. Fanali, Chromatographia 58 (2003) 283.
[174] L. Muller, P. Bednar, P. Bartak, K. Lemr, J. Sevcik, J. Sep. Sci. 28 (2005) 1285.
[175] D.J. Watson, A.A. Bushway, R.J. Bushway, J. Liq. Chromatogr. Related Technol. 27 (2004) 113.
[176] R. Saenz-Lopez, P. Fernandez-Zurbano, M.T. Tena, J. Chromatogr. A 990 (2003) 247.
[177] T. Ichiyanagi, Y. Kashiwada, Y. Ikeshiro, Y. Hatano, Y. Shida, M. Horie, S. Matsugo, T. Konishi, Chem. Pharm. Bull. 52 (2004) 226.
[178] R. Saenz-Lopez, P. Fernandez-Zurbano, M.T. Tena, J. Chromatogr. A 1052 (2004) 191.
[179] Z. Guadalupe, A. Soldevilla, M.-P. Saenz-Navajas, B. Ayestaran, J. Chromatogr. A 1112 (2006) 112.
[180] M.-P. Saenz-Navajas, M.T. Tena, P. Fernandez-Zurbano, Food Chem. 115 (2009) 766.
[181] E.V. Petersson, A. Puerta, J. Bergquist, C. Turner, Electrophoresis 29 (2008) 2723.
[182] P. Bednar, B. Papouskova, L. Muller, P. Bartak, J. Stavek, P. Pavlousek, K. Lemr, J. Sep. Sci. 28 (2005) 1291.
[183] A. Segura-Carretero, M.A. Puertas-Mejia, S. Cortacero-Ramirez, R. Beltran, C. Alonso-Villaverde, J. Joven, G. Dinelli, A. Fernandez-Gutierrez, Electrophoresis 29 (2008) 2852.
[184] P. Comandini, G. Blanda, A. Cardinali, L. Cerretani, A. Bendini, M.F. Caboni, J. Sep. Sci 31 (2008) 3257.
[185] P. Bridle, C. Garcia-Viguera, Food Chem 59 (1997) 299.
[186] D. Calvo, R. Saenz-Lopez, P. Fernandez-Zurbano, M.T. Tena, Anal. Chim. Acta 524 (2004) 207.
[187] J.M. Herrero-Martinez, F.Z. Oumada, M. Roses, E. Bosch, C. Rafols, J. Sep. Sci. 30 (2007) 2493.
[188] Y. Sun, N. Fang, D.D.Y. Chen, K.K. Donkor, Food Chem. 106 (2007) 415.
[189] J. Pazourek, G. Gonzalez, A.L. Revilla, J. Havel, J. Chromatogr. A 874 (2000) 111.
[190] L. Arce, M. Teresa Tena, A. Rios, M. Valcarcel, Anal. Chim. Acta 359 (1998) 27.
[191] A. Cifuentes, B. Bartolome, C. Gomez-Cordoves, Electrophoresis 22 (2001) 1561.
[192] H. Horie, T. Mukai, K. Kohata, J. Chromatogr. A 758 (1997) 332.
[193] P.J. Larger, A.D. Jones, C. Dacombe, J. Chromatogr. A 799 (1998) 309.
[194] M.B. Barroso, G. Van de Werken, J. High Resolut. Chromatogr. 22 (1999) 225.
[195] S. Kodama, A. Yamamoto, A. Matsunaga, H. Yanai, Electrophoresis 25 (2004) 2892.
[196] J.M. Herrero-Martinez, C. Rafols, M. Roses, E. Bosch, C. Lozano, J.L. Torres, Electrophoresis 24 (2003) 1404.
[197] H.-Y. Huang, W.-C. Lien, C.-W. Chiu, J. Sep. Sci. 28 (2005) 973.
[198] Y. Peng, Q. Chu, F. Liu, J. Ye, Food Chem. 87 (2004) 135.
[199] Y.Y. Peng, J.N. Ye, Fitoterapia 77 (2006) 171.
[200] Z. Chen, J. Zhang, G. Chen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 863 (2008) 101.
[201] Q. Chu, L. Fu, T. Wu, J. Ye, Biomed. Chromatogr. 19 (2005) 149.
[202] C. Fang, X. Wan, H. Tan, C. Jiang, Ann. Chim. (Rome, Italy) 96 (2006) 117.
[203] G. Dinelli, I. Aloisio, A. Bonetti, I. Marotti, A. Cifuentes, J. Sep. Sci. 30 (2007) 604.
[204] C. Li, J. Zhang, A. Chen, J. Xie, X. Chen, Z. Hu, Biomed. Chromatogr. 19 (2005) 369.
[205] G.A. Micke, N.M. Fujiya, F.G. Tonin, A.C.O. Costa, M.F.M. Tavares, J. Pharm. Biomed. Anal. 41 (2006) 1625.
[206] Y. Zhang, J. Chen, L. Zhao, Y.-P. Shi, Biomed. Chromatogr. 21 (2007) 987.
[207] G. Chen, J. Zhang, J. Ye, J. Chromatogr. A 923 (2001) 255.