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Review

Extraction, separation, and detection methods for phenolic acids and flavonoids

The impetus for developing analytical methods for phenolic compounds in natural products has proved to be multifaceted. Hundreds of publications on the analysis of this category of compounds have appeared over the past two decades. Traditional and more advanced techniques have come to prominence for sample preparation, separation, detection, and identification. This review provides an updated and extensive overview of methods and their applications in natural product matrices and samples of biological origin. In addition, it critically appraises recent developments and trends, and provides selected representative bibliographic examples.
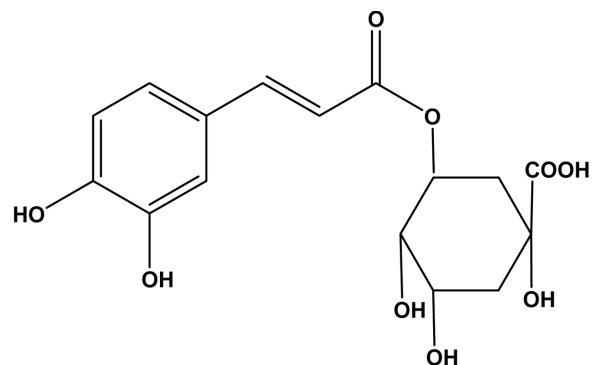
1 Introduction

Vascular plants are able to synthesise a multitude of organic molecules/phytochemicals, referred to as "secondary metabolites" [1, 2]. These molecules are involved in a variety of roles in the life span of plants, ranging from structural ones to protection. Phenolic compounds are regarded as one such group that are synthesised by plants during development [1, 3] and in response to conditions such as infection, wounding, UV radiation, *etc.* [4, 5]. Approximately 8000 naturally occurring compounds belong to the category of "phenolics", all of which share a common structural feature: an aromatic ring bearing at least one hydroxyl substituent, *i.e.* a phenol [6]. A straightforward classification attempts to divide the broad category of phenolics into simple phenols and polyphenols, based exclusively on the number of phenol subunits present [7]. Thus, the term "plant phenolics" encompasses simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, up to hydrolysable and condensed tannins, lignans, and lignins.

Phenolic acids are aromatic secondary plant metabolites widely distributed throughout the plant kingdom

Figure 1. Structure of 5-caffeoylquinic acid (chlorogenic acid)

[8]. The term "phenolic acids", in general, designates phenols that possess one carboxylic acid functionality. However, when talking about plant metabolites, it refers to a distinct group of organic acids. These naturally occurring phenolic acids contain two distinctive carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures (Table 1). Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring make the difference and establish the variety. Caffeic, *p*-coumaric, vanillic, ferulic, and protocatechuic are acids present in nearly all plants [9, 10]. Other acids are found in selected natural sources (*e.g.*, gentisic, syringic). Pre-eminent amongst cinnamic acids, as far as natural occurrence is concerned, is chlorogenic acid (5-0-caffeoylquinic acid) which is caffeic acid esterified with quinic acid (Fig. 1).

The polyphenols, to which the flavonoids belong, possess at least two phenol subunits; compounds possessing

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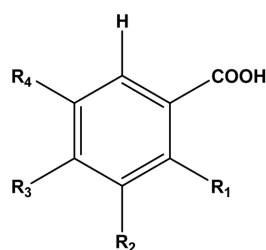**E-mail:** cstalika@cc.uoi.gr
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Abbreviations: **ACN**, acetonitrile; **CCC**, counter-current chromatography; **ECD**, electron capture detection; **EI**, electron ionisation; **FID**, flame ionisation detection; **LLE**, liquid-liquid extraction; **MeOH**, methanol; **MSPD**, matrix solid-phase dispersion; **PDA**, photodiode array; **SFE**, supercritical fluid extraction; **SPME**, solid-phase microextraction; **TMS**, trimethylsilyl (derivatives); **UPLC**, ultra-performance liquid chromatography

Table 1. Structures of the prominent naturally occurring phenolic acids.

Hydroxybenzoic Acids

Name	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H
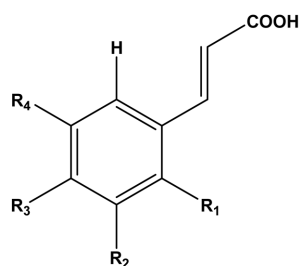
Hydroxycinnamic Acids

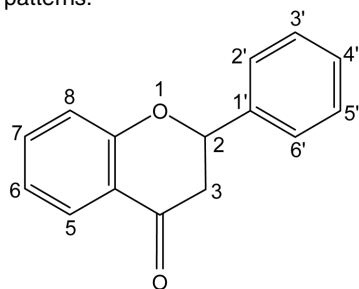| Name	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

Figure 2. Basic structure of flavonoids

nols, flavanonols, flavan-3-ols, anthocyanidins, and anthocyanins (acylglycosides and glycosides of anthocyanidins) (see Table 2).

## 1.1 Sources and role of phenolic acids and flavonoids in plants

Insoluble phenolics are distributed in the cell walls, while soluble phenolics are compartmentalised within the plant cell vacuoles [12–15]. Various phenolic acids have been found during the different stages of maturation [16] while growing conditions are known to have an impact on the phenolic acid content [17]. Many of the phenolic acids like cinnamic and benzoic acid derivatives exist in all plant and plant-derived foods (*e.g.*, fruits, vegetables, and grains) [18]. However, only a minor fraction exists in the free acid form. The major fraction is linked through ester, ether, or acetal bonds to cellulose, proteins, lignin [19, 20], flavonoids, glucose, terpenes, *etc.* [21, 22]. This diversity is one of the factors contributing to the complexity of the analysis of phenolic acids.

Although much knowledge is to be obtained with respect to the role of phenolic acids in plants, they have been associated with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy [23–25].

Flavonoids are universal within the plant kingdom; they are the most common pigments next to chlorophyll and carotenoids. They generally occur in plants as glycosylated derivatives and their physiological roles in the ecology of plants are diverse. Due to their attractive colours, flavones, flavonols, and anthocyanidins may act as visual signals for pollinating insects. In consideration of their astringency, catechins and other flavanols can represent a defence system against insects harmful to the plants [26]. Moreover, flavonoids act as catalysts in the light phase of photosynthesis and/or as regulators of ion channels involved in phosphorylation [27]. They also function as stress protectants in plant cells by scavenging reactive oxygen species produced by the photosynthetic electron transport system [28]. Finally, because of their UV-absorbing properties, flavonoids protect plants from

three or more phenol subunits are referred to as tannins (hydrolysable and non-hydrolysable). Flavonoids are planar molecules ubiquitous in plants, formed from the aromatic amino acids phenylalanine, tyrosine, and malonate [11]. The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6–C3–C6), which are labelled A, B, and C (Fig. 2). Their structural variation emanates, in part, from the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation. Among the many classes of flavonoids, those of particular interest to this review article are flavones, flavanones, isoflavones, flavo-

Table 2. Different classes of flavonoids and their substitution patterns.

Flavones

Position Compound	5	7	3′	4′
Apigenin	OH	OH	-	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	–	–
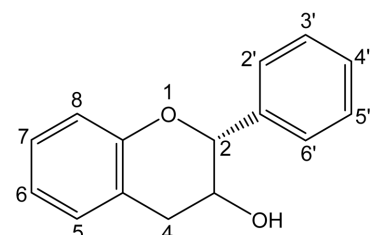
Flavan-3-ols

Position Compound	3	5	7	3′	4′	5′
(+)-Catechin	βOH	OH	OH	OH	OH	–
(−)-Epicatechin	αOH	OH	OH	OH	OH	-
(−)-Epigallocatechin	αOH	OH	OH	OH	OH	OH
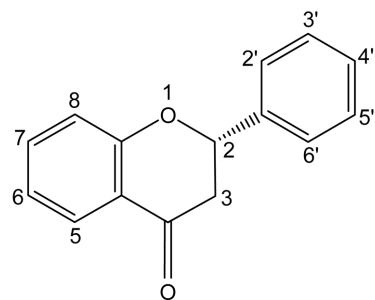
Flavanones

Position Compound	5	7	3′	4′
Naringenin	OH	OH	-	OH
Naringin	OH	0-Rha-Glu	–	OH
Hesperetin	OH	OH	OH	OCH$_3$
Hesperidin	OH	0-Rha-Glu	OH	OCH$_3$

Table 2. Continued
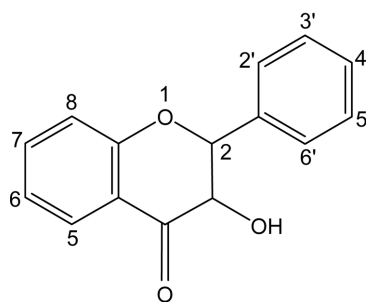
Flavonols

Position Compound	5	7	3′	4′	5′
Quercetin	OH	OH	OH	OH	–
Kaempferol	OH	OH	–	OH	–
Galangin	OH	OH	–	–	–
Fisetin	–	OH	OH	OH	–
Myricetin	OH	OH	OH	OH	OH
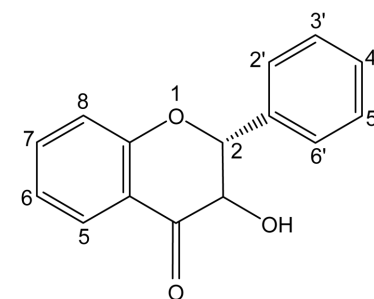
Flavanonol

Position Compound	5	7	3′	4′
Taxifolin	OH	OH	OH	OH
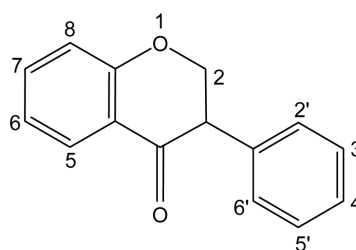
Isoflavones

Position Compound	5	7	4′
Genistein	OH	OH	OH
Genistin	OH	0-Glu	OH
Daidzein	–	OH	OH
Daidzin	–	0-Glu	OH
Ononin	OH	0-Glu	CH$_3$

Table 2. Continued
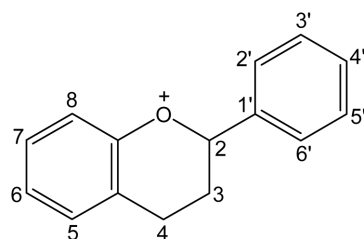
Anthocyanidins

Position Compound	3	5	7	3′	4′	5′
Cyanidin	OH	OH	OH	OH	OH	–
Cyanin	O-Glu	OH	OH	OH	OH	–
Peonidin	OH	OH	OH	OCH$_3$	OH	–
Delphinidin	–	OH	OH	OH	–	OH
Pelargonidin	OH	OH	OH	–	OH	–
Malvidin	OH	OH	OH	OCH$_3$	OH	OCH$_3$

Glu: Glucoside, Rha-Glu: Rhamnoglucosyl

the UV radiation of the sun and scavenge UV-generated reactive oxygen species [29].

1.2 Phenolic acids and flavonoids in human health

Phenolics have an array of health-promoting benefits; they are of current interest due to their important biological and pharmacological properties, especially the antiinflammatory [30], antioxidant [31], and antimutagenic and anticarcinogenic activities [32, 33]. Since they are widespread in plant-based foods, humans consume phenolic acids on a daily basis. The estimated range of consumption is 25 mg to 1 g a day, depending on diet (fruit, vegetables, grains, teas, coffees, spices, *etc.*) [34]. Phenolic acids are amenable to conjugation reactions *in vivo* with sulphate, glucuronate, S-adenosylmethionine, or a combination thereof [35].

Apart from their physiological roles in the plants, flavonoids are considered as important components in the human diet, although they are generally considered as non-nutrients. Flavonoid intake can range between 50 and 800 mg a day, depending on the diet – consumption of vegetables, fruit, red wine, tea, unfiltered beer, *etc*. Another significant source of flavonoids is different medicinal plants and related phytomedicines [36]. With the burgeoning interest in alternative medicine, herbal products are ingested by at least 10% of the general population [37]. Flavonoid glycosides are poorly absorbed until they have undergone hydrolysis by bacterial enzymes in the intestine, whereupon their aglycones can be absorbed. However, recent studies suggest that a fair degree of absorption of flavonol glycosides can also occur in the small intestine [38, 39].

As alluded to earlier, phenolics are structurally assorted and are generally part of a complex mixture isolated from matrices of plant and biological origin. A wide gamut of natural products have been the main focus of study for phenolic compounds while urine and blood are the two main biological fluids that have been analysed for metabolism studies. The rapid and systematic measurement of phenolic acids and flavonoids is a serious challenge for analytical chemists, phytochemists, and biochemists because of their inherent structural diversity and dietary impact. In this context, in 2000, the US Food and Drug Administration (FDA) published a draft of Guidance for Industry Botanical Drug Products (available at http://www.fda.gov/OHRMS/DOCKETS/98fr/001392gd.pdf) [40]. According to this guidance, before a plant drug can be legally marketed, its spectroscopic or chromatographic fingerprints and chemical assay of characteristic markers are required. Because of the complex nature of a typical botanical drug and the lack of knowledge of its active constituent(s), the FDA may rely on a combination of tests and controls to ensure the identity, purity, quality, strength, potency, and consistency of these drugs. Aside from the tests pertaining to the plant drugs themselves, tests and controls should also include botanical raw material and in-process controls and process validation.

The aim of this review article is to pull together the key characteristics of phenolic acids and flavonoids in terms of their extraction, separation, and detection methods in natural products such as plants, plant-derived matrices, and biological samples. Figure 3 illustrates most of the common steps/procedures for the determination of phenolic compounds. These possibilities are critically discussed and future trends are indicated in the present review.

2 Sample preparation

Sample preparation is of paramount importance to any reliable analysis. Many sample preparation methods have been developed to determine polyphenolics and simple phenolics in a wide gamut of sample types. The occurrence of three main types of phenolic-containing matrices, *i.e.* plants, foods, and liquid samples (including biological fluids and beverages), necessitates detailed elaboration of the subject of sample preparation.

Sample preparation procedures for the analysis of phenolic acids and flavonoids can vary a great deal; from the simple filter-and-shoot in the case of several beverages and urine to the more complicated work-up routines, such as hydrolysis of glycosides and extraction/clean-up prior to analysis. Because of the great assortment of phenolics with respect to polarity, acidity, number of hydroxyl groups and aromatic rings, concentration lev-
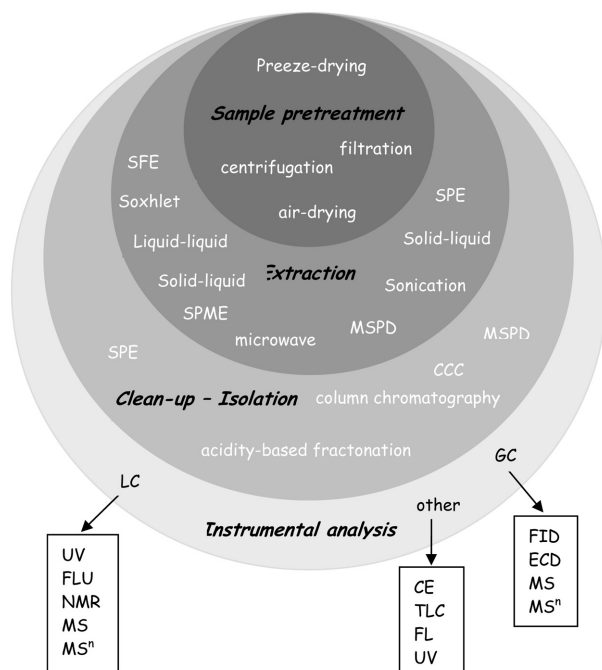
Figure 3. Schematic of strategies for the determination of phenolic acids and flavonoids in biological fluids, beverages, plants, and food. Abbreviations: SFE, supercritical fluid extraction; MSPD, matrix solid-phase dispersion; SPME, solid-phase microextraction; CCC, counter-current chromatography; FL, fluorescence; FID, flame ionisation detection; ECD, electron capture detection.

els, and complexity of the matrix, there is no coherence in the choice of pretreatment procedures. Therefore, it is appropriate to choose the optimal pretreatment method according to the chemical structures and properties of the analysed compounds. The most commonly described assay methods include two or more steps of sample preparation. Each step aims at leveraging the sensitivity and selectivity, but at the same time increases the number of errors through introducing interferents and artifacts and decreases the recovery of the method. It is the analyst's responsibility to control the entire preparation and to evaluate the influence of such effects on the analytical results.

In general, solid samples are usually subjected to milling, grinding, and homogenisation, which may be preceded by air-drying or freeze-drying. Liquid samples are first filtered or centrifuged, after which they are either directly injected into the separation system or the analytes are isolated via additional steps using relevant techniques, described below. Wines, spirits, and clear juice samples have minimal manipulation requirements. Alcohol is most often removed from the sample via rotary evaporation and the residue is taken up in a small volume of the solvent subsequently used in the chromatographic separation. In an attempt to simplify sample preparations for wine analysis and to prevent loss or

decomposition of components, some investigators have succeeded in directly injecting the wine sample after filtration through a 0.45-µm membrane [41, 42].

An important aspect of phenolic analysis is whether the determination focuses on the target analytes in their various conjugated forms or as aglycones. In plants, food products, and biological matrices, researchers usually look at the intact conjugates [43]. When the flavonoids are to be determined in their glycosylated form, digestion is by-passed. In biological fluids (serum, plasma, and urine), flavonoids exist as glucuronide and sulphate conjugates. In many cases, the total aglycone content is determined; therefore, a hydrolysis-digestion step is used to disrupt glycoside or sulphur linkages. The hydrolysis process should be chosen to achieve the highest possible release of aglycones.

2.1 Hydrolysis of phenolic acids

Acidic hydrolysis and saponification are the most common means of releasing the acids, although they may decompose under these conditions. Enzymatic release is an alternative but less prevalent technique.

The acidic hydrolysis method involves treating the plant extract or the food sample itself with inorganic acid (*e.g.* HCl) at reflux or above reflux temperatures in aqueous or alcoholic solvents (methanol being the most common). Acid ranges from 1 to 2 N HCl and the reaction times range from 30 min to 1 h. Aqueous HCl is reported to have destroyed the hydroxycinnamic acids. Krygier *et al.* reported that losses under acidic conditions vary with the form of phenolic acid, ranging from 15 to 95% for *o*-coumaric acid and sinapic acid, respectively [44].

Saponification entails treating the sample with a solution of NaOH at concentrations from 1 to 4 M. Most of the reactions are left to proceed at room temperature for 15 min up to overnight. Some investigations report that the reactions are carried out in the dark, as well as under an inert atmosphere such as argon or nitrogen gas [45].

Enzymatic reactions have been said to release phenolic acids. Enzymes such as pectinases, cellulases, and amylases are employed for the degradation of carbohydrate linkages. The mode of action by which these acids are released is known. Andreasen *et al.* discussed and compared several different enzyme preparations for the release of phenolic acids from the cell wall of rye grains [46]. Yu *et al.* reported that a sequential acid, α-amylase, and cellulose hydrolysis might be applicable to the release of phenolic acids from barley [47].

2.2 Hydrolysis of flavonoids

Hydrolysis, frequently used to remove the sugar moieties from glycosides, may be acidic, basic, or enzymatic. Numerous papers have been cited in an earlier extensive

study relating to the hydrolysis conditions for flavonol glucuronides, flavonol glucosides, and flavone glucosides, for six food samples [48]. These data proved to be different indicating the fact that consensus on the conditions could barely be reached.

Hydrolysis of anthocyanins to anthocyanidins is often indispensable as anthocyanin standards are scarce. Hydrolysis of anthocyanin is typically done by refluxing in MeOH–2 N HCl (aq) [49] or 2 M HCl [50]. Alkaline hydrolysis cleaves the acylated portions of acylated anthocyanins.

Other researchers reported that the phenolic extract of sunflower honey was hydrolysed in 2 N NaOH [51] while the glycosides of flavones and flavonols were hydrolysed by refluxing in 1–2 M HCl in 50% MeOH–H$_2$O v/v [52, 53].

For physiological fluids (bile, plasma, serum, or urine), flavonoids may first be submitted to enzymatic hydrolysis with β-glucuronidase and sulfatase, separately or sequentially [54]. ^{13}C-labeled flavonoid conjugates have been prepared and are available to ensure that these enzymes are active in the incubates [55].

2.3 Extraction

As noted earlier, after proper sample handling, the first steps of a preparation procedure are milling, grinding, and homogenisation. Extraction is the main step for the recovery and isolation of bioactive phytochemicals from plant materials, before analysis. It is influenced by their chemical nature, the extraction method employed, sample particle size, as well as the presence of interfering substances. Additional steps may be called for if the removal of unwanted phenolics and non-phenolic substances such as waxes, fats, terpenes, and chlorophylls is of interest.

Liquid-liquid and solid-liquid extraction are the most commonly used procedures prior to analysis of polyphenolics and simple phenolics in natural plants. They are still the most widely used techniques, mainly because of their ease of use, efficiency, and wide-ranging applicability. Commonly used extraction solvents are alcohols (methanol, ethanol), acetone, diethyl ether, and ethyl acetate. However, very polar phenolic acids (benzoic, cinnamic acids) could not be extracted completely with pure organic solvents, and mixtures of alcohol–water or acetone–water are recommended. Less polar solvents (dichloromethane, chloroform, hexane, benzene) are suitable for the extraction of nonpolar extraneous compounds (waxes, oils, sterols, chlorophyll) from the plant matrix. Other factors, such as pH, temperature, sample-to-solvent volume ratio, and the number and time intervals of individual extraction steps, also play an important role in the extraction procedure. Extractions are,

almost invariably, repeated two to three times and extracts are combined.

Extraction of flavonoids from biological matrices is usually one of the fastest and less time consuming tasks [56, 57]. In addition, due to the simple manipulation of relatively small amount of samples to be extracted, analytical characteristics, such as the relative standard deviation proved to be satisfactory. To quote an example, for the simultaneous quantification of multiple flavonoids in rat plasma, the matrix was treated as follows [58]: The plasma (50 µL) was acidified with 0.25 M HCl (10 µL), mixed with ethyl acetate (1 mL), vortexed, and centrifuged. The upper organic phase (850 µL) was evaporated to dryness; the residue was reconstituted in CH$_3$CN–H$_2$O (24:76, v/v, containing 0.01% HCOONH$_4$) and centrifuged. The supernatant was subsequently used for liquid chromatographic analysis.

Soxhlet extraction is frequently used to isolate flavonoids from solid samples. In most cases, aqueous methanol or acetonitrile is used as solvent. In the literature, reported extraction times vary up to 12 h using this extraction mode. Various flavonoids were extracted from *Tilia europea, Urtica dioica, Mentha spicata*, and *Hypericum perforatum* after 12 h Soxhlet extraction with methanol [59]. Also, phenolic acids were quantitatively obtained by the same extraction technique from the aerial parts of *Echinacea purpurea* [60].

Flavonoids are considered favoured constituents as chemotaxonomic markers in plants because they show large structural diversity and are chemically stable. To distinguish rapidly between various birch species, leaf surface flavonoids were extracted from a single fresh leaf by immersing the whole leaf (without crushing the tissue) for 60 s in 1.5 mL of 95% ethanol contained in an Eppendorf tube [61].

Supercritical fluid extraction (SFE) provides relatively clean extracts, free from certain degradation compounds which may emanate from lengthy exposure to high temperatures and oxygen. Moreover, extracts contain no chlorophyll and other nonpolar compounds which are insoluble in supercritical CO$_2$. This technique is applicable to plant samples and can also be combined with other sample preparation techniques. All samples are usually dried before the SFE assay. As expected, highly polar flavonoids are not extracted by 100% CO$_2$. The solvating power of a supercritical fluid is varied and extraction efficiency is markedly improved by controlling the pressure or by adding organic modifiers, such as methanol. In the SFE of flavonoids from *Scutellaria radix*, Lin *et al.* observed that, for 1 g of sample, adding 3 mL of 70% methanol in 20 mL of CO$_2$ gave much better extraction than pure methanol [62]. This might be because 30% of water would further increase the polarity of the modifier, and polar constituents would thus be extracted more easily. In another report, SFE was compared with Soxhlet extrac-

tion, steam distillation, and maceration for the isolation of the active components present in chamomile flower heads [63]. The recovery of the flavonoid apigenin obtained by supercritical CO_2 after a 30-min extraction at 200 atm and 40°C, was 71.4% compared to Soxhlet extraction performed for 6 h and 125% compared to maceration performed for three days. For some phenolic compounds, the extraction recoveries are not sufficiently high because the content of the organic modifier is not sufficient for their complete isolation, especially in the case of very polar phenolic acids.

Pressurised fluid extraction utilises conventional solvents at controlled temperatures and pressures and has been widely applied as a routine tool in natural product extraction. As it uses less solvent in a shorter period of time, can be automated, and retains the sample in an oxygen- and light-free environment, it has the potential to be a powerful tool in the nutraceutical industry. This kind of extraction was proposed for the isolation of catechin and epicatechin from tealeaves and from grape seeds [64].

A microwave-assisted extraction procedure was developed for the simultaneous determination of isoflavonoids in *Radix Astragali* [65]. The procedure showed the highest extraction efficiency when compared to Soxhlet, reflux, and ultrasonic extraction. A feature of conventional extraction is that it influences the integrity of flavonoid glycosides during prolonged extraction, thus affecting reproducibility. According to this report, the researchers overcame this drawback by using microwave-assisted extraction.

An approach for automated, continuous, and rapid extraction of flavonoids from *Saussurea medusa* Maxim dried cell cultures has been developed in a newly-designed dynamic microwave-assisted extraction system [66]. The main factors affecting the extraction process, namely power of microwave irradiation, liquid/solid ratio, flow rate of solvent, and irradiation time, were optimised. By comparing dynamic microwave-assisted extraction with dynamic solvent extraction without microwave assistance, the former showed obvious advantages of short extraction time and high efficiency. Finally, microwave irradiation and sonication have been successfully used to enhance the extraction of phenolic acids from *Echinacea purpurea* [67].

In addition to the aforementioned techniques, mechanical means are sometimes employed to enhance molecular interaction: vortexing followed by centrifugation [68], mechanical stirring, and continuous rotary extraction [69].

2.4 Clean-up – isolation

Food samples contain both polyphenolics and simple phenolics. The most commonly reported, albeit less efficient, technique of fractionation is based on acidity. The pK$_a$ of the phenolic hydrogen is around 10, whereas that of the phenolic carboxylic acid proton is between 4 and 5. Removal of neutral compounds is performed after treatment with NaOH. A sequence of acidification, treatment with $NaHCO_3$, and extraction step then isolates the phenolic acids [70].

Queiroz *et al.* fractionated phenolics by elution of *Blumea gariepina* extract through silica gel and RP-18 column chromatography with a solvent gradient to yield the fractions [71]. The method is labour-intensive and solvent-consuming; however, it ensures that great amounts of fractions can be obtained for use in subsequent isolation and identification of pure substances.

Solid-phase extraction is a good choice for the clean-up procedure of crude plant extracts or biological samples. The SPE method is fast and reproducible, and fairly clean extracts are obtained; it is essentially emulsion-free and small sample volumes can be used. A very simple SPE method is required for all acidic and basic analyte isolation from the "crude" plant extract and high recoveries are common for this simple procedure. There is a consistency in the choice of sorbents for isolating the phenolic acids and flavonoids. Most frequently, the sorbent is C_{18} bonded silica and the sample solution and solvents are usually slightly acidified to prevent ionisation of the phenolics, which could greatly reduce their retention.

In a recent study, different sample preparation methods for human plasma phenolic compounds (six phenolic acids, five flavonoids, *trans*-resveratrol, and tyrosol) were compared [72]. The recovery values of sample treatments (SPE, extraction with methanol, deproteination, and inhibition of enzymatic plasma activity) were assessed. Given the aim of quantitating the whole set of compounds, the most suitable approach was to inhibit enzymatic activity and then deproteinate with acidified ethanol.

Hydrolysed and non-hydrolysed acidified urine samples were analysed, elsewhere, by passage through Amberlite XAD-2 particles and stirring to retain the phenolic compounds on the surface of the nonionic Amberlite particles [73].

The great advantage of SPE is the possibility of combining on-line extraction with HPLC, and thus realizing the so-called direct sample analysis. This means that the "crude" extract of plant material is injected directly into this SPE–HPLC system [74].

In a more straightforward mode, solid-phase microextraction was employed to extract genistein and daidzein from human urine in combination with LC–MS analysis. A Carbowax-templated poly(divinylbenzene) resin proved to be the best fibre type, with a 5-min extraction at pH 4 and a temperature of 35°C, negating completely the need for organic solvent [75].

Molecular imprinting is an emerging approach expected to offer a quick, simple, and selective alterna-

tive for extracting certain active components from natural products. Even though the molecular structures of the flavonoids are not ideal for molecular imprinting, as the presence of several OH groups in the polyphenolic structure could be the source of hydrogen bonding and nonlocalised electrostatic interactions between the OH groups, some attempts have been made in this direction. The objective of the work of Theodoridis *et al.* was to fabricate molecularly imprinted polymers for the specific adsorption of rutin and quercetin [76]. The two flavonoids were used as the template molecules for the preparation of polymer phases. Relatively high imprinting factors for target flavonoids were obtained with the polymeric materials although highly nonspecific binding was observed.

Xie *et al.* demonstrated that a molecularly imprinted polymer cartridge was able to trap a specific class of compounds including quercetin and kaempferol from the hydrolysate of ginkgo leaves [77]. The polymer was prepared using quercetin, a typical active compound of the flavonoid family in ginkgo leaves, as the template. It exhibited high selectivity for quercetin and good affinity to its structural analogues. Another molecularly imprinted polymer was evaluated toward six phenolic acids [78]. The polymer was prepared with protocatechuic acid as template, acrylamide as functional monomer, ethylene glycol dimethacrylate as crosslinking monomer, and ACN as porogen. Selective extraction of the analytes from the plant extract of *Melissa officinalis* was presented, although poor recoveries of 56.3–82.1% were attained.

Matrix solid-phase dispersion (MSPD) is another alternative for sample preparation of fruits, vegetables, herbs, and other plant matrices. This technique consists of distinct steps in a single process: matrix homogenisation with a silica-based phase, cellular disruption, extraction, and purification. Sample extraction and clean-up are carried out simultaneously with, generally, good recoveries and precision. MSPD is frequently used to determine pesticides in, *e.g.* fruits, vegetables, beverages, and foods, and application to flavonoid analysis was reported only recently. For the determination of isoflavone aglycones and glycosides in *Radix Astragali,* MSPD was compared in terms of its extraction capacity to Soxhlet and ultrasonic extraction [79]. For aglycones, MSPD yielded the best extraction efficiency but for glycosides Soxhlet extraction proved to be more efficient. MSPD was also used for sample preparation of *Melissa officinalis* prior to liquid chromatography of rosmarinic, caffeic, and protocatechuic acids present in this herb [80]. Different MSPD sorbents and various elution agents were tested and the optimal extraction conditions were determined with the aim of obtaining extraction recoveries greater than 90% for all analytes. MSPD has been demonstrated to be a suitable preparation technique, a simple alternative to

liquid-liquid, solid-liquid extraction, SPE, and SFE, for the isolation of phenolics from plant material.

Counter-current chromatography (CCC) is an all-liquid method, representing an alternative chromatographic technique for fractionation without solid phases. It relies on the partitioning of a sample between two immiscible solvents to achieve separation. CCC is gaining popularity as a purification tool for natural products and especially in the bioassay-guided fractionation of plant-derived compounds. High-speed centrifugal CCC has been explored for the fractionation of red wine phenolics [81]. Phenolics were extracted first from red wine into ethyl acetate. Subsequently, the phenolic extract was chromatographed using a cation-exchange column and non-phenolic constituents were washed out from the column with water. Phenolics were eluted with aqueous methanol (75%, v/v) and the extract was fractionated using high-speed CCC in a water–ethanol–hexane–ethyl acetate solvent mixture.

Baumann *et al.* developed a simple and efficient procedure for the separation of catechin gallates from spray-dried tea extract [82]. Tea phenolic extract was first subjected to liquid–liquid partitioning between ethyl acetate and water. The organic layer containing catechins was then submitted to high-speed CCC operating in an ascending mode. Partitioning was achieved using *n*-hexane–ethyl acetate–water (1:5:5, v/v/v) or ethyl acetate–methanol–water (5:1:5 and 5:2:5, v/v/v). A Sephadex LH-20 column with methanol as a mobile phase was used for the final purification of catechin gallates.

By the same method, several milligrams of flavonoids were obtained from 1 kg of seeds of *Vernonia anthelmintica* Willd using two different types of solvent systems, *i.e.* chloroform–dichloromethane–methanol–water (2:2:3:2, v/v) and 1,2-dichloroethane–methanol–acetonitrile–water (4:1.1:0.25:2, v/v) [83]. Each isolated component showed 95–97% purity, as determined by HPLC.

3 Separation – detection

3.1 Thin-layer chromatography

Although less used in analysis, since the early 1960s, thin-layer chromatography has been in vogue in phenolic analysis and still plays a distinct role in the determination of phenolic acids in natural products [84, 85]. It is especially useful for the rapid screening of plant extracts for pharmacologically active substances prior to detailed analysis by instrumental techniques because of its capacity for high sample throughput. In most cases, TLC entails using silica as stationary phase and plates are developed with either a combination of 2-(diphenylboryloxy)ethylamine and polyethylene glycol or with $AlCl_3$. Detection is mainly performed using UV light at 350–

365 or 250–260 nm or with densitometry at the same wavelengths.

Soczewinski *et al.* used double-development TLC to separate a mixture of flavonoids containing nine glucosides and seven aglycones [86]. The more polar glycosides were separated using an eluent with high solvent strength. After solvent evaporation, the aglycones were separated in a subsequent step in the same direction with another, relatively weak, eluent.

Quantification generally is not the main goal of TLC studies. However, densitometry is used in several studies to achieve this goal. Kaempferol and quercetin were determined in an extract of *Ginkgo biloba* leaves by scanning the high performance TLC silica plates in the reflectance mode at 254 nm [87]. The recoveries using a standard spiking procedure were above 94%. In like manner, Janeczko *et al.* determined genistin and daidzin at 260 nm in various soy cultivars [88].

Two-dimensional TLC on cyanopropyl-bonded silica method was employed to separate eight flavonoids and three phenolic acids in *Flos sambuci* [89]. A normal-phase separation was the first dimension where seven binary eluents were tested, and a reversed-phase separation was the second one, studied by using three binary eluents. The three best combinations contained *n*-hexane in the first, and water in the second dimension. More than 12 spots were discriminated and nine flavonoids and three phenolic acids were (tentatively) identified in the *Flos sambuci* L. extract.

Lewis *et al.* determined anthocyanins, flavonoids and phenolic acids in potatoes [90]. They reported that chromatography was carried out on cellulose thin layers using the following developing solvents: (i) 15% (v/v) acetic acid in water; (ii) *n*-butanol, acetic acid, water (4:1:2); and (iii) acetic acid, HCl, water (30:3:10). Analysis of the various tissue extracts was carried out by two-dimensional TLC on cellulose plates developed with *n*-butanol, acetic acid, water followed by acetic acid in water. The dry TLC plates were observed under UV light before and after exposure to ammonia fumes or after spraying with chromogenic spray reagents to visualise spots and characterise groups of compounds.

A rapid high-performance TLC densitometric method has been proposed for the simultaneous quantification of gallic and ellagic acids in herbal raw materials [91]. The method was validated for precision, repeatability and accuracy.

Maleš and Medic-Saric presented an optimised TLC method for the analysis of flavonoids and phenolic acids on silica gel 60 F$_{254}$ [92]. The most suitable chromatographic system for the separation of investigated compounds was ethyl acetate-formic acid-water (65:15:20, v/v/v). Visualisation of the flavonoids and phenolic acids was achieved by spraying the sheets with 1% methanolic diphenylboryloxyethylamine followed by 5% ethanolic

polyethylene glycol 4000. The chromatograms were evaluated in UV light at λ = 366 nm (flavonoids appeared as orange-yellow bands and phenolic acids as blue fluorescent bands). The separation power of thirteen thin-layer chromatographic systems was evaluated by chemometric approaches.

Special attention is to be drawn on the native fluorescence of flavonoids [93, 94]. Fluorescence properties of flavonoids were examined applying TLC separation with fluorodensitometric detection [93]. The native fluorescence of fourteen flavone and twenty six flavonol type compounds were enhanced by their *in situ* reaction on the plate with diphenylboric acid 2-aminoethyl ester leading to lower detection limits. It was recommended that for the sake of reproducibility, reference standards at appropriate concentrations should be measured on the same plate. A correlation between fluorescence and molecular structure was feasible.

3.2 Gas chromatography

Many volatile compounds are directly amenable to analysis by gas chromatography, a technique of unsurpassed separation capacity. In particular, when combined with mass spectrometry it offers high sensitivity and selectivity. One chemical characteristic of the OH group in phenolic compounds is the hydrogen bonding capability, which increases the melting point. Consequently, the significant concern with this technique is the low volatility of phenolic compounds. Gas chromatography is a major chromatographic technique employed for the analysis especially of phenolic acids in plants.

Preparation of samples for GC may include the removal of lipids from the extract, and as mentioned earlier, liberation of phenolics from ester and glycosidic bonds by alkali, acid, or enzymatic hydrolysis. Traditionally, analysis in the gas phase requires a chemical derivatisation step, in addition to sample extraction, isolation, and clean-up.

Hyphenation of chromatographic and spectroscopic methods is important in analytical chemistry and is of great value in modern natural product analysis. But early work with derivatised phenolics was typically performed with flame ionisation detection (FID). Mass spectrometry later became widespread. Most of the GC–MS work is performed in the electron impact ionisation mode, with the ionisation voltage set to a standard 70 eV. The spectra are collected up to m/z 650 in the scan mode.

There are a variety of reagents used to modify and generate volatile derivatives via converting hydroxyl groups to ethers or esters. Prior to chromatography, phenolics are usually transformed into more volatile derivatives by methylation, conversion into trimethylsilyl (TMS) derivatives, or derivatisation with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide. Typically, in GC analysis, fla-
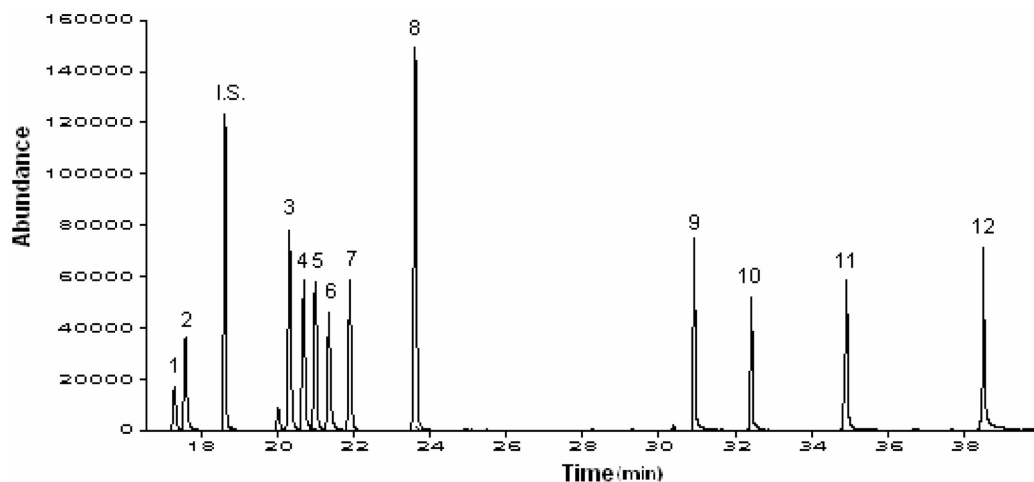
Figure 4. GC-MS (SIM) chromatogram of a *Mentha spicata* fortified extract after derivatisation with methyl iodide and phase-transfer catalysis. Peak assignment: 1. *p*-hydroxy benzoic acid, 2. *trans*-cinnamic acid, 3. homovanillic acid, 4. vanillic acid, 5. 2-hydroxy cinnamic acid, 6. 4-hydroxy cinnamic acid, 7. syringic acid, 8. ferulic acid, 9. naringenin, 10. galangin, 11. kaempferol, 12. luteolin, I.S. internal standard [59].

vonoids are hydrolysed and converted into their derivatives, injected onto a non-polar column (1% phenyl–99% methyl polysiloxane or 5% phenyl–95% methyl polysiloxane) in the split or splitless mode, and separated with a linear 30–90 min temperature programme up to 300°C. GC in the identification of aglycones as silylated derivatives completed by mass selective detection can be regarded as fairly acceptable in the identification of phenolics. Care is taken to ensure anhydrous conditions during the preparation and derivatisation process because of the high sensitivity of TMS derivatives to moisture.

The early GC–MS study of perdeuteromethylated flavonoid aglycones is of theoretical importance. Methylation was carried out with C_2H_3I and NaH in dimethylformamide [95]. This method provided information about the sugar sequence, their interglycosidic linkages, and the sugar attachment to the aglycone.

Diazomethane is often used for the generation of methyl esters. Although solutions of diazomethane react efficiently with carboxylic acids, it must be generated in the laboratory, and is explosive and harmful. Hušek made use of ethyl and methyl chloroformate for the formation of ethyl and methyl esters, respectively [96]. Dimethyl sulphoxide with methyl iodide in an alkaline medium is another alternative to methylation. However, methyl esters can lead to some confusion, as they are naturally occurring in some plant-based material.

An improved derivatisation procedure using in-vial derivatisation–extraction for the GC–MS analysis of methylated flavonoids and phenolic acids in various herbs has been proposed by Stalikas *et al.* [59, 97, 98]. Derivatisation takes place under basic conditions so that the hydroxyl groups of the analytes are deprotonated. The anionic nucleophiles are transferred to the organic phase as ion pairs using a phase-transfer catalyst and are next subjected to reaction with methyl iodide. Polymer-bound tri-*n*-butylmethylphosphonium chloride proved to be the best catalyst. In the SIM mode, the LODs of the flavonoids in the extracts were 4–40 ng/mL. A relevant chromatogram of a *Mentha spicata* extract treated under these conditions is shown in Fig. 4.

There are many advantages to generating the silylated derivatives instead of using other derivatising agents. Phenols and carboxylic acids are relatively reactive and are easy functional groups to silylate. However, in some instances, the derivatives can be unstable once removed from the silylating medium and exposed to the moisture in the air. A common step taken to prevent decomposition is to cover the silylated derivatives with an organic solvent (*e.g.*, hexane, isooctane) after removal of the solvent from the derivatisation reaction step. The derivatisation reaction involves dissolving the dried sample in pyridine or ethylamine (*i.e.* base), adding the TMS reagent, and then heating the reaction vial for 20–60 min. Both functional groups (*i.e.* carboxyl and phendic) are derivatised in a single step. Moreover, many of the minor products or artifacts have been well described and documented, are extremely volatile, elute very early, and do not interfere with the analysis [99].

Although there have been some endeavours to speed up the silylation procedure, most of the literature indicates that heating is still the predominant technique. Chu *et al.*, reasoning that the heat transfer was a slow process, devised a microwave derivatisation procedure cutting the time to 30 s [100].

An ultra-sensitive GC–MS method was developed for the quantitation of catechin, quercetin and resveratrol in biological fluids applying fisetin as internal standard

Table 3. Representative examples of sample preparation and gas-chromatographic methods for the analysis of phenolic acids and flavonoids.

Analytes	Sample source	Sample preparation	Derivatisation conditions	Chromatographic method details	Detector	Ref.
gallic acid, *p*-hydroxyben-zoic acid, gentisic acid, *p*-coumaric acid, vanillic acid, ferulic acid, syringic acid, catechin, quercetin, apigenin, naringenin, luteolin, caffeic acid, epicatechin, rutin, hydroxytyrosol	plant extracts	stirred, sonicated and refluxed in aqueous MeOH containing BHT and HCl, at 90°C for 2 h; filtered and extracted with ethyl acetate; evaporation of the organic layer after removal of moisture with anhydrous Na_2SO_4	TMCS and BSTFA in screw cap glass tubes (deactivated with 5% DMDCS in toluene), at 80°C for 45 min	CP-Sil 8 capillary column, 30 m × 0.32 mm id, 0.25-µm film thickness; injector temperature: 280°C; transfer line temperature: 290°C; column temperature program: from 70°C to 135°C at 2°C/min, held for 10 min, to 220°C at 4°C/min, held for 10 min, to 270°C at 3.5°C/min, held for 20 min	MS	105
catechin, taxifolin, epicatechin, methyl ethers of catechin	wine, plasma	incubated at 37°C in a shaking water bath, for 45 min, in nitrogen-flushed tubes containing sulfatase and β-glucuronidase; extracted with methylene chloride and water, vortexed for 1 min and centrifuged at 4°C; extraction of aqueous supernatant with ethyl acetate; removal of moisture and drying under nitrogen	pyridine and BSTFA at 65 – 75°C, for 2 h	DB-5 capillary column, 30 m × 0.25 mm id, 0.25-µm film thickness; column temperature programme: 100°C for 3 min, to 260°C at 30°C/min, held for 30 min	MS (SIM)	106
caffeic acid, pinocembrin, galangin	propolis	extracted with 70% ethanol, overnight, at room temperature; evaporation of the extracts to dryness	large excess BSTFA at 65°C, for 30 min	SE-54 capillary column, 9 m × 0.25 mm id; injector temperature: 300°C; detector temperature: 320°C; column temperature programme: from 80 to 280°C at 20°C/min, from 280 to 300°C at 2°C/min, held for 10 min	FID	107
benzoic acid, *p*-coumaric acid, 3,4-dimethoxy-cinnamic acid, ferulic acid, isoferulic acid, caffeic acid	propolis	extracted with 70% ethanol, at room temperature; filtered and evaporated to dryness (the procedure leads to a minimum of waxes and a maximum of active substances)	BSTFA at 60°C, for 60 min	capillary column (15% phenyl –85% methylpolysiloxane), 20 m × 0.30 mm id, 0.1-µm film thickness, connected to a 2 m piece of 0.25 mm id high temperature fused silica served as an interface; cold on-column injector; transfer line temperature: 320°C; column temperature programme: 40°C, at 15°C/min to 390°C, held for 20 min	MS	108
kaempferol, quercetin	urine	hydrolysed with: **a.** sulphatase in acetate buffer pH 5.0, **b.** β-glucuronidase in phosphate buffer pH 6.8, at 37°C, for 1 h and **c.** 3 M HCl at 80°C for 1 h; extracted with SPE ENV cartridges using ACN in water (8:2) and blown to dryness by a nitrogen stream	BSA	RTX-5 capillary column, 30 m × 0.32 id, 0.5-µm film thickness; injector temperature: 250°C; transfer line temperature: 280°C; column temperature programme: 160°C for 1 min, to 290°C at 20°C/min and to 320°C at 5°C/min	MS	109
kaempferol, quercetin, isorhamnetin	*Ginkgo biloba* extract and pharmaceutical preparations	hydrolysed with 1 M HCl in 20% MeOH under sonication, for 15 min; heated at 85°C, for 1 h; extracted with ethyl acetate and derivatised	BSTFA containing 1% TMCS in *N,N*-dimethyl-formamide, at 115°C, for 45 min	HP Ultra1 capillary column, 25 m × 0.20 mm id, 0.33-µm film thickness; injector temperature: 275°C; transfer line temperature: 290°C; column temperature programme: 80°C for 0.1 min, to 245°C at 25°C/min, held for 25.5 min, to 270°C at 60°C/min, held for 8 min	MS	110
flavone, flavonol, isoflavone, flavanone aglycones	standard solutions	–	pyridine, 1,1,1.3,3,3 HMDS, TMCS	RSL 200BP non-polar bonded phase fused silica capillary column, 50 m × 0.25 mm id, 0.2-µm film thickness; column temperature programme: 235°C (for 2 min) to 290°C at 1°C/min	FID	111
ferrulic acid, *p*-coumaric acid, 4-hydroxy-3-meth-oxy-benzoic acid, 4-hydroxybenzoic acid	*Lupinus albus*	(roots and seedlings) homogenised with 80% MeOH in an ultrasonic bath for 30 min; filtered under vacuum and eluted from SCX and C_{18} columns with MeOH	BSTFA containing 1% TMCS	DB-5 capillary column, 30 m × 0.25 mm id; injector temperature: 250°C; column temperature programme: 200°C for 2 min, at 5°C/min to 300°C, held for 12 min	MS	112

Table 3. Continued

Analytes	Sample source	Sample preparation	Derivatisation conditions	Chromatographic method details	Detector	Ref.
benzoic acid, *o*-hydroxybenzoic, cinnamic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, *p*-hydroxyphenyl acetic, phthalic, 2,3-dihydroxybenzoic, vanillic, *o*-hydroxycinnamic, 2,4-dihydroxybenzoic, *p*-coumaric, ferulic, caffeic, sinapic acid	cranberry fruit	ground in distilled-deionised water; acidified with 1 N HCl to pH 2; extracted with diethyl ether. Extraction of ethereal phase with 5% NaHCO₃, acidification with 1 N HCl to pH 2 and extraction with ether; evaporation of ethereal extract to dryness	pyridine and large excess of BSTFA and TMCS with heating at 60°C, for 30 min	DB-5 capillary column, 30 m × 0.35 mm id, 0.25-µm film thickness; injector temperature: 280°C; transfer line temperature: 280°C; column temperature programme: 80°C for 1 min, to 120°C at 5°C/min, to 240°C at 10°C/min, to 280°C at 20°C/min, held for 5 min	MS	113
benzoic acid, *o*-hydroxybenzoic acid, *trans*-cinnamic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, vanillic acid, *o*-hydroxycinnamic acid, 2,4-dihydroxybenzoic acid, *p*-coumaric acid, ferulic acid, caffeic acid, sinapic acid, *trans*-resveratrol, epicatechin, catechin, quercetin, myricetin	plasma	extracted with ethyl acetate by vortexing for 1 min at pH 2.0; dried through anhydrous MgSO₄ and evaporated to dryness	BSTFA and TMCS and heated at 70°C, for 4 h	DB-5 capillary column, 30 m × 0.35 mm id, 0.25-µm film thickness; injector temperature: 280°C, transfer line temperature: 280°C, column temperature programme: 80°C (for 1 min), to 220°C at 10°C/min, up to 310°C at 20°C/min, held for 6 min	MS	114
cinnamic acid, *o*-coumaric acid, caffeic acid	apple and pomegranate juices	apple peel was dried at 50°C for 48 h. After grinding, 80% aqueous MeOH was added, the mixture was centrifuged and the supernatant was taken to dryness. The solid residue was transferred to a volumetric flask and filled to capacity with 20% aqueous MeOH. An aliquot was transferred to a silicone septum capped vial, a microsyringe was then positioned in the extraction vial at a depth of about 1 cm below the surface of the aqueous solution. A solvent drop was formed and the sample solution was stirred during the extraction. After 20 min of extraction, the organic solvent was retracted into the syringe.	BSA was withdrawn into the syringe and mixed with the solvent by successive movements of the plunger through, for 10 min	OV-1 capillary column, 25 m × 32 mm id, 0.1-µm film thickness; injector temperature: 240°C, transfer line temperature: 230°C; column temperature programme: 60°C for 1 min, to 220°C at 20°C/min, held for 1 min, to 240°C at 30°C/min held for 5 min	MS	115
pelargonidin, cyanidin, malvidin, quercetin, apigenin, luteolin, naringenin, hesperetin, trimethoxybenzoic, 4-hydroxybenzoic, vanillic, quinic, chlorogenic and rosmarinic acids	orange, grapefruit, lemon juices	evaporated to dryness at 50–60°C; hydrolysed with TFA for various periods of times; evaporation to dryness	pyridine (containing hydroxylamine hydrochloride), HMDS and TFA at 100°C, for 60 min	BPX5 capillary column, 30m × 0.25 mm id, 0.25-µm film thickness; septum-equipped programmable injector: 150°C for 2 min, to 330°C within 1 min held for 5 min; transfer line temperature: 280°C; column temperature program: 150°C, held for 2 min, to 330°C at 10°C/min, held for 7 min	MS	116
caffeic acid, 3,4-dihydroxyphenylacetic acid, ferulic acid, vanillic acid, sinapic acid, protocatechuic acid, 4-hydroxy-benzoic acid, *p*-hydroxy-phenylacetic acid, *p*-coumaric acid, syringic acid, gallic acid, catechin, epicatechin, tyrosol, vanillin, quercetin, resveratrol, kaempferol	*Vitis vinifera*	homogenised and subjected to freeze drying; sonicated and stirred with MeOH for 15 min and left for 24 h under stirring at room temperature; centrifuged and evaporated; reconstituted in bi-distilled water; isolation of polyphenols from an Isolute C₈ SPE column eluted with ethyl acetate; evaporated to dryness under a stream of nitrogen	BSTFA at 75°C, for 20 min	HP-5 MS capillary column, 30 m × 0.25 mm, 250-µm thickness; injector temperature: 280°C; transfer line temperature 300°C; column temperature program: 70°C for 5 min, to 130°C at 15°C/min, to 160°C at 4°C/min, for 15 min, to 300°C at 10°C/min, held for 15 min	MS	117

Abbreviations: DMDCS, dimethyldichlorosilane; HMDS, hexamethyldisilazane; TFA, trifluoroacetic acid; BSTFA, *N,O*-bis(trimethylsilyl)trifluoracetamide; TMCS, trimethylchlorosilane; BSA, *N,O*-bis(trimethylsilyl)acetamide

[101]. Selective-ion monitoring elutions were based on the quantitation of target ions as follows: $[M + 3TMS]^+ = m/z$ 444 (*trans*-resveratrol), $[M + 5TMS - 248 - CH_3]^+ = m/z$ 369 (catechin), and $[M + 5TMS - CH_3]^+ = 647$ (quercetin), respectively.

In conventional GC, it is very difficult to analyse flavonoid glycosides even after derivatisation. Therefore, Pereira _et al._ used high-temperature–high-resolution GC–MS, with columns that can withstand temperatures up to 400°C, for the glucoside hesperidin [102]. Although GC performed on underivatised phenols and acids is rare, there exist some methodological investigations. Christov _et al._ described flame ionisation as detection method in one such analysis of underivatised acids [103]. The stability of eleven methoxyflavones under certain pyrolytic conditions used allowed for their analysis in _Kaempferia parviflora_ by GC without derivatisation on an HP50+ (crosslinked (50% phenyl)-methylpolysiloxane) column [104].

Other important gas chromatographic methods for the analysis of phenolic acids and flavonoids are presented in Table 3.

3.3 HPLC

In the last twenty years, HPLC has been the analytical technique that has dominated the separation and characterisation of phenolic compounds. Due to the relatively high-molecular mass and intrinsic features of hydrophobic flavonoid aglycones and hydrophilic flavonoid glycosides, the overwhelming majority of chromatographic methods in the literature fall in the realm of HPLC and related technologies. HPLC techniques offer a unique chance to separate simultaneously all analysed components together with their possible derivatives or degradation products. In many cases, they enable the determination of low concentrations of analytes in the presence of many other interfering and coeluting components. There are many advantages dictating the widespread use of HPLC in the analysis of phenolic compounds in plant-derived and biological matrices, such as (i) the wide range of commercially available columns, including those using new generation sorbents with fit-for-purpose properties and (ii) the possibility of combining two or more columns in a switching mode.

3.3.1 HPLC columns

Several reviews have been published on the application of HPLC methodologies to the analysis of phenolics [10, 118]. From a critical appraisal, it seems that there is a great deal of uniformity in column choice. The introduction of reversed-phase (RP) columns has considerably enhanced HPLC separation of different classes of phenolic compounds. Almost exclusively, RP C_{18} phases ranging from 100 to 250 mm in length and usually with an internal diameter of 3.9 to 4.6 mm are employed. Particle sizes are in the usual range of 3–10 μm. Special silica sorbents with reduced metallic residue contents and minimum residual silanol groups on the surface could positively influence peak symmetry as chromatographic resolution and the efficiency of the column are better for columns with very good free silanol group covering, endcapping, or embedding. To a lesser extent, other silica-based chemically bound phases, and non-silica polymers or mixed inorganic–organic phases are employed.

Narrow-bore columns (internal diameter 2 mm) are recommended especially for HPLC–MS applications [119]. When columns with lower diameters and particle sizes are used, the adaptation of HPLC equipment is necessary, _e.g._, UV detection cells with reduced volume, low injection volumes, pumps with accurately-controlled low flow-rate, and low diameter capillary connections [120].

Different RP columns of conventional dimensions were applied for the analysis of flavonoid glycosides [121]. An end-capped column is to be preferred because residual silanol groups appear to impair the separation. For the analysis of phenolic compounds in beer by LC, separation conditions were optimised for a standard mixture of several flavone aglycones and glycosides [122]. Eleven different stationary phases (all C_{18} bonded silicas) were compared with column dimensions of (100–250) mm × (2.0–4.6) mm, inner diameter. On the basis of the experimental evidence, four columns qualified as most appropriate although the variation of many parameters makes it difficult to reach a rational consensus with respect to the pros and cons of the various columns.

Most HPLC analyses of phenolic compounds are performed at ambient column temperature, but moderately higher temperatures between 30 and 40°C have also been recommended. Roggero _et al._, examining phenolic acids and polyphenols in one run, maintained the column temperature at 22.5°C [123]. The researchers emphasised the unquestionable point that the long analysis time (150 min) required constant temperature for reproducibility. Gioacchini _et al._ [124] reported that, in order to achieve highly reproducible retention times, their column was thermostated at 10 ± 0.1°C.

The need for fast separation of bioflavonoids fostered the synthesis of a highly hydrophilic poly(7-oxonorbornene-5,6-dicarboxylic acid-_block_-norbornene)-coated silica for their liquid chromatographic determination in plant extracts [125]. Compared to the most commonly used octadecyl derivatised silica this sorbent allowed fast separation even at extreme pH values.

The importance of monolithic (continuous) beds is connected with their ease of preparation and the far-reaching possibilities of modification of their surface and porous properties. These properties make them particularly attractive for the analysis of biologically important compounds characterised by a wide spectrum of physicochemical properties [126]. So far, there are not many reports about the separation of phenolic compounds on monolithic columns. Tolstikov _et al._ used a

90 cm long and 0.2 mm id capillary monolithic octadecyl silica column for probing the metabolome of a model plant. From amongst the several classes of compounds separated in a single run, flavonoids were prominent peaks in the chromatogram [127].

A fast RP-HPLC method for the simultaneous separation of eleven flavonoid aglycones was developed using another monolithic type column (Chromolith Performance C_{18} endcapped, from Merck). The method was successfully applied to the analysis of these compounds in complex natural samples such as propolis and *Ginkgo biloba* [128]. Stereochemistry in the studied field has rarely been an issue in the recent literature [129]. A number of different chiral stationary phases have been utilised to resolve and separately quantify the enantiomers of chiral flavonoids including chiral polymer phases. These chiral polymer phases can further be sub-divided into polysaccharide-derived columns and cyclodextrin and "mixed" cyclodextrin columns. It is to be reported that Cyclobond I, a β-cyclodextrin stationary phase made up of cyclic glucoamyloses, was the most widely used column in the RP mode and in the normal phase mode to separate the 2*R* and 2*S* diastereomers of flavanone glycosides and benzoylated flavanone glycosides, respectively [130]. Work on the enantiomeric separation of flavanones and the diastereomeric separation of flavanone glycosides has also been reported by Ficarra *et al.* who studied the performance of four chiral liquid chromatographic columns utilizing polysaccharide derivatives (*i.e.* Chiralcel OA, OJ, OC, OD) [131]. A good enantioseparation (*a* up to 1.45) was feasible for most of the racemates.

Two-dimensional LC enables improving the separation quality of various complex samples. In comparison to a single C_{18} column, serially coupled PEG and C_{18} columns show significant improvement in resolution of phenolic antioxidants in beer or wine samples [132, 133]. Figure 5A illustrates the two-dimensional separation of standard phenolic compounds. Figure 5B depicts the separation of a preconcentrated beer extract in a two-dimensional setup with a PEG column in the first dimension and a monolithic C_{18} column in the second dimension, with aqueous ACN buffered at pH = 3 as the mobile phase.

Recently, a greatly improved chromatographic performance has been achieved by the introduction of ultra-performance liquid chromatography (UPLC). As the particle size decreases to less than 2.5 μm, there is a significant gain in efficiency. In addition, efficiency does not diminish at increased flow rates or linear velocities (van Deemter equation). Thus, UPLC capitalises on basic chromatographic principles to perform separations using columns packed with smaller particles and/or at higher flow rates resulting in a shorter analysis time, with superior peak capacity (number of peaks resolved per unit time in gradient separations) and sensitivity. Four flavo-
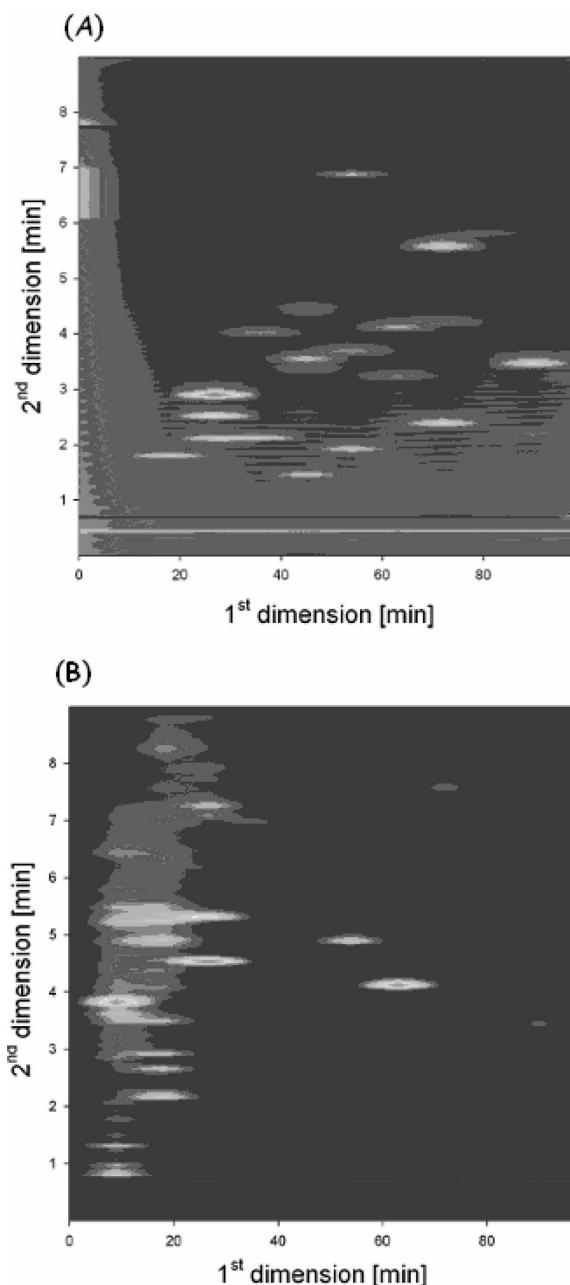
Figure 5. Two-dimensional PEG-C_{18} RP × RP separation of (A) a mixture of phenolic standard solution and (B) of phenolics in concentrated beer sample [133].

noids were determined in the flower of *Trollius ledibouri* from different sources. The analysis was performed on an AcQuity UPLC BEH C_{18} column using gradient elution with a mobile phase of 0.1% acetic acid and acetonitrile over 20 min [134].

The choice of columns depends on the developed sample preparation technique because fairly crude plant extracts could decrease the lifetime of or cause damage to the column. Protection of the main column by adding,

in line, a small guard column containing the same adsorbent as in the column is almost always mandatory and can increase the effective life of a column many times over.

3.3.2 HPLC mobile phases

Both isocratic and gradient elution are applied for analyses of phenolic compounds. The choice depends on the number and type of the analytes and the nature of the matrix. Acetonitrile and methanol are the most commonly used organic modifiers. In some cases, acetonitrile leads to better resolution in a shorter analysis time than methanol and, generally, acetonitrile gives sharper peak shapes, resulting in a higher plate number. However, methanol is often preferable to acetonitrile because of its nontoxic properties and the possibility of using higher percentages in the mobile phase which could protect the HPLC column. Occasionally, tetrahydrofuran and 2-propanol as less polar solvents with their high elution strength have also been used.

A great alteration observed in the mobile phases was the type of acid used as modifier to minimise peak tailing. Most phenolic acids have pK_a of about 4 while flavonoids presenting several ionisable hydroxyl groups have pK_a values relatively close to each other but certainly greater than 4. It is important to avoid the ionisation of analytes during analysis to improve the resolution and reproducibility of the retention characteristics. Therefore, the recommended pH range for the HPLC assay is pH = 2–4. The pH value is controlled by adding small amounts of acids to the water–organic mixture. Aqueous acidified solvents such as acetic, formic, phosphoric, and most rarely perchloric acid were employed. However, phosphate, citrate, and ammonium acetate buffer at low pH were recommended instead of the addition of acid. The buffer concentration can vary from 5 to 50 mM. Dallüge found that deactivated RP-18 columns and the use of trifluoroacetic acid as the acidic modifier of the mobile phase greatly improved peak shape and reproducibility of retention times of catechins in green tea [135].

On using a LiChrosorb RP C_{18} column and a 5% aqueous formic acid and methanol, Casteele et al. have demonstrated the separation of 141 flavonoids from polar triglycosides to relatively nonpolar polymethoxylated aglycones belonging to the classes of flavones, flavonols, flavanones, etc. [136].

Free phenolic acids (chlorogenic, protocatechuic, p-hydroxybenzoic, caffeic, vanillic, syringic, p-coumaric, and ferulic) could be separated in medicinal plants or pharmaceutical preparations using a simple isocratic mobile phase (methanol–water–acetic acid) [137]. In the case when phenolic acids of different chemical structures and different polarities have to be analysed simultaneously, gradient elution is necessary. Generally, phenolic acids are eluted from RP columns according to

decreasing polarities. The loss of polar hydroxy groups and the presence of the methoxy groups or ethylene side chains could decrease the polarity and increase the retention time. The derivatives of common phenolic acids with two or more aromatic rings are less polar and are eluted much later than others. So, the gradient program has to be managed in a case-dependent manner, according to the number and chemical properties of the analysed compounds.

Some phenolic acids could be present in natural plants as geometric isomers. The greatest number of phenolic acids occurs in nature as trans-isomers, but on exposure to UV radiation or daylight they are gradually transformed into cis-isomers, which elute, usually, before trans-isomers. Their simultaneous separation is usually possible using RP stationary phases.

The inclusion of an optically active molecule in the mobile phase can facilitate separation of enantiomers on conventional stationary phases [129]. There are no generally applicable mobile phase additives for flavonoid glycosides separation and assays must be developed individually. However, β- and γ-cyclodextrin and neutral and charged cyclodextrin derivatives were all successful as chiral selectors as they may interact with the enantiomers resulting in diastereomeric pairs which distribute between the achiral stationary phase and the mobile phase.

3.3.3 HPLC detection

Phenolics are commonly detected using ultraviolet/visible (UV/VIS), photodiode array (PDA), and UV-fluorescence detectors. Other methods used for the detection of phenolics include electrochemical coulometric array detection, on-line connected PDA and electroarray detection, chemical reaction detection techniques, mass spectrometric and NMR detection.

Given the intrinsic existence of conjugated double and aromatic bonds, every phenol exhibits a higher or lower absorption in the UV or UV/VIS region. Phenolic acids with the benzoic acid carbon framework have their maxima in the 200 to 290 nm range. The only exception is gentisic acid, which has an absorbance that extends to 355 nm. The cinnamate derivatives, due to the additional conjugation, show a broad absorbance band from 270 to 360 nm.

All flavonoid aglycones contain at least one aromatic ring and, consequently, efficiently absorb UV light. The first maximum, which is found in the 240–285 nm range, is due to the A-ring and the second maximum, which is in the 300–550 nm range, is attributed to the substitution pattern and conjugation of the C-ring [138]. Simple substituents such as methyl, methoxy, and non-dissociated hydroxyl groups generally effect only minor changes in the position of the absorption maxima. Detec-

tion and characterisation of the aglycones also holds true for their conjugates.

It is evident that phenolics absorb well in the UV range and UV detection is therefore a convenient method to localise a phenol in the effluent of a column. However, no single wavelength is sufficient for their simultaneous monitoring in various natural plant extracts. Detection at 280 nm is most generally used for the simultaneous separation of mixtures of phenolic acids although for dual monitoring 254 and 280 nm, or 280 and 320 nm, can be ideal wavelengths. On the other hand, PDA is the most prevalent method, as it allows for scanning real time UV/Vis spectra of all solutes passing through the detector. It could help in the identification of individual compounds in the extracts of complex mixtures, such as extracts of natural plants. Superimposed upon the above advantage is the possibility of PDA giving important information about the purities of all analytes.

HPLC–PDA with post-column addition of UV shift reagents provides rapid information about the polyphenol aglycone and its substitution pattern. Shift reagents induce a displacement of the absorption maxima, which can be used to determine the position of free hydroxyl groups. The application of these shift reagents to the structural elucidation of flavonoids has been extensively described and successfully applied to different polyphenol families [139, 140]. The identification and quantitation of the derivatised flavonol content of beverages, based on the chemical reaction between flavonols and p-dimethylaminocinnamal aldehyde, was utilised in the postcolumn chemical reaction detection version [141], subsequent to their HPLC separation and UV detection at 280 nm. Thus, on the basis of this combined detection system, monitored before (A_{280}) and after derivatisation (A_{640}), both the flavanol profile and the spectral characteristics obtained upon double monitoring served as additional confirmation tools.

The nature of the functional groups and their substitution pattern determine whether a particular flavonoid is fluorescent or not. For example, from amongst the isoflavones, only those that do not have an OH-group in the 5-position show strong native fluorescence. To extend the application range of fluorescence detection, derivatisation has been used. For example, quercetin, kaempferol, and morin, with their 3-OH, 4-keto substituents, can form complexes with metal cations, some of which are highly fluorescent [142, 143].

Fluorescence detection is not very often used in phenolic acid analysis, but, in cases when fluorescence detection is used in combination with UV, it offers the possibility to discriminate between fluorescent and non-fluorescent co-eluting compounds [144]. However, the same problems could arise as in the UV detection, i.e., establishing the correct excitation and emission wavelengths, as large differences were observed for several phenolic

compounds. In this case, the rapid scanning fluorescence detector, in combination with PDA, is available for applying multiple excitation and emission wavelengths [137].

Electrochemical detection is very sensitive for the compounds that can be oxidised or reduced at low-voltage potentials. Phenolic acids in food and human plasma extracts are routinely detected by HPLC-electrochemical coulometric detection +600 mV [145]. Amperometric and conventional coulometric electrochemical detection are generally not compatible with the gradient elution mode. With the recent advances in electrochemical detection, multi-electrode array detection is becoming a powerful tool, compatible with gradient elution, for detecting phenolic acids and flavonoids in a wide range of samples. The multi-channel coulometric detection system may offer a highly sensitive method for the overall characterisation of antioxidants [146, 147].

A combination of HPLC technique and voltammetry has successfully been employed for the detection, identification, and quantification of flavonoid and non-flavonoid phenolics in wine. Positive identification may be obtained by comparing the capacity factor (k') and electrochemical behaviour of wine phenols with those of standard solutions containing pure phenolics [148]. Using a carbon polyethylene electrode and an Ag/AgCl reference electrode, Malher et al. employed electrochemical detection for the analysis of Vidal Blanc wines [149]. Electrochemical behaviour provided additional information for the identification of phenolic acids not previously identified with LC–UV.

In the determination of quercetin levels in the cells of the immune system [150] and in the validated assay of six metabolites originated from artichoke leaf extract in human plasma [151], HPLC combined with coulometric-array detection furnished spectacular selectivity and sensitivity. Recently, Romani et al. compared electrochemical (differential pulse voltammetry and amperometric biosensor) and PDA detection methods for the analysis of phenolics in natural matrices [152]. Of these, HPLC–PDA technique gave the most accurate results, while the differential pulse voltammetry technique which employed graphite screen-printed electrodes was considered as a good and quick method for screening polyphenols in natural extracts.

HPLC–PDA provides valuable information on polyphenol structures in crude or semi-purified plant extracts. However, the identities of non-phenolic moieties in molecules, such as sugars or aliphatic acyl groups, are not revealed by HPLC–PDA, because these groups do not have strong UV chromophores. HPLC coupled with other more powerful detectors can provide this information on-line without previous isolation or hydrolysis of the compounds.

For structure elucidation especially of flavonoids, special attention has been devoted to the use of mass spec-

trometric techniques for the characterisation of several important sub-classes, and to the potential of combined diode-array UV, tandem-MS, and NMR detection for unambiguous identification. The electrospray ionisation and atmospheric pressure chemical ionisation interfaces dominate the analysis of phenolics in herbs, fruits, vegetables, peels, seeds, and other natural samples by LC–MS. In some cases, HPLC, with different sensitivity detectors (UV, electrochemical, fluorescence), and HPLC–MS are simultaneously used for the identification and determination of phenolic acids in natural products and related food products.

The ESI-MS has been employed for structural confirmation of phenolics in plums, peaches, grape seeds, soy food, cocoa, olive oil, and walnut leaves. Complexation of flavonoids with Cu(II) resulted in more intense and simpler to interpret complexes than those of the corresponding flavonoids, a fact that enhances the detection and identification of flavonoids by ESI-MS [153]. Conventional RP columns were coupled to PDA/UV detector and a magnetic sector-type MS equipped with an ESI source was applied to the analysis of flavonoid glycosides in Crataegus extract [121]. It was demonstrated that UV spectra and first-order ESI mass spectra allowed a fast characterisation of flavonoids even if reference compounds were not at hand or available. Identification of phenolics separated after HPLC was also carried out using fast atom bombardment mass spectrometry [154, 155] and electron impact mass spectrometry [154]. On the other hand, matrix-assisted laser desorption/ionisation mass spectrometry has been employed for qualitative and quantitative analysis of anthocyanins in foods [156].

NMR is, no doubt, the technique that generates more information about a molecule. HPLC–NMR is an excellent approach to search for novel biologically active structures and new sources of rare natural products, to identify known compounds without isolation, and to avoid unwanted re-isolation of known constituents. Direct, on-line coupling of an NMR spectrometer to HPLC has required the development of special interfaces called flow probes. These systems can basically work in "on-flow" mode – the solute passes through the system as it passes out of the column and is analysed – or with a valve that causes flow of the effluent to stop when a compound is detected by a UV detector (which is always used in connection with these systems). In the second case, the flow is stopped in order to have enough time for acquisition of the NMR spectrum. A more practical way to perform LC–NMR is to use a loop collector, which automatically collects the peaks of interest without stopping the LC flow. Off-line post-chromatographic analysis of the content of the loops is then automatically performed [157]. In a recent study by de Rijke et al. on the flavonoid constituents of a red clover extract, stopped-flow reversed-phase LC–NMR was performed using a gradient

of deuterated water and deuterated acetonitrile and stand-alone NMR were used to identify structural isomers that could not be distinguished on the basis of MS/MS information [158]. The recently developed cryoflow NMR probe exhibits a detectability four-fold better than with conventional probes or, alternatively, the scan time is 16-fold shorter for the same amount of sample. The probe has been applied for the analysis of an oregano extract where five flavonoids were identified using an LC–UV–SPE–NMR–MS set-up [159]. In Fig. 6, two-dimensional NMR spectra show up for the identification of naringenin and apigenin after trapping on the same SPE cartridge.

Other less common means of detection, coupled to LC, have been refractive index and evaporative light scattering techniques. These two types of detection provide limited selectivity and sensitivity. However, both of them have been successfully used: the HPLC–refractive index system in the quantification of (3,3′,4,4′,5,7-hexahydroxyflavan) in unripe banana pulp [160], and the HPLC–evaporative light scattering detection one in the determination of Ginkgo biloba and Radix Astragali flavonoids [161, 162].

Table 4 gives an overview of relevant information on a selection of typical modern LC examples of sample preparation, separation, and detection conditions reported in the recent literature.

3.4 Capillary electrophoretic and capillary electrochromatographic methods

The electromigration modes primarily used are capillary electrophoresis (CE), capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) with, typically, phosphate or borate buffers, capillaries of 50–100 μm id, voltages of 10–30 kV, and injection volumes of 10–50 nL. Detection is usually performed with UV, but electrochemical and MS detectors are also used. Most studies that use capillary electrophoretic methods for the analysis of phenolics fall in the field of natural product research, including the analysis of plants, vegetables, herbs, and other plant or fruit-derived products [173].

Tea catechin and theaflavins from tea infusions were analysed, by CE and HPLC in parallel, with UV detection [174]. The reproducibility figures were approximately the same with the two methods, however, the analysis time for CE was three times shorter (10 min versus 27 min) but the sensitivity five times lower in comparison to HPLC. Other authors used an amperometric system based on the end-column wall-jet configuration in which the working electrode is placed at the outlet of the separation capillary. This configuration allows the use of normal size (i.e., >100-μm diameter) working electrodes without introducing significant postcapillary zone

Table 4. Representative examples of sample preparation and HPLC detection methods for the analysis of phenolic acids and flavonoids.

Analytes	Sample source	Sample preparation	Stationary phase/mobile phase	Detector	Ref.
gallic, caffeic, sinapic, p-coumaric, chlorogenic, 3,4,5-trimethoxycinnamic acids, myricetin, quercetin	cranberry juice	extraction with Sep-Pak C$_{18}$ cartridge to clean and fractionate free phenolic acids and flavonoids; acid-catalysed hydrolysis process to liberate flavonoids and phenolic acids from their bound forms (adjusted to pH 2.0 with 2.0 M HCl).	Eclipse XDR-C RP column (150 mm × 4.6 mm, 5 µm)/A: H$_2$O-acetic acid (97:3; v/v); B: MeOH; gradient: 100% A to 90% A, 0–10 min; to 30% A, 10–40 min; to 100% A, 40–47 min flow rate: 0.9–1.0 mL/min	UV 280 nm and 360 nm	[163]
myricetin, luteolin, apigenin, kaempferol	vegetables and fruits	extracted and hydrolysed to their aglycones with HCl in 50% aqueous MeOH	Nova-Pak C$_{18}$ column/Isocratic: ACN/phosphate buffer (25:75, v/v, pH 2.4)	UV 370 nm	[164]
catechin, vanillic, syringic acids, epicatechin, trans-resveratrol	wine	adjusted to pH 2, extracted with diethyl ether; the organic layer was evaporated to dryness and redissolved in MeOH-H$_2$O$_2$ (1:1)	Nova-Pak C$_{18}$ column (150 × 3.9 mm, 4 µm)/A: MeOH-acetic acid-H$_2$O (10:2:88, v/v); B: MeOH-acetic acid-H$_2$O (90:2:8, v/v); gradient: 100% A to 85% A, 0-15 min; to 50% A, 15-25 min; to 30% A, 25-34 min. flow rate: 1.0 mL/min	UV 280 nm Fluorescence: ex: 280 nm, em: 360 nm and ex: 330 nm, em: 374 nm	[144]
vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, hyperoside	hawthorn leaves	extracted with ethanol, filtered and extracted with ethyl acetate; concentrated and dissolved in MeOH	Diamonsil C18 column (250 × 4.6 mm id, 5 µm)/Isocratic: THF/ACN/MeOH/0.05% phosphoric acid solution (pH 5.0) (18:1:1:80, v/v/v/v) flow-rate: 1.0 mL/min	UV 340 nm	[165]
20 flavonoids of two different subclasses (flavanone and flavone glycosides), eriocitrin, neoeriocitrin, naringin, narirutin, rutin, hesperidin, neoesperidin, diosmin, neodiosmin, didimin, poncirin	citrus juices	centrifuged at 4500 rpm for 15 min; filtered and kept at temperature of −4°C until analysis	1. Conventional HPLC Ultra C$_{18}$ 250 × 2.1 mm, 5 µm, narrow-bore column/flow-rate: 0.2 mL/min. 2. Micro-HPLC Discovery C$_{18}$ 100 × 1 mm, 3 µm, microbore column. A: water-formic acid (99.9:0.1, v/v); B: water-ACN–isopropanol–formic acid (39.9:20:40:0.1, v/v); gradient: 0–3 min 10% B; 3–43 min from 10% B to 42% B; 43–47 min 42% B, 47–57 min from 42% B to 100% B; 57-60 min from 100% B to 10% B; flow rate: 40 µL/min.	UV 280 nm, MS	[166]
puerarin, daidzin, daidzein baicalin, baicalein	Gegen Qinlian (decoction, granule, pill)	extracted in an ultrasonic bath with MeOH	C18 RP-ODS column (250 × 4.6 mm id, 5 µm)/A: 1% triethylamine, 1% acetic acid, adjusted to pH 3 using phosphoric acid B: MeOH; gradient:: 0–12 min: isocratic 26% B; 12–13 min: linear 26–28% of B; 13–19 min: isocratic 28% B; 19–20 min: linear 28–33.8% of B; 20–37.5 min: isocratic 33.8% B; 37.5–38.5 min: linear 33.8–41% of B; 38.5–49 min: isocratic 41% B; 49–50 min: linear 41–48% of B; 50–59 min: isocratic 48% B; 59–60 min: linear 48–55% of B; 60–71 min: linear 55–70% of B; 71–80 min: isocratic 70% B. flow-rate: 1.0 mL/min	PDA 270 and 346 nm	[167]
catechin, epicatechin	sorrel leaf	refluxed with 50% MeOH for 30 min at 90°C	LUNA C$_{18}$ column (250 × 2 mm id, 5 µm)/1. 10 mm H$_3$PO$_4$/ ACN (88:12 = v/v) and 2. ammonium acetate buffer (10 mm, pH 5.5)/ACN (88:12 = v/v) flow rate: 0.2 mL/min	UV 205 nm, 279 nm ESI-MS/MS Fluorescence: ex: 279 nm, em: 307 nm	[168]
gallic, protocatechuic, gentisic, chlorogenic, 4-hydroxybenzoic, 3-hydroxybenzoic, syringic, vanillic, caffeic, p-coumaric, ferulic, salicylic acids	leaves of yacon (Smallanthus sonchifolius, Asteraceae)	extracted as follows: 1. Soxhlet extraction MeOH; chlorophyll removal with petroleum ether; acidification and extraction with ethyl acetate. 2. Boiled in water (decoction) under reflux and freeze-drying. 3. Boiling water poured onto leaves (infusion) and then allowed to extract for 20 min	Tessek Separon SGX C$_{18}$ column (250 × 4 mm id, 5 µm)/KH$_2$PO$_4$ (25 mm, pH 3.0)/ACN (90:10 or 80:20, v/v). flow rate: 1.0 mL/min.	amperometric detection +550 mV to +1200 mV versus SCE	[169]
myricetin, scutellarein, quercetin, luteolin, kaempferol, apigenin, baicalein	health tea and green tea	extracted with boiling water, acidified with 6 N HCl, liquid-liquid extraction	Inertsil ODS-3 column (250 × 4.5 mm, 5 µm)/isocratic: 0.5% H$_3$PO$_4$/MeOH (1:1, v/v) flow rate: 1 mL/min	UV 349 nm	[170]

Table 4. Continued

Analytes	Sample source	Sample preparation	Stationary phase/mobile phase	Detector	Ref.
chlorogenic acid, quercetin, quercitrin, isoquercitrin, rutin, hyperoside, I3,II8-biapigenin	*Hypericum perforatum*	extracted with hot MeOH	201 TP 54 RP-18 (250 × 4 mm, 5 µm/A: H_2O/85% H_3PO_4 (99.7:0.3, v/v); B: CH_3CN; C: MeOH; gradient: 100% A – 85% A, 15% B, 10 min; to 70% A, 20% B, 10% C, 30 min; to 10% A, 75% B, 15% C, 40 min; to 5% A, 80% B, 15% C, 55 min; to 100% A, 56 min; 100% A, 65 min; flow rate: 1 mL/min	thermospray – triple quadrupole mass spectrometry, PDA	[171]
chlorogenic, protocatechuic, *p*-hydroxybenzoic, caffeic, vanillic, syringic, *p*-coumaric, ferulic acids	*Eleutherococcus senticosus*	refluxion of plant material in CH_3OH; pH adjustment to 7.0 – 7.2 with 5% $NaHCO_3$, followed by SPE (quaternary amine); final elution with 0.2 M H_3PO_4 – CH_3OH (1:1, v/v)	1. ODS-Hypersil C_{18} column (200 × 4.6 mm, 5 µm) and 2. Symmetry C_{18} column (250 × 4.6 mm, 5 µm)/1. CH_3OH-acetic acid-H_2O (23:1:77, v/v/v) and 2. CH_3OH-0.001 M H_3PO_4 (23:77, v/v) flow rate: 1 mL/min	UV 254 nm PDA 254 nm and 280 nm fluorescence: ex: 230 nm, em: 350 nm	[137]
caffeic, ferulic, and chlorogenic acids	human plasma, urine	(plasma) incubated in sulfatase and glucoronidase; acidified with H_3PO_4, extracted with methylene chloride, vortexed and centrifuged. (urine) mixed with sodium acetate buffer (pH 5.5) and $CaCl_2$ solution; incubated in a manner similar to that of the plasma samples; acidified with 6 N HCl; extracted with ethyl acetate, vortexed, and centrifuged	Hamilton PRP-1 column/A: 1% acetic acid in water; B: ACN; gradient: 20% B, 4 min; 50% B, 6 min, 100% B, 15 min; flow rate: 0.5 mL/min	ESI-MS	[172]

broadening which could compromise separation efficiency [175]. A three-electrode cell consisting of a carbon disc working electrode, a platinum auxiliary electrode, and a saturated calomel electrode as the reference electrode was utilised. The working potentials were optimised by hydrodynamic cyclic or pulse differential voltammetry [176–178].

Operated in a wall-jet configuration, a 300 µm diameter carbon disc electrode was used as the working electrode exhibiting a good response at +0.85 V (*versus* saturated calomel electrode) as electrochemical detector [179]. Under optimum conditions, four major flavonoids were baseline separated within 20 min in a 80-mM/L borax buffer. The method was successfully used in the analysis of *Frucus aurantii* of different geographical origin.

CE–electrochemical detection has proved to be a powerful technique for the chemical markers and fingerprint study of natural products and has become an alternative, competitive, and supplementary method for HPLC, because of its special attributes.

Rather few papers discuss the use of CE–MS for the determination of flavonoids and phenolic acids [180]. The dearth of applications may evidence that the technique is not considered sufficiently robust and user-friendly by many researchers. In the CE–ESI–MS study by Lafont *et al.*, a standard mixture of seven phenolic acids was analysed [181]. With selected-ion-monitoring MS the authors were able to identify all eight compounds based on their retention times and characteristic fragment ions and obtained LODs of 0.1 – 40 µg/L. Huck *et al.* established a CE method that has been for the analysis of a flavonoid mixture consisting of 5-methoxyflavone, biochanin A, hesperetin, and naringenin obtained from plant extracts [182]. They concluded that although CE–MS is not a technique that will replace other methods, it appears to be a complementary tool.

Although it is generally believed that MEKC possesses higher separation efficiency than CZE, the utilisation of MEKC in the determination of phenolics was about three times less frequent than that of CZE in recent years [183]. It seems that the use of borate-based buffers in conventional CZE allows sufficient resolution of polyphenols in relatively complicated mixtures due to the complex formation effects of borate. In the case of compounds with similar structure but different lipophilicity, the use of micelles is advantageous since the separation process is affected by more factors compared to CZE (*e.g.*, unsaturation of C-ring lowers the migration time; methylation of hydroxyl group increases the hydrophobicity of analyte and consequently its affinity to the micelles, which results in increase in migration times; glycosylation and higher number of –OH groups increases hydrophilicity and therefore migration times are decreased) [184].

In the MEKC of polyphenols, sodium dodecyl sulphate is the most widely used surfactant [185–188]. Sodium cholate was reported as an auxiliary additive to sodium dodecyl sulphate-containing background electrolyte in so-called "mixed MEKC". This separation technique utilises the formation of mixed micelles formed from both surfactants to improve the resolution of either polar or non-polar analytes that could not be separated by conventional MEKC [189].
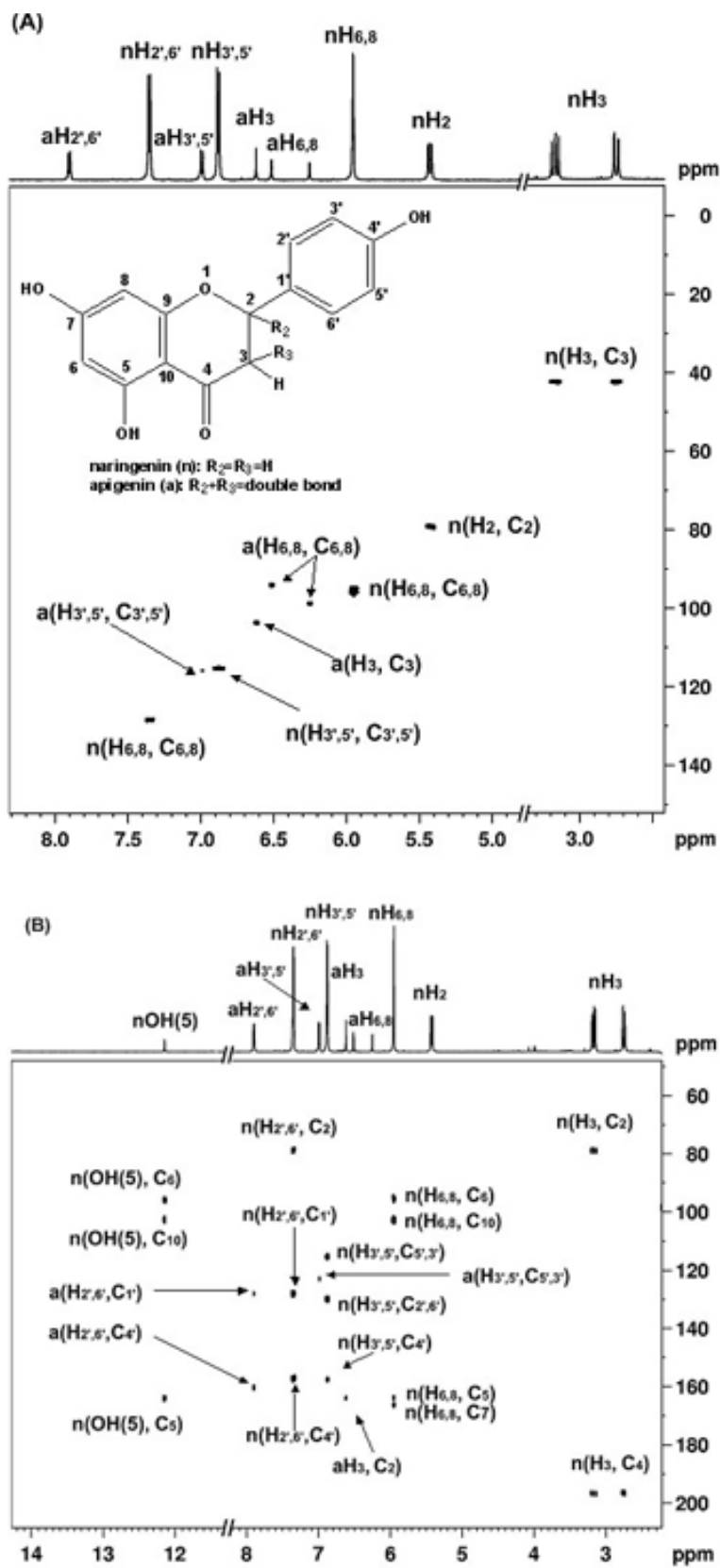
Figure 6. Two-dimensional (A) ^{1}H-^{13}C HMQC and (B) ^{1}H-^{13}C HMBC NMR spectra for the identification of naringenin (n) and apigenin (a) trapped on the same SPE cartridge [159].

The merits and drawbacks of HPLC and electromigration methods, as utilised in routine analysis of polyphenols, have been discussed [190, 191]. Bonoli *et al.* validated RP-HPLC and MEKC methods employed for the determination of tea catechins [192]. They arrived at the conclusion that the MEKC surpassed HPLC in its higher sensitivity.

Separation of flavonoid-3-*O*-glycosides differing in their sugar moiety and flavonoid-7-*O*-glycosides differing in their aglycones were separated as borate complexes by CE [193] and MEKC [194], applying UV detection. In both cases, the authors decided that CE and MEKC gave higher efficiency, selectivity, and speed, compared to HPLC. To separate flavonoid-3-*O*-glycosides and flavonoid-7-*O*-glycosides by CE, 0.2 M borate buffer (pH 10.5) was employed, while for MEKC separation of the flavonoid-7-*O*-glycosides, in the presence of sodium dodecyl sulphate, neutral conditions (pH 7.1) proved to be the optimum.

Zhang *et al.* applied capillary electrochromatography on monolithic columns combined with stepwise gradient elution to analyse flavonoids in leaves of *Adinandra nitida*. By this means, good resolution was obtained within a short time [195]. Huang *et al.* compared microemulsion electrokinetic chromatography with MEKC methods for the analysis of phenolic compounds [196]. A higher voltage and a higher column temperature improved the separation efficiency without any noticeable reduction in resolution for microemulsion electrokinetic chromatography whereas they caused a poor resolution for the MEKC system. Although separations with baseline resolution were achieved by the optimised microemulsion electrokinetic chromatography and MEKC methods, the separation selectivity resulting from the proposed microemulsion electrokinetic chromatography method was completely different from that of MEKC.

Room-temperature ionic liquids are liquids that are constituted entirely of ions and can provide a solvent environment quite unlike any other available at room temperature. A CZE method was established for resolving natural flavonoids in a Chinese herbal extract using 1-alkyl-3-methyl-imidazolium-based ionic liquids as additives [197]. Baseline separation, high efficiencies, and symmetrical peaks were obtained for the flavonoids. The hydrogen-bonding interaction between the ionic liquid cation and the analytes is conceived to be the main separation mechanism. Electropherograms of a mixture of flavonoids and extracts of *H. rhamnoides* and a tablet are portrayed in Fig. 7.

Chiral separation of diastereomeric flavonoids can be performed by CE with cyclodextrins added to the running buffer. Gel-Moreto *et al.* have reported the separation of the diastereomers of six major flavanone-7-*O*-glycosides by chiral CE using 0.2 M borate buffer at pH 10.0 and with 5 mM β-cyclodextrin as chiral selector [198].
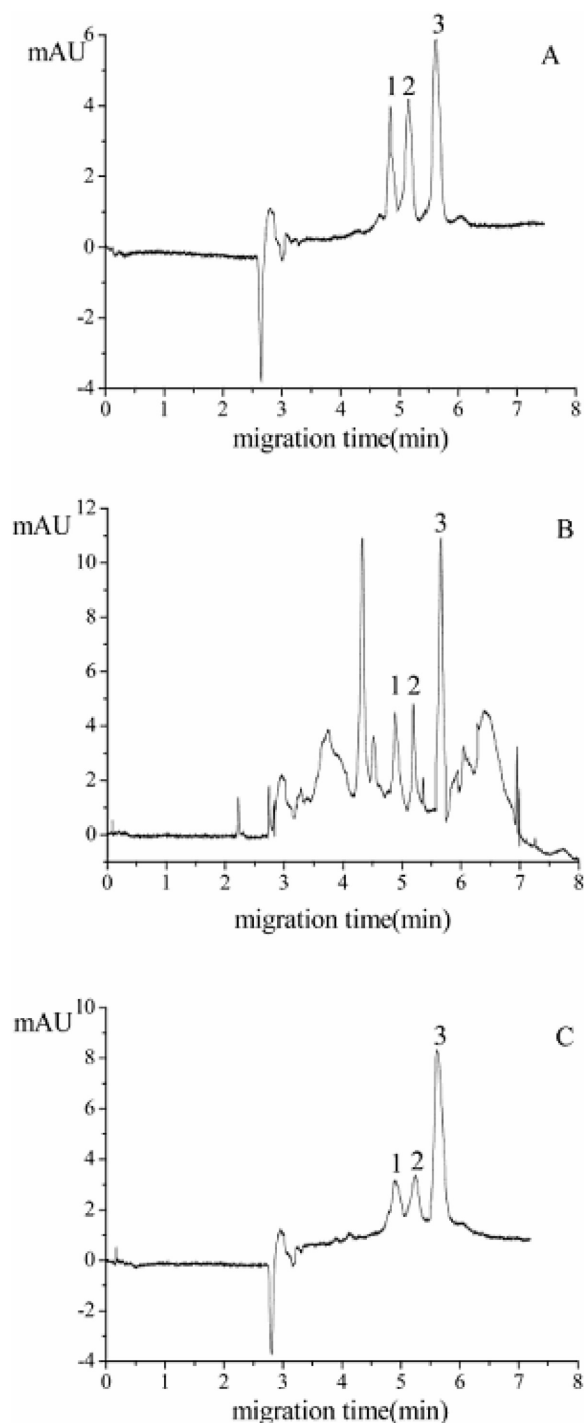
Figure 7. Electropherograms of (A) a mixture of flavonoids, (B) an extract of *Hippophae rhamnoides* and (C) an extract of medicinal preparation, Sindacon tablet, using 1-alkyl-3-methyl-imidazolium-based ionic liquids, as the additive in 20 mM borate buffer, pH 10. 1: IS, isorhamnetin; 2: KA, kaempferol; 3: QU, quercetin [197].

Another recent study reported the enantiomeric separation of naringenin by capillary electrophoresis using the various cyclodextrins as selectors and demonstrated sep-

aration with the best resolution of R_s = 4.85 with hydroxy-propyl-γ-cyclodextrin and baseline resolution with methyl-γ-cyclodextrin (R_s = 3.81), carboxymethyl-γ-cyclodextrin (R_s = 2.26), and sulphate-β-cyclodextrin (R_s = 3.63) [199].

The on-line combination of capillary isotachophoresis and the CZE technique allows the limits of detection to be substantially improved and at the same time involves a pre-separation step [200]. The exact timing of the introduction of the isotachophoretically stacked flavonoid isotachophoresis zones into the CZE capillary enables the removal of unwanted matrix from the minor analytes of interest when analysing complex natural samples. Detection limits were fairly low in the range of ng/mL; a single analysis took 45 min. Other authors devised the integration of a flow-injection system with a CE analyser for the on-line pre-concentration of analytes leading to the improvement of limits of quantification. The flow-injection system conducted automated C_{18} minicolumn solid-phase extraction of analytes and elution by methanol before the CE analysis of wine samples for flavonoids [201].

A summary of other recently reported capillary electrophoretic and capillary electrochromatographic methods is given in Table 5. It should be mentioned that almost invariably all background electrolytes in CZE are alkaline in order to maintain a degree of ionisation and sufficient separation of polyphenols thanks to the complex-formation ability of borate [183]. It was found that the migration times of the analytes increased with increasing borate concentration, due to the stronger interaction of borate and flavonoids at high borate concentration. For the analysis of reducing compounds such as phenolics which can be oxidised by dissolved oxygen at such pH values, it remains necessary to address this risk issue.

3.5 Spectrophotometric detection

Detailed information on molecular absorption characteristics has been provided in Subsection 3.3.3. Simple phenolics have absorption maxima between 220 and 320 nm but their absorption is affected by the nature of the solvent and the pH of the solution [213]. On top of this, the possibility of interference by UV-absorbing substances such as proteins, nucleic acids, and amino acids should also be considered. Although the development of a satisfactory UV assay is a rather cumbersome and tough task and highly dependent on the material to be analysed, both UV and visible spectroscopic techniques are used for the identification of isolated phenolic compounds, particularly flavonoids [138, 214].

The Folin–Denis assay is the first and most widely used rapid reaction procedure for the quantification of total phenolics in plant materials [215]. The method relies on the reduction of phosphomolybdic–phosphotungstic acid (Folin–Denis) reagent to a blue coloured complex in alkaline solution. The generated phosphomolybdic-phosphotungstic-phenol complex gives an easily detected absorbance at 760 nm. The Folin–Ciocalteu assay is also used for the determination of the total content of plant food phenolics [216, 217]. Neither Folin–Denis nor Folin–Ciocalteu reagents are very specific and they do not detect all phenolic groups found in extracts. Another disadvantage of this assay is the interference of components in the food extracts, such as ascorbic acid, that behave as reducing agents.

The vanillin method is specific for flavan-3-ols, dihydrochalcones, and proanthocyanidins which have a single bond at the 2,3-position and possess free *meta*-hydroxy groups on the B ring [218]. Catechin, a monomeric flavan-3-ol, is often used as a standard in the vanillin assay. This assay in methanol is more sensitive toward polymeric proanthocyanidins than monomeric flavan-3-ols. This is the reason that this assay is more recognised as a useful method for the detection and quantification of proanthocyanidins in plant materials.

The complexation of phenolics with aluminium ion, Al(III), has been reported for the spectrophotometric determination of total caffeic acid, total flavonoids, and total tannins [219, 220]. The method is based on the formation of a complex between Al(III) and the carbonyl and hydroxyl groups of the flavonoid. According to the pharmacopoeia method originally designed for flavonols, absorption is measured at 425 nm [221]. Modification of this assay proposed by Zhishen *et al.* included the reaction of phenolic extract with sodium nitrate followed by the formation of flavonoid–aluminium complex [222]. The absorbance of the solution was then read at 510 nm. On the other hand, total caffeic acid was measured by adding a solution of $AlCl_3$ to the methanolic extract of phenolics and adjusting the pH of this mixture to 4.8 with a solution of NH_4Cl. The absorbance of this solution was then measured at 355 nm.

Determination of total flavonoid content in buckwheat seed was described by Oomah and Mazza [223]. A 2-aminoethyl-diphenylborate methanol solution was added to the extract and the absorption was measured at 404 nm and compared to that of a standard rutin curve.

For quantification of flavanones and dihydroflavonols, the spectrophotometric method of *Das Deutsche Arzneibuch 9* [224], as modified by Nagy and Grancai [225], was used. The method is based on the interaction of these compounds with 2,4-dinitrophenylhydrazine in acidic media (sulphuric acid) to form coloured phenylhydrazones. The absorbance is measured at 486 nm. Calibration is performed using pinocembrin as reference compound.

Quantification of anthocyanins takes advantage of their characteristic behaviour in acidic media. The ana-

Table 5. Electrophoretic procedures for the determination of phenolic compounds.

Analytes	Sample source	Sample preparation	Background electrolytes	Capillary / voltage	Detector	Ref.
catechin, epicatechin, myricetin, quercetin, trans-resveratrol	wine	evaporated under nitrogen stream at 50°C, reconstituted in 2 mL of MeOH, filtered, and injected into the CE	5 mM malonate – 9.6 mM tetrabutyl-ammonium hydroxide, in 100% MeOH	poly(GMA-co-NVP)-coated 58.5 cm (8.5 cm) × 50 µm/30 kV	UV 230 nm	[202]
catechin, epicatechin, catechin gallate, epicatechin gallate, epigallocatechin, epigallocatechin gallate	tea leaves, tea beverage	leaves brewed with hot water (85°C) for 1 min and filtered; diluted with water and analysed; tea beverages diluted five times with water and analysed	200 mM borate – 20 mM phosphate – 240 mM SDS – 25 mM 6-O-α-D-glucosyl-β-CD (pH 6.4)	64.5 cm (56 cm) × 50 µm/25 kV	UV 210 nm	[203]
chlorogenic acid, hyperoside, isoquercitrin, quercetin, quercitrin, rutin	*Hypericum perforatum*	extracted from dried pulverised leaves and flowers, into MeOH by sonication; filtered, diluted with water and analysed by ITP-CZE	25 mM MOPSO – 50 mM TRIS – 55 mM H₃BO₃ – 0.2% HEC – 20% MeOH (pH 8.3 – 8.7)	fluorinated ethylene-propylene copolymer, 16 cm × 0.3 mm/–	UV 254 nm, Conductivity	[204]
apigenin, caffeic acid, ferulic acid, luteolin, quercetin, rutin	propolis	extracted with MeOH in an ultrasonic bath and centrifuged; diluted with MeOH-running buffer; filtered and injected to the CE system.	50 mM borate (pH 9.2)	60 cm (50 cm) × 75 µm/ 23 kV	UV 262 nm	[205]
catechin, epicatechin, quercetin, rutin, ferulic acid, p-coumaric acid, vanillic acid, myricetin, kaempferol, caffeic acid	wine	extracted with diethyl ether; separation of the ether layer, evaporation to dryness; dissolved in MeOH and injected into the CE system	150 mM boric acid (pH 8.5) – 50 mM SDS – 5% MeOH	57 cm (50 cm) × 375 µm/20 kV	UV 280 nm	[206]
eriodictyol, naringenin, hesperetin, pinobanksin, pinocembrin, myricetin, quercetin, kaempferol, 8-methoxykaempferol, luteolin, apigenin, chrysin, galangin	honeys	(Honey) diluted with water (pH 2 – 3, adjusted with HCl) and filtered; passed through a column of Amberlite XAD-2 and eluted with MeOH; purified by passing through a Sephadex LH-20 column; concentrated, redissolved in MeOH and analysed by MECC	0.2 M sodium borate buffer (pH 8.0) – 50 mM SDS – 10% MeOH	75 cm × 75 µm/20 kV	280 nm	[207]
baicalin, wogonin 7-O-glucuronide, oroxylin A, 7-O-glucuronide, baicalein, wogonin, oroxylin A	herbal extracts of *Coptidis Rhizoma* and *Scutellariae Radix*	extracted with 70% MeOH by stirring at room temperature for 30 min; centrifuged, filtered and injected into the capillary electrophoresis system	5 mM sodium borate – 15 mM sodium dihydrogenphosphate – 50 mM sodium cholate in 65% ACN	80 cm (71.5 cm) × 50 µm/30 kV	UV 270 nm	[208]
rutin, vanillin, quercetin, taxifolin, luteolin, apigenin, gallic, vanillic, protocatechuic, caffeic, p-coumaric, sinapinic, 2,5-dihydroxybenzoic, trans-cinnamic, 4-hydroxybenzoic, o-coumaric, ferulic, gentisic acid	virgin olive oil	dissolved in hexane and passed through Diol SPE cartridges; recovered with MeOH and brought to dryness; dissolved with MeOH/water (50:50 v/v) and filtered before the CE analysis	45 mM sodium tetraborate buffer (pH 9.3)	47 cm (40 cm) × 50 µm/28 kV	UV 200 nm, 240 nm, 280 nm, 330 nm	[209]
catechin, hyperoside, quercitrin, quercetin, rutin	*Agrimonia pilosa* Ledeb.	pulverised and extracted with ethanol under sonication; concentrated and diluted with the running buffer prior to analysis	60 mM Na₂B₄O₇ – 120 mM NaH₂PO₄ (pH 8.8)	60 cm × 25 µm /19.5 kV	electrochemical, carbon disc electrodes +0.95V	[177]
chrysoeirol-7-O-neohesperidoside, tricin-7-O-neohesperidoside, lonicerin, luteolin-7-O-galactoside, rutin, hyperoside, quercetin, luteolin	*Flos Lonicerae*	suspended in 70% ACN ultrasonically extracted; diluted with water and passing through a SPE cartridge; eluted with ACN, filtered and CZE analysis	80 mM tetraborate – 20 mM phosphate (pH 8.1) – 15% ACN	72.5 cm (64 cm) × 50 µm/28 kV	UV 380 nm	[210]
naringin, hesperidin, neohesperidin, narirutin, eriocitrin	lemon, orange, grapefruit	extracted with Sep-Pak C₁₈ cartridge	20 mM tetraborate buffer (pH 7) – 5 mg/mL SBE-β-CD – 10% MeOH	48.5 cm (40 cm) × 50 µm/20 kV	UV 205 nm	[211]
caffeic acid, chlorogenic acid	Echinacea/tablets, capsules	extracted in an ultrasonic bath, at room temperature, with 70% MeOH in water; filtered, and injected	110 mM SDS – 100 mM HP-β-CD – 10 mM Britton-Robinson buffer (pH 8.0)	48.5 cm (40 cm) × 50 µm/20 kV	UV 260 nm, 320 nm	[212]

Abbreviations: SDS, sodium dodecyl sulphate; SBE-β-CD, sulphobutyl ether-β-cyclodextrin; HP-β-CD, hydroxypropyl-β-cyclodextrin; TRIS, tris(hydroxymethylamino)methane; HEC, 2-hydroxyethylcellulose; MOPSO, β-hydroxy-4-morpholinopropanesulphonic acid; poly-(GMA-co-NVP), poly(glycidylmethacrylate-co-N-vinylpyrrolidone).

lytical procedure for the spectrophotometric quantification of anthocyanins was first developed by Sondheimer and Kertesz many decades ago [226]. This procedure was later modified by Swain and Hillis who suggested expressing the concentration of pigments in terms of the change in the absorbance at λ_{max} between pH 3.5 and pH = 1.0 [227].

Chemometrics are powerful techniques for overcoming the overestimation of polyphenol contents arising from overlapping of spectral responses. Partial least squares or principal component analysis has been employed for this purpose [228]. Edelmann *et al.* developed a rapid method of discrimination of Austrian red wines based on mid-infrared spectroscopy of phenolic extracts of wine [229]. Subsequently, Brenna and Pagliarini employed a multivariate analysis for establishing a correlation between the polyphenolic composition and the antioxidant power of red wines [230]. Briandet *et al.* applied principal component analysis to differentiate between Arabica and Robusta instant coffees based on their FTIR spectra [231]. The discrimination between different species of coffee was based on their different contents of chlorogenic acid and caffeine. Schulz *et al.* used a near-infrared reflectance spectroscopic method for prediction of polyphenols in the leaves of green tea (*Camelia sinensis* L.) [232].

4 Concluding remarks

The huge number of publications appearing on the analysis of flavonoids and phenolic acids over the past two decades testifies to the significance of the subject. The overall analytical method to be used for these compounds is not cut-and-dried but is highly dependent upon the matrix characteristics, the availability of the techniques, the selectivity, and the interest in structure elucidation and unambiguous identification.

Because of the complexity of most of natural plant samples and biological matrices, the sample preparation procedure is a critical step of the entire assay. Many techniques offer the real possibility of preparing the sample before analysis with sufficient specificity. Nonetheless, there is still no standardised procedure available for sample preparation and extraction. Liquid extraction has to be, in many cases, the first step of the preparation stage. Attention should be devoted to effective clean-up methods for plant and biological extracts, such as SPE in off-line or on-line mode. Microwave-assisted extraction, SFE, and MSPD could also be suitable extraction/clean-up alternatives.

Special emphasis should be placed on the hydrolysis step of flavonoids which are present in natural matrices in their various conjugated forms or as free aglycones.

The same holds for phenolic acids, albeit to a lesser extent.

Complex extracts of plant constituents often require very effective separation techniques to allow the identification of different compounds. The TLC separation of plant extracts is described as a method of analysis in different pharmacopoeias. It can provide a chromatographic "fingerprint" of a plant extract, which is very useful for identification purposes. The focus is on screening for the main phenolics in real-life samples. TLC can also be considered with other methods such MS or NMR in order to provide conclusive evidence for the identification of compounds, or coupled with densitometry for quantification purposes.

For the analysis of phenolic acids and flavonoids, GC can hardly replace HPLC which can overcome specific separation problems, especially when emphasis is placed on both aglycones and glycosides. The possibility of coupling HPLC to several detection devices has turned it into an even more valuable and indispensable tool for the separation of phenolics. This, however, does not rule out the usefulness of GC and its outstanding separation capabilities.

UV detection became the preferred tool in LC-based analyses and, even today, LC with multiple-wavelength or UV/PDA detection is a fairly satisfactory tool in studies dealing with, *e.g.* screening, quantification, and provisional sub-group classification.

The high informative power and throughput capabilities of analytical techniques such as MS and NMR have led to identification and/or quantification applications. Analysis of biologically active flavonoids and phenolic acids has taken giant steps forward as a result of the application of MS techniques. The development of LC–MS has extended the scope of MS coupling techniques to allow analysis and identification of natural product compounds. For a few years LC–MS systems have been applied for the detection and identification of flavonoid glycosides in plants extracts or various biological fluids. A particular advantage of LC–MS is its capability to determine both free and conjugated forms. Neither GC nor GC–MS can match the speed of direct LC–MS(/MS) procedures and their possibility of easily screening samples for target analytes as well as unknowns. Additionally, in contrast to GC–MS, in LC–MS it is often unnecessary to use any extraction procedure. Urine samples, for instance, can be analysed directly with the only work-up required being centrifugation or filtration of the urine to remove particles that would otherwise clog up the HPLC column. Finally, the coupling of micro- and/or nano-LC, to tandem MS instruments facilitates the analysis of minute samples. However, for a more detailed structure elucidation of conjugates, the complementary information derived from LC–NMR is indispensable. In this area of application, LC–NMR outperforms the competition for

purposes of identification but quantification is rather doubtful.

For high-throughput analysis, the emergence of UPLC coupled to MS has appeared as an alternative to traditional HPLC techniques. The strengths of UPLC technology promote the ability to separate and identify drug compounds with significant gains in resolution and sensitivity, and marked reductions in overall analysis time.

Capillary electrophoresis (CE) coupled to mass spectrometry (MS) detection can provide structure-selective information about the analytes in such matrices. However, compared with LC, CE analysis of phenolics shows no dramatic difference in run times, and the limited consumption of sample and solvents does not appear to balance the low repeatability of retention/migration times and restricted sensitivity. Therefore, CEC and MEKC have turned out to be complementary to LC methods. The importance of CE–MS techniques may assume greater significance for the analysis of phenolic compounds and metabolites from physiological samples. High preconcentration may, however, be required to ameliorate sensitivity.

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