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Review

Sample handling strategies for the determination of biophenols in food and plants

Debora Tura, Kevin Robards*

School of Science and Technology, Charles Stuart University, P.O. Box 588, Wagga Wagga, New South Wales 2678, Australia

Abstract

The analysis of phenols in samples of plant and food origin attracts considerable attention. However, sample handling is often an ignored feature of the analysis. This review highlights the importance of sample extraction in an analysis and the problems that can arise during this step. Many questions remain unanswered and there is a need to more carefully validate extraction efficiencies. Although many new procedures have been developed the use of traditional techniques still dominates.

Keywords: Reviews; Sample handling; Food; Plants; Biophenols

Contents

1. Introduction

There is intense interest in plant phenols as witnessed by the numerous papers devoted to various aspects of these compounds. Knowledge of their levels and forms in plants, foods and increasingly in human diets and in the environment is of considerable interest. Rhodes and Price [1] observed that analysis of phenolic species in foods is an important outstanding problem that has been hindered by problems of analysis. There are several distinct aspects to an analysis and, of these, sample handling has been relatively neglected [1–3]. Indeed, in comparison with the quantification step, sample handling is in its infancy and, yet, it is a critical step in the procedure. The demands are even greater in

*Corresponding author. Tel.: +61-2-6933-2539; fax: +61-2-6933-2737.

E-mail address: krobards@csu.edu.au (K. Robards).

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the analysis of plant and food phenols where the number and diversity of analytes (many of unknown structure) further complicates sample handling.

Phenolic compounds [4] embrace a considerable range of substances that differ in the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton plus the number and position of hydroxy substituents. Moreover, the phenolics may occur as the free compounds, in glycosidic linkage or in some instances as acylated derivatives. The range of known phenolics is thus vast and also includes polymeric lignins and condensed tannins plus oligomeric species (e.g. dimeric states such as *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-D-xylopyranose) [5–7].

Isolation of phenolic compounds from the sample matrix is generally a prerequisite to any comprehensive analysis scheme although enhanced selectivity in the subsequent quantification step may reduce the need for sample manipulation. The ultimate goal is the preparation of a sample extract uniformly enriched in all components of interest and free from interfering matrix components. It encompasses a series of steps ranging from exhaustive solvent extraction and pre-concentration procedures to simple liquid–liquid extraction or filtration. The effect of structural diversity of the phenolics on physicochemical behaviour such as solubility and partitioning presents a challenging analytical problem. The task of recovery is further complicated as many foods and plants have a high enzyme activity, and hence extreme care must be taken to ensure correct extraction, devoid of chemical modification, which will invariably result in artefacts [4]. Artefactual changes, for example, hydrolysis, oxidation [8,9] and isomerisation [10] during the extraction process are a constant concern. A specific example is the photochemical isomerisation of *trans*-resveratrol to the *cis*-isomer [11] although this is of general concern with all naturally occurring *trans*-isomers [12,13]. Methods of protecting the compounds from these deteriorative processes have included the addition of antioxidants (one presumes of higher ''activity'' than the compounds themselves) during the extraction, the use of inert atmospheres and absence of light [14]. The fidelity between the phenolic profile of the starting material and that of the isolated extract provides the theoretical basis for judging analytical techniques. Hence, the conditions employed should be as mild as possible to avoid oxidation, thermal degradation and other chemical and biochemical changes in the sample.

Quantification procedures typically involve the separation sciences and are more universally applicable whereas sample handling is generally dependent on the nature and type of sample. Soleas et al. [15] illustrate this point. They developed a derivatisation procedure for determination of 15 phenolic constituents in solid vitaceous plant materials and concluded that the method ''should be suitable to measure polyphenols in fruit, vegetables, and other foods provided that *efficient extraction techniques are employed*''. Such statements underline the critical importance of sample preparation. This is further illustrated by Merken and Beecher [16] who developed an high-performance liquid chromatography (HPLC) system that separated major phenolics from each of the five sub-classes of flavonoids (flavones, flavonols, flavanones, catechins and anthocyanidins). However, their extraction procedure involved acid hydrolysis (at reflux) in *tert*.-butylhydroquinone (TBHQ) stabilised aqueous methanol under which conditions anthocyanidins and catechins are unstable. Thus, the method did not permit quantification of these substances in blueberries or tea, respectively.

Sample handling covers both sample collection/storage and sample pre-treatment/clean-up. This review is restricted to the latter aspect of sample handling in relation to the analysis of phenolic compounds in food and plant materials and specifically excludes the synthetic phenolic antioxidants such as BHA and BHT [17–21]. Sample pre-treatment cannot be treated in isolation from quantification steps as some procedures (e.g. capillary electrophoresis) [22] require more rigorous sample pretreatment than others. This is generally related to low selectivity of the subsequent method as in spectrophotometric measurement of total phenols [23]. Other factors are also important in some situations such as gas chromatography–mass spectrometry (GC–MS) where the effects of non-volatile matrix components on column lifetime are an important consideration.

A further review could usefully be devoted to other aspects of sample handling; namely, sample

collection and storage. In many instances, definitive procedures for storage and collection have not been established and procedures remain controversial. For example, several papers have mentioned the use of refrigeration of juice samples prior to analysis (e.g. Ref. [24]) and yet we observed significant precipitation of hesperidin complex [25] during overnight storage of orange juice at refrigerator temperatures. Moreover, at Groupe Polyphenols in 1998 [26] there was extensive debate regarding the use of fresh versus freeze-dried samples and no definitive conclusions were reached. Fundamental issues of analysis related to sample handling must be addressed. For instance, current sample recovery procedures involving spiking of extracts are inadequate answers to the challenges of the Third Millenium.

2. Background considerations

Interest in the phenolic profiles of plants can be attributed to their use as fingerprints for authentication of wines [27,28], citrus juices [29,30], olive oils [31] and other commercial products [32–36]. In this application area, identification of the phenols is not critical. Characterisation of phenolic components of various plants and assessing their physiological activity has also attracted considerable attention. There has been considerably less ''interest'' in quantifying the phenolic components presumably due to the limited range of phenols commercially available as suitable reference compounds [37]. This situation is changing rapidly and the need to quantify the levels of phenols is being addressed. It is now clear that the range of concentrations of phenols in plants is as diverse [38,39] as the number and type. For example, flavonoids were not detected in cultivated mushrooms [40] whilst they were present in orange juice at levels up to 500 mg l^{-1} [41,42]. In orange juice, flavanone glycosides (e.g. hesperidin, 100–500 mg l^{-1}) were present in much higher concentrations than polymethoxylated flavones with typical values of 0.1 mg l^{-1} [41,42]. Total flavanol contents varied from non-detectable in most vegetables to 1840 mg kg^{-1} in a broad bean sample [43].

The shift from essentially qualitative to quantitative analysis [44] has significant implications for sample recovery procedures. Plant phenols are ionisable with typical pK_a values ranging from 8 to 12 and oil:water partition coefficients ranging from 6×10^{-4} to 1.5 [45]. Thus, they exhibit considerable diversity in terms of acidity as well as polarity ranging from hydrophobic to hydrophilic in character. These aspects of their chemistry must be considered when determining sample handling strategies as, for example, in pH control to ensure favourable partitioning behaviour during extraction [45]. The situation with respect to pH dependent equilibria is especially complex in the case of anthocyanin extraction [46]. The extraction procedure is somewhat simplified in analyses where a single specific phenolic compound is to be determined. Here the conflicting stabilities, solubilities, etc. of the target compounds are not an issue. For example, hydroxytyrosol has only recently been reported in wine [44] using a method specifically targetting this compound. The analyte was eluted from a C_{18} cartridge with ethyl acetate prior to derivatisation and quantification by gas chromatography (GC).

At the tissue level, there are significant qualitative and quantitative differences in phenolic contents [47]. For example, anthocyanins are located primarily in the skin of grapes but are present throughout the fruit in strawberry and blueberry. At the subcellular level, phenolic compounds may accumulate in the vacuoles or in the cell walls. Limited data suggests they are located mainly in the vacuoles [48] with small amounts in free space and none in the cytoplasm. Sample handling procedures for various tissues and cellular fractions have been relatively neglected. The occurrence of phenolics in soluble, suspended and colloidal forms and in covalent combination with cell wall components [49] has significant impact on their extraction. For instance, during winemaking mainly soluble phenolic compounds present in the vacuoles of the grape plant cells are extracted leaving behind a large amount of phenols associated with the cell walls [50]. Analytical procedures for phenolics typically require a liquid extract of the analytes. However, study of their spatial distribution at the cellular and tissue level generally requires transverse sections [51] and analyses of this type are not covered in this review.

The extractable phenolic fraction has been studied in a wide group of representative samples from the main food sources of phenolic compounds: fruit and

beverages (juice, wine, tea, coffee, chocolate, beer) and, to a lesser extent, vegetables, dry legumes and cereals [52]. The most significant dietary phenols (in terms of quantitative intake) are phenolic acids and flavonoids. The major dietary sources of various classes of phenols plus the main phenolic constituents of various foods are given in Tables 1 and 2. Caution is necessary in the interpretation of such compilations. Main dietary sources will obviously depend on culture and the precise dietary details. For example, the albedo in citrus is a relatively rich source of flavanones but is generally discarded although the amount remaining on fruit segments varies greatly between cultivars [55]. Comparisons of data between authors are difficult due to normal biological variations relating to cultivar, seasonal, environmental and agronomic practices. Processing also impacts on levels in foods; for example, pressing may result in solubilisation of phenols in juices otherwise present in unconsumed parts of the fruit. Phenols in grains are mainly located in the outer layer and thus milling degree has a significant effect on the phenolic content [74]. Intercomparisons of food sources can also lead to incorrect conclusions. For example, the major phenols in blueberries and gooseberries are phenolic acids and flavonols, respectively but the total phenol content is 3500 mg kg^{-1} (seedless dry mass) in blueberries but only 100 mg kg^{-1} in gooseberries [39]. The analytical method must also be considered in any comparisons. Thus, flavanones were identified in onions [16] but using a procedure that would induce hydrolysis of flavanone glycosides.

Typical liquid samples include beverages such as wine, fruit juices, tea, coffee, chocolate and beer and olive oil. Urine, plasma and breast milk are also encountered as a result of interest in bioavailability [72]. Solid samples include fruits, vegetables, legumes and cereals with increasing interest in other plant tissues such as leaves and seeds and phytopharmaceutical products [75]. Materials traditionally regarded as waste such as food by-products (seed [76–78], peel [79], skin [80] of fruits and vegetables) and effluents from food processing (grape marc [81], olive mill wastewater [82], soaking water from lentils [12]) are attracting attention as rich sources of often unique phenols. New phytochemicals are also sought in traditional medicines [83] and in the ''non-edible'' portions of plants such as roots [84], flowers [85,86] and leaves [87–91]. Extraction procedures vary between the different sample types but a common theme here is the extension of the analytical extraction to pilot-scale and ultimately commercial recovery. In the case of olive mill wastewater [82], sample was diluted with aqueous formic acid and then fractionated by solid-phase extraction (SPE). The SPE cartridges were exhaustively washed with hexane and the phenols recovered by elution with ethyl acetate followed by aqueous acidic methanol. Ethyl acetate and butanol extraction have also been used [92,93] to recover phenols from olive mill wastewater. Extraction combined acid and alkaline hydrolyses to determine the amounts of both free and bound phenolic acids in *Ginkgo biloba* L. leaves [94] and flavonoids, phenolic acids, and coumarins in seven medicinal species [95]. Ferulic acid and *p*-coumaric acid were extracted with methanol [96] from fourteen forbs and fractionated into free phen-

Table 1
Dietary sources of the main classes of plant phenols

Class of phenol	Main dietary source	Ref.
Phenolic acids	Wheat bran, potato	[52]
Flavonols	Onions (red/yellow), tea	[52]
Flavones	Sweet red peppers, celery	[52]
Flavan-3-ols and proanthocyanidins	Apples, pears, plums, grapes, red wine, tea	[43,52–54]
Flavanones	Citrus	[55]
Chalcones	Tomatoes	[55]
Dihydrochalcones	Apples and particularly apple juices and ciders	[55]
Anthocyanidins	Cherries, plums, strawberries, blackberries, grapes, currants	[52]

Table 2
Major classes of phenols identified in various foods

Food	Major classes of phenols	Ref.
Beverages		
Coffee	Phenolic acids	[56]
Tea	Flavan-3-ols, theaflavins	[16,53,57–59]
Fruits		
Bilberry	Anthocyanins, flavonols	[39,60]
Blueberry	Phenolic acids	[39]
Cowberry	Flavanols, procyanidins	[60]
Cranberry	Flavonols, hydroxycinnamic acids	[39]
Currant (red, black)	Anthocyanins, flavonols	[39,52,60,61]
Gooseberry	Flavonols	[39]
Raspberry (red)	Ellagitannins	[39,60]
Rowanberry	Phenolic acids	[39,60]
Strawberry	Anthocyanins, ellagitannins	[39,60]
Apple	Hydroxycinnamic acids, proanthocyanidins	[52,60,62]
Cherry	Anthocyanins	[52]
Citrus	Flavanones, polymethoxylated flavones	[52]
Grape	Proanthocyanidins	[52]
Olive	Secoiridoids	[63]
Pear		[52]
Persimmon	Flavanol, phenolic acids	[38]
Plum	Proanthocyanidins, anthocyanins	[52]
Vegetables		
Lettuce	Phenolic acids, flavonols	[52]
Onion	Flavanone (glycosides), phenolic acids, flavonols	[16,52]
Potato	Phenolic acids	[52]
Tomato	Phenolic acids, flavonols	[52]
Cereals		
Oats, rice, rye	Phenolic acids	[13,64–66]
Wheat bran	Phenolic acids	[52]
Other		
Canola	Phenolic acids	[67]
Chocolate	Procyanidins, flavan-3-ols	[52,68]
Hazelnut	Phenolic acids	[69]
Herbs	Flavanone glycosides	[70]
Mushroom	Phenolic acids	[40]
Pollen (honeybee collected)	Flavonol glycosides	[71]
Soybean	Isoflavonoids	[72,73]

olic acids by partitioning into ether, ester-bound phenolic acids after alkaline hydrolysis, glycoside-bound phenolic acids after acid hydrolysis, and cell wall-bound phenolic acids after alkaline hydrolysis of the solid residue from the extraction with methanol. The cell wall-bound phenols were quantitatively the most important fraction contributing approximately 50% to the total of both ferulic and *p*-coumaric acids. Polymethoxylated flavones were isolated [79] from the peel of oranges and tangerines by Soxhlet extraction with benzene for 4 h. The extracts were concentrated in vacuo and analysed without further purification by normal-phase HPLC. However, alcoholic extraction (methanol or ethanol) has been the usual approach to handling solid samples. The methods differ in the use of fresh [87–91] or freeze-dried sample [77,78], extraction at room temperature or by refluxing [97] and in the

addition or otherwise of an antioxidant such as metabisulfite to the extractant [77,78]. Lipids and chlorophyll can be eliminated from the extracts, if necessary, by partitioning into chloroform or hexane and the extracts may be analysed directly or further processed [77] by partitioning of the phenolic compounds into ethyl acetate prior to analysis.

Thus, sample handling strategies depend on the nature of both the analyte (e.g. total phenols, *o*-diphenols versus other phenols, specific phenolic classes such as flavonone glycosides or individual compounds; bound versus free phenols; monomeric, oligomeric or polymeric species) and sample and particularly its physical state. Sample preparation procedures that have been devised to cope with this diversity include mincing, homogenisation, filtration/centrifugation, distillation, simple solvent extraction, headspace analysis, supercritical fluid extraction (SFE), pressurised-fluid extraction, microwave-assisted extraction (MAE) and Soxhlet extraction. Derivatisation of the analyte may also be incorporated in the recovery step. Some procedures have limited application such as headspace analysis whereas others are more broadly applicable (e.g. solvent extraction).

The significance of phenolic extraction is not limited to the analytical context for it has important practical applications in the food industry. For instance, the mechanism and kinetics of the extraction of phenols from wood to wine during aging in barrels [98] has significant consequences for the production of quality wines. There has been considerable interest in comparing the phenol content of olive oils [99] and olive mill wastewater [92] produced by different extraction technologies. This is a further instance where the same set of considerations important in analytical methodology has important implications for processing technology.

The applications and merits of various recovery procedures (Tables 3–6) are examined in the remainder of this review. It has been convenient to treat the various pre-treatment/clean/up procedures separately, but there is often no clear distinction [152]. For instance, a procedure involving liquid extraction with subsequent clean-up by SPE might be considered under either heading. In such cases, we have endeavoured to interpret what we consider to be the most significant aspect of the procedure.

3. Hydrolysis

Preliminary hydrolysis of samples has been used as an aid to structural elucidation and characterisation of glycosides [153], to minimise interferences in subsequent chromatography [154] and as an aid to simplifying chromatographic data [155–158] particularly in instances where appropriate standards are commercially unavailable. Enzymatic, acidic, and alkaline treatment are used. Enzyme-assisted treatment of the press residue (grape pomace) from wine production [50] was efficient in degrading the grape pomace polysaccharides and thus releasing phenols. Total phenols ranged from 820 to 6055 mg l^{-1} gallic acid equivalents (GAE) and varied in response to enzyme type, time of enzyme treatment, particle size of the pomace, and type of extraction solvent employed. The yield of total phenols was correlated to the degree of plant cell wall breakdown of grape pomace ($r>0.6$, $P<0.01$). These data are relevant to both the analytical and commercial-scale recovery of phenols.

Chemical treatment has been more common in the role of simplifying chromatographic data because it is more exhaustive and less selective. However, there is considerable variation in the lability of the glycosidic bond under hydrolytic conditions and structural rearrangements can occur [159,160]. For example, flavanones with appropriate hydroxyl-substitution can be easily converted to isomeric chalcones in alkaline media (or vice versa in acidic media) [55]. Furthermore, methods employing hydrolysis when used for purposes other than characterisation/structural elucidation of unknown phenols result in a reduction in information content. Consider the example of a sample extract containing several *O*-glucosides of a single aglycone plus the free aglycone which will theoretically produce a single HPLC peak after acid hydrolysis. The advantages in terms of simplicity of interpretation and quantification are apparent as seen in HPLC of red raspberry juices [145] where acid and base hydrolysis simplified the complex phenolic profiles dramatically. Minor differences were observed in the profiles resulting from the two treatments following sample preparation on C_{18} cartridges.

Acid hydrolysis has been the traditional approach to measurement of flavonoid aglycones

Table 3
Representative examples of the use of hydrolysis as a pre-treatment method in the determination of biophenols

Details	Sample	Quantification[a,b]	Ref.
Hydrolysis in acidic methanol	Wine and fruit juice	HPLC–DAD	[100]
Homogenise, enzyme treatment, filter	Strawberry and raspberry juices	HPLC–DAD; 520 nm (cyanidin), 370–600 nm (anthocyanidins), 505 nm (pelargidin), 280 nm (flavonol), 355 nm (ellagic acid)	[101]
Free acids: acidify, ethyl acetate extraction; Total acids: alkaline hydrolysis in dark and ethyl acetate extraction	Blood orange juice	HPLC, 280 nm	[102]
Acid hydrolysis of methanolic extract	Passionfruit	HPLC	[103]
Pulp filtered, centrifuged to produce a clear juice; column chromatography and enzymatic hydrolysis	Purple passionfruit	GC–MS of trifluoroacetylated derivatives	[104]
Freeze-dried, acid hydrolysis containing TBHQ and liquid extraction	Fruits	HPLC, UV detection	[105]
Aqueous alcohol extraction followed by alkaline hydrolysis	Orange and grapefruit	HPLC, 300 nm	[106]
(Enzymatic pectinase extraction), followed by aqueous methanol or aqueous acetone extraction	Berries	Colorimetry; HPLC, 280 nm (flavan-3-ols as catechin equivalents, benzoic acid derivatives as GAE), 316 nm (hydroxycinnamates as caffeic acid equivalents), 365 nm (flavonols as rutin equivalents), 520 nm (anthocyanins as malvin equivalents)	[107]
3 extraction and hydrolysis procedures using freeze dried berries	Berries	HPLC, 260 nm (ellagic and *p*-hydroxybenzoic acids), 280 nm (catechins), 320 nm (hydroxycinnamic acids), 360 nm (flavonols)	[39,108]
Hydrolysis in acidified aqueous methanol containing TBHQ	Berries	LC–MS; HPLC–DAD	[109]
Aqueous methanol extraction from hexane solution of oil followed by SPE fractionation; acid and base hydrolysis	Olive oil	Colorimetry; HPLC	[110]
Boiled in 2 *M* HCl and ethyl acetate extraction	Olive leaf and root	GC of TMS[b] derivatives	[111]
Petroleum ether extraction of powdered mesocarp; alkaline hydrolysis and ethyl acetate extraction	*Diospyros lotus* L. fruit	GC–MS of TMS derivatives	[112]
Petroleum ether extraction of powdered mesocarp, followed by alkaline hydrolysis of residue under nitrogen and ethyl acetate recovery	Cherry laurel	GC–MS of TMS derivatives	[113]

[a] DAD, photodiode array detection.
[b] TMS, trimethylsilyl.

Table 4
Representative examples of the use of simple dilution/filtration for the recovery of biophenols from plants and foods

Details	Sample	Quantification	Ref.
Filtration and direct injection except for procyanidins where isolation on Sephadex LH-20 column	Grape juice	Colorimetry; HPLC–DAD, 280 and 320 nm	[114]
Nil	Wine	Colorimetry	[98]
Nil	Wine	HPLC, 288 nm (*cis*-isomer), 308 nm (*trans*-isomer)	[115]
Filtration	Red wine, beer, apple cider, and sour cherry and blackthorn fruit liqueurs	HPLC, UV at 280 nm and post-column reactor with absorption at 640 nm	[116]
Filtration	Wine	HPLC–DAD, 280 nm	[117]
Centrifugation and dilution	Fruit	HPLC, coulometric array detector	[118]

[16,158,161,162] and a kinetic method involving its use has been devised [163]. It appears that acid hydrolysis is seen to more closely reflect dietary intakes although it is evident that absorption, metabolism and bioavailability of plant phenols is complex and that knowledge of these is still very limited [52]. The five major flavonoid aglycones—quercetin, kaempferol, myricetin, luteolin, and apigenin—were determined [105] in freeze-dried fruits and vegetables, after acid hydrolysis of the samples. The recovery of non-anthocyanic phenols from frozen non-grape berries has been systematically investigated [39,61,108,109] by three extraction and hydrolysis procedures for the recovery of flavonols (kaempferol, quercetin, myricetin) and phenolic acids (*p*-coumaric, caffeic, ferulic, *p*-hydroxybenzoic, gallic and ellagic acids). The thawing method (refrigerator, room temperature or microwave) showed differential effects on the level of various flavonols. Microwave thawing produced the most reliable results and was also the most practical approach for routine analyses. Flavonols were extracted and hydrolysed to aglycones by refluxing in aqueous methanol containing hydrochloric acid and an antioxidant. Compounds that have been used for this purpose include BHA [162], TBHQ [163] and ascorbic acid [39,108]. The choice of stabiliser can be influenced by the subsequent procedure as co-elution with plant phenols can occur [108]. Recoveries of flavonols were critically dependent [39,61,108,109] on the concentration of the aqueous methanol extractant. The authors concluded [108] that it ''is not an easy task to find a single method which is adequate for an analysis of a diverse group of phenolics because of the differing chemical structures and the varying sensitivity of the compounds to the conditions of hydrolysis and extraction''.

Two forces have driven the use of alkaline hydrolysis. Firstly, commercial processing of many plant-derived foods now involves alkali-treatment and the stability of plant phenols under these conditions becomes of interest [164]. For instance, the major characteristic phenols of olive are secoiridoids and their reactivity in alkali has been examined [165–167]. The complexity of their chemistry has been demonstrated [168] and four phenolic fractions were identified [169] as simple biophenols, soluble biophenols and soluble esterified derivatives of these compounds; cytoplasmatic soluble biophenols; and soluble, glucosidic, esterified and cell-wall bound biophenols. The same group isolated three fractions in a subsequent paper [170]. Secondly, many phenols

Table 5
Representative examples of the use of liquid extraction for the recovery of biophenols from plants and foods

Details	Sample	Quantification	Ref.
Methanol extraction of pulp and skins; column chromatography or TLC	Grape and citrus fruit	Spectrophotometry; GC of TMS derivatives	[119,120]
Extraction with acidic aqueous methanol	Sweet cherry	HPLC, 280 and 525 nm, (and GC)	[10]
Extraction of dried material with dimethylsulfoxide	Grapefruit and pummelo	HPLC–DAD	[121]
Aqueous ethanol extraction	Sour orange	LC–DAD–MS	[122]
Aqueous acidic methanol extraction and filtration	Blueberries	HPLC–DAD; GC of anthocyanidins as TMS derivatives	[123]
Extraction with hot methanol or, for anthocyanins, acidified methanol. Extracts stored at −40 °C	Berries	Spectrophotometry	[124]
Methanol extraction	Apple and pear	HPLC–DAD, 280 nm	[37]
Aqueous methanol extraction of freeze dried material (no pericarp)	Apple	HPLC–DAD 280 nm, 350 nm; 200–600 nm post run scan	[125]
Aqueous methanol extraction	Apple and grape	HPLC, UV (270 nm) or fluorescence (280/310 nm excitation/emission)	[126]
Ethanol extraction with metabisulfite and ethyl acetate partitioning	Apple	HPLC, 280 nm (flavan-3-ols, dihydrochalcones), 320 nm (hydroxycinnamic derivatives and flavonols)	[127]
Aqueous ethanol extraction of powdered fruit; clean-up by liquid–liquid extraction	Pear	HPLC–DAD, 325 nm (hydroxycinnamic acids); 280 nm (flavanols); 360 nm (flavonols)	[128]
Aqueous methanol or ethanol extraction of pulp	Apricot	GC–MS of TMS derivatives	[129]
Aqueous acetone extraction	Areca fruit	Total phenols—colorimetry at 735 nm by Folin Ciocalteu; Condensed tannins—colorimetry at 500 nm by vanillin–HCl	[130]
Methanol extraction	Peach and nectarine skin	Colorimetry; HPLC	[131]
Methanol extraction	Apple pulp and peel	HPLC–DAD, 280 nm	[132]
Freeze dried, successive methanol and aqueous acetone extraction, thiolysis. Butanol–hydrochloric acid hydrolysis for procyanidins	Cider apple tissues	Colorimetry; HPLC–DAD, 540 nm (procyanidins), 280 nm (other phenols); LC–MS	[133,134]
Extraction of ground apple peel with acidified methanol	Apple skin	HPLC, 350 nm (flavonols), 530 nm (anthocyanins), 280 nm (proanthocyanidins), 313 nm (phenolic acids)	[80]

Table 5. Continued

Details	Sample	Quantification	Ref.
Aqueous methanol extraction	Grape skin	HPLC, 520 nm; spectrophotometry, 280 nm, 355 nm, 535 nm	[135]
Homogenised in aqueous methanol, dried and extracted with ethyl acetate	Peach and apple purees and concentrates	HPLC–DAD, 210–360 nm	[136]
Dilution and column chromatography	Quince, pear and apple purees	HPLC–DAD, 280 nm, 350 nm	[137]
Aqueous methanol extraction of hexane solution	Olive oil	Colorimetry, 725 nm (total phenols), 370 nm (*o*-diphenols)	[138]
Methanol extraction	Olive oil	GC and GC–MS of TMS derivatives; HPLC, 232 and 278 nm	[139]
Methanol extraction followed by partitioning between acetonitrile and hexane	Olive oil	GC–MS of TMS derivatives	[140]
Methanol extraction	Olive leaf	LC–MS–MS	[90]
Petroleum ether wash followed by aqueous methanol extraction and fractionation by column chromatography	Grapevine leaf	HPLC, 340 nm	[141]

and particularly the phenolic acids exist in a wide range of conjugated forms and the free phenols are liberated following alkaline hydrolysis. Thus, alkaline conditions are employed in the isolation of phenolic acids from certain fruits, fruit products and cereals in order to determine ''bound'' phenols. In some instances, the phenolic acids were unstable in alkaline ambient conditions [171] and it was necessary to hydrolyse the samples under argon. The loss of *o*-diphenols by oxidation via the corresponding quinones is a constant concern under alkaline conditions. This is seen in significant losses of caffeic acid [113] in conditions where other phenols were relatively stable. Artefacts have also been reported [41] for the alkaline extraction of some polymethoxylated flavones. Similarly, flavanones and 3-hydroxyflavanones were sensitive to alkali under which conditions the dihydro-γ-pyrone ring was broken forming chalcones that decomposed to phenols and cinnamic acid derivatives [172]. Under these circumstances, hydrolysis has been performed in acidic conditions or using specific enzymes for known glycosides or technical enzymes when samples contain a mixture of glycosides. Nevertheless, alkaline hydrolysis has been widely used for samples such as citrus (juices) [173], grape and cherry juices [171], coffee [56], cereals [13] and oilseeds [67].

Orange juice was hydrolysed with sodium hydroxide for 4 h at room temperature under nitrogen [102,103] and the total phenolic acids were recovered by ethyl acetate extraction followed by silica gel column chromatography. Phenolic acids including caffeic, chlorogenic, ferulic and gallic acids were also determined in grape and cherry juices [171] following recovery by extraction with ethyl acetate from fresh or hydrolysed juices. Hydrolysis was performed in hydroxide solution at pH 12.5 and required 48 or 62 h for cherry and grape juices, respectively. Cherry juice contained a high concentration of chlorogenic acid that was hydrolysed rapidly to caffeic acid. Phenolic acids were recovered from cherry laurel in a similar fashion [113] by

Table 6
Representative examples of the use of SPE for the recovery of biophenols from plants and foods

Details	Sample	Quantification	Ref.
Tandem SPE	Wine	LC–MS	[142]
Dilution and SPE	Wine	GC–MS of TMS derivatives	[143]
SPE on C_{18}	Wine	GC–MS of TMS derivatives	[44]
Diluted, SPE on C_8 cartridge eluting with ethyl acetate	Wine	GC–MS of TMS derivatives	[15]
Dealcoholised (wines) and SPE to remove sugars	Berry and fruit wines and liquors	Colorimetry	[144]
SPE, acid and base hydrolysis	Raspberry juice	HPLC (see Ref. [104])	[145]
SPE C_{18}	Kiwifruit juice	HPLC	[146]
SPE fractionation into neutral and acidic fractions	Apple musts and ciders	HPLC–DAD; 280 nm (polyphenols), 320 nm (cinnamic acids), 360 nm (flavonols)	[147]
Acetone extraction due to high pectin content, SPE	Strawberry	HPLC–DAD, 240–550 nm	[148]
Extraction with methanol/dimethylsulfoxide, SPE	Citrus	HPLC–DAD, 285 nm	[149]
Dilution in dimethylformamide/ammonium oxalate solution and centrifugation; SPE concentration for *trans*-cinnamic acid	Blood orange	HPLC, 280 nm	[150]
Aqueous ethanol extraction with bisulfite; hexane partitioning and SPE	Olive	LC–MS; HPLC–DAD	[151]
Various, e.g. aqueous methanol extraction containing diethyldithiocarbamate followed by SPE	Olive fruit, virgin olive oil, vegetation waters, and pomace	HPLC–DAD	[92]
Acidified aqueous methanol extraction, SPE	Fruit jams	HPLC, 520 nm	[36]

extraction of dried mesocarp with petroleum ether. The residue was hydrolysed with sodium hydroxide, acidified and extracted into ethyl acetate prior to formation of oxime TMS derivatives that were analysed by GC–MS. Similar procedures have been adopted for the analysis of the fruit. For example, the distribution of free and bound phenolic acids was determined in fruit cuticles of several varieties of apple, using either cuticular wax scraped from fruit peel or enzyme-isolated cuticles [174] and in the fruit of orange and grapefruit [113] by extraction with ethyl acetate, silica gel column chromatography clean-up and HPLC analyses of samples before and after alkaline hydrolysis (24 h). Phenolic acids, which account for approximately 2% of the oil-free meal mass in rapeseed have been recovered [67]

following alkaline hydrolysis of an acetone extract of hexane-defatted samples. In all cases, the level of bound phenolic acids determined after alkaline hydrolysis [102,103,113,171,173] was significantly higher than that of the corresponding free acids.

Apart from the normally encountered conjugated forms, a significant portion of the hydroxycinnamate fraction of grasses and cereals exists as a bridge between lignin and polysaccharides in which ether and ester linkages, respectively are involved. The recovery of this bridged material involves a laborious extraction [175]. The hydroxycinnamate exists as dehydrodimers [13,176] that have been quantified after alkaline hydrolysis. Esterified phenols were hydrolysed under mild alkaline conditions whereas ether-linked phenols were hydrolysed under pressure [176]. Alternatively, dehydrodimers were identified in rye [13] and insoluble cereal fibres [177] following enzyme treatment (to degrade starch) and saponification under nitrogen in the dark. Ferulic acid (ca. 1000 mg kg^{-1} dry mass) was the most abundant phenolic acid [13] with lesser amounts of *p*-coumaric and sinapic acids. The most abundant dehydrodimer ranged from 130 to 200 mg kg^{-1} (dry mass).

4. Filtration/dilution

Some liquid samples are amenable to direct analysis requiring no treatment other than centrifugation, filtration and/or dilution. Fruit juices and wines often fall into this category. For instance, flavan-3-ols, anthocyanins, cinnamic acid derivatives and flavonol derivatives were determined [178–180] in wines by direct injection without sample pre-treatment. Apple juices were filtered through polytetrafluoroethylene filters and several classes of phenolic compounds were quantified in commercial juices by HPLC [181] and absorption at characteristic wavelengths as hydroxycinnamates (316 nm), anthocyanins (520 nm), flavan-3-ols (280 nm) and flavonols (365 nm). Cloudy juices such as citrus juices are also amenable to direct analysis following filtration and centrifugation [29,30,182,183] although poor recoveries have been attributed to low solubility of certain phenolics [184] and/or to sorptive losses on the filtration medium [185].

Simple dilution of citrus peel oils with ethyl acetate provided adequate sample treatment for determination of polymethoxylated flavones [186]. Analyte solubility presents a problem in some instances as in the case of hesperidin (the major flavonoid of sweet orange) which has low solubility in aqueous media. Addition of dimethylformamide to orange juice [187,188] and heating of the juice [189] have been used in an effort to improve solubility. Buffering of the juice in the pH range from 4.5 to 5.0 prior to extraction has been recommended [190] to overcome more general problems of the pH dependence of flavanone glycoside recovery. In this instance, oranges were hand-squeezed and the extract filtered through a stainless steel sieve (1.25 mm) to remove seed and pulp [24,190] although a double layer of cheese-cloth has also been used for this purpose [191]. The separated juice was mixed with dimethylformamide and ammonium oxalate (to maintain pH) and heated for 10 min. The cooled juice was centrifuged and filtered prior to analysis by HPLC.

5. Liquid extraction

In many instances, simple filtration is ineffective in recovering a broad range of phenols and liquid extraction represents a simple and convenient alternative [192–199]. The advantages of liquid extraction versus direct injection have been demonstrated [192] for HPLC analysis of a wine sample. Ethyl acetate and aqueous methanol are often the solvent(s) of choice for recovery of a wide range of phenolics from diverse sample types [106,184,195–197] including pollen [65,71], oats [74], fruits and vegetables [14], olive oil [198], onion and soybean extracts [199] and herbs [70].

Simple aqueous extraction involving percolation or infusion is useful for samples such as coffee and tea, respectively where the extracts provide data on probable dietary intakes [57–59]. However, caution is necessary as some flavan-3-ols (catechins) are unstable [193] in neutral or alkaline solutions but were precipitated with aluminium chloride [194] which reduced pH and stabilised the extract.

Various solvents have been described for the extraction of phenols from juices as demonstrated by a study on the recovery of polymethoxylated

flavones from citrus juices [195] and juices treated with sodium hydroxide to eliminate possible interfering lactones. In terms of total flavones, methyl isobutyl ketone was only slightly less efficient than benzene but was more effective for specific flavones. These data demonstrate the need to carefully consider any recovery problem [200,201] on an individual basis. Polarity differences in citrus juice components [202] were exploited in a comprehensive recovery scheme for carotenoids, polymethoxylated flavones and flavanone glycosides based on extraction with solvents of graded polarity. Citrus juice was diluted with methanol, centrifuged and aqueous sodium chloride added to the supernatant to minimise formation of troublesome emulsions. The solution was then sequentially extracted with hexane and dichloromethane to isolate the carotenoids and polymethoxylated flavones, respectively leaving the flavanone glycosides in solution.

Commercial juices and nectars of orange, apple, peach, apricot, pear and pineapple [200] were concentrated using a rotary evaporator prior to sequential extraction with ethoxyethane and ethyl acetate. The extraction time and temperature were evidently critical and this may reflect conflicting actions of solubilisation and analyte degradation by, for example, oxidation. The two extracts were combined to provide quantitative data on the contents of benzoic acids and aldehydes, flavan-3-ols, flavonols, chalcones, cinnamic acids and their esters, glycosidic derivatives and flavonoids. Differences in levels of flavanols were attributed to different degrees of pressing of the fruit as these phenols are found mainly in the skin and seeds. Similarly, phenolic composition of fruit purees and concentrates [136] was quantified by homogenising samples in acidified methanol and partitioning the phenolic components into ethyl acetate. Phenols were identified by HPLC as various benzoic acids and aldehydes, cinnamic acids and their derivatives, flavan-3-ols, procyanidins, flavonols, and dihydrochalcones. Peach-based products were completely devoid of flavonol and dihydrochalcone derivatives and this was attributed to the removal of the skin and stone from the fruit during the manufacturing process.

Extra virgin olive oil represents the juice from the olive and it contains several distinctive phenols [139,203–211] such as verbascoside, ligstroside and oleuropein. Isolation of the phenols from lipid components is generally mandatory. Phenols have been recovered from olive oil by extraction of the unsaponifiable matter with aqueous methanol [206] and by direct extraction from neat oil with methanol [211] in an ultra-turrax apparatus [140]. The methanol was removed and the residue dissolved in acetonitrile and washed with hexane. After evaporation of the acetonitrile under vacuum, the residue was dissolved in acetone prior to derivatisation with BSTFA and separation by GC. The more usual procedure has involved dissolution of the oil in hexane [138,204,208,209] or ethoxyethane [139] followed by liquid–liquid extraction using various mixtures of water and alcohol [139,203,204,208,209] in order to isolate the desired analytes from unsaturated, interfering species. Residual oil must be removed by overnight storage at subambient temperature [139], by centrifugation [208] or solvent extraction with hexane [209] although Sephadex column chromatography has also been used [8,9] to effect further clean-up. An internal standard is included in most procedures. Of several solvents examined, methanol–water (80:20, v/v) provided highest recoveries of phenolic species [8] measured as Folin Ciocalteu total phenols. Montedoro et al. [8] have compared the various methods of extraction (from neat oil vs. a solution of oil) using different solvent combinations and also concluded that aqueous methanol provided optimum results. It has also been claimed [139] that extraction with neat methanol improved yields of a number of phenols and eliminated formation of troublesome emulsions seen with aqueous methanol.

In the case of fruit, hot or cold extraction with aqueous mixtures of ethanol, methanol, acetone or dimethyl formamide has been common [212,213]. Extraction method and solvent choice are generally critical [213] and extractions have been performed on freeze-dried ground extracts of the fruit or, alternatively, by maceration of the fresh, undried fruit with the extracting solvent [210]. In the latter case, the required proportion of water in the extractant is lower. The optimum alcohol content will differ for phenols of diverse structures [212]. Grapefruit portions and peel were dried at 50 °C in a fan forced air oven [121]. The residue was ground to a fine powder, filtered, extracted with dimethylsulfox-

ide and analysed by HPLC. In the more usual approach, flavonoids were extracted with aqueous ethanol [122] from a dried extract of sour orange. The ethanolic extract was filtered and evaporated to dryness under vacuum prior to analysis by LC–MS. Isoflavones, flavanones and flavonol glycosides were isolated [214] from lyophilised ground tart cherries by partitioning of a methanolic extract with ethyl acetate while anthocyanins were recovered in a separate fraction. In a similar procedure, lyophilised apple peel or flesh was extracted with methanol and sonicated for 30 min prior to centrifugation and HPLC [62]. Alternatively, whole apples, peel or flesh were homogenised with aqueous methanol [181] using a Waring blender. The extracts were filtered and the methanol removed by rotary evaporation prior to analysis by HPLC. Flavan-3-ols comprised 31–54% of the phenolic content with lesser amounts of flavonols, 1–10%; phloridzin, 11–17%; cinnamates, 3–27%; and anthocyanins, n.d.–42%. Parenchyma (62% by mass), epidermis (18%), core (11%) and seeds (1%) of mature cider apples [134] were isolated while spraying with aqueous formic acid to avoid oxidation. The tissue samples were then frozen, freeze-dried and extracted sequentially with hexane (to remove lipids, carotenoids and chlorophyll), methanol (sugars, organic acids and low molecular mass phenols) and acetone (polymeric phenols). Hydroxycinnamic acid derivatives, flavan-3-ols, flavonols and dihydrochalcones were identified in the extracts. Procyanidins were the predominant phenolic constituents in the fruits, much of them corresponding to highly polymerised structures.

The major flavan-3-ols are (+)-catechin and (−)-epicatechin and whereas most flavonoids occur as glycosides, the catechins are an exception in that they occur only as aglycones and gallate esters. The recovery of flavan-3-ols from fresh and freeze-dried samples was examined [126] in three model foods: apples, black grapes, and canned kidney beans. The levels of flavan-3-ols were not affected [126] by the drying process but recoveries were dependent on the type (ethanol, methanol, or acetone) and concentration (40–100% in water) of extraction solvent. Maximum recovery required a minimum of 70% methanol in the extractant and this was attributed to the need to inactivate polyphenol oxidases, which are widely distributed in plants. Using the optimised procedure, flavan-3-ols were recovered with aqueous methanol from freeze-dried sample by room temperature extraction using a mechanical shaker (60 min). The extracts were filtered and analysed without further processing using HPLC and fluorescence or ultraviolet detection.

There is also a group of oligomeric and polymeric species based on the flavan-3-ols that are termed proanthocyanidins. These compounds are one of the two groups of tannins and are an extremely important phenolic fraction both quantitatively and in their properties [54]. The precise structure of many of these substances is unclear and Santos-Buelga and Scalbert [54] defined them as ‘‘polymeric flavan-3-ols’’ but then proceeded to discuss proanthocyanidins of ‘‘low polymerisation degree (dimers and trimers)’’. We prefer the definition to include all substances that yield anthocyanidins upon heating in acidic media. This is an important point and acidic extraction may lead to underestimation of the oligomeric and polymeric species. Thus, the average polymerisation degree varies widely with the species, tissue and method of extraction [54]. Proanthocyanidins have been extracted by various procedures [12,116] including gel permeation chromatography [77] and solvent extraction [43]. Aqueous methanol was chosen for extraction from diverse food samples because of its efficiency in recovering flavanols of low polymerisation degree. However, extraction varies with the solvent system used and also the polymerisation degree of the analyte [54]. Methyl acetate was used for selective extraction of monomeric flavan-3-ols and low molecular mass oligomers [152] from lyophilised powder of apple condensed tannins. In cider apple extracts, proanthocyanidins with the highest polymerisation degree were better extracted by aqueous acetone than by aqueous methanol [134]. Aqueous acetone generally gives the best yields but a variable proportion of proanthocyanidins resist extraction particularly in aged or oxidised tissues [54]. The most common proanthocyanidins in foods are procyanidins which exhibit a specific dihydroxy-substitution pattern [54]. Catechins and procyanidins were extracted with ethyl acetate from an aqueous acetone extract of pomace and pear fruits adjusted to pH 7.0 [33] whereas all other phenols were recovered by extraction at pH 1.5. This procedure was necessary since catechin,

epicatechin and the procyanidins were not detectable following acidic extraction. In the case of apple juice, the incorporation of an additional clean-up step was mandatory. This was achieved by SPE or, alternatively, by drying over sodium sulfate.

Partitioning into hexane has been used to eliminate non-polar components from fruit extracts [134] but this becomes mandatory with olives due to the relatively high oil content of the fruit and the extraction procedure developed by Amiot et al. [215] has been widely adopted [151,165,167,216,217]. The details differ but sample preparation has generally entailed extraction with aqueous ethanol in the presence (or absence) of metabisulfite of freeze dried olives powdered with the aid of liquid nitrogen. The extracts were concentrated under reduced pressure, acidified (in some instances) and washed with hexane to remove lipophilic compounds [215]. The phenolic compounds were partitioned into ethyl acetate [166] in the presence of ammonium sulfite, metaphosphoric acids and methanol [167,216,217]. Minor phenolic components of olive such as flavonoids were extracted with aqueous methanol [218] while simple phenols were recovered [219] from olives by homogenising in a blender with water. The homogenate was evaporated to dryness under reduced pressure, redissolved in water and the solution partitioned into ethyl acetate to retrieve the phenolic substances. Extracts have typically been analysed by HPLC.

Anthocyanins comprise a major portion of the phenolic content of dark-coloured berries [10,123,220–222]. They were traditionally recovered as the flavylium cation by extraction with cold methanol containing hydrochloric acid [223]. However, the acylated anthocyanins are frequently labile under such conditions [16,224] but may be recovered by replacing hydrochloric acid with weaker acids, either formic or acetic acid [225–227]. With the most labile anthocyanins, the use of nonacidified solvents is probably a sensible precaution. Grape anthocyanins were extracted [228] at room temperature using a mixture of formic acid in aqueous methanol. A similar extraction procedure was applied [10] to sweet cherries in which anthocyanins comprise the major phenolics particularly in dark-coloured genotypes. Mature sweet cherries were pitted and homogenised with extracting solvent, filtered and analysed by HPLC for separation and quantification of both anthocyanins and other phenolic compounds predominantly neochlorogenic acid and *p*-coumaroylquinic acid.

The phenolic profile of fruit juices often differs from that of the fruit [169,181] and arises from the effects of enzymatic activity during processing. This is also seen during extraction [62] where enzymatic activity in green olive drupes [229] was inhibited by refluxing in boiling methanol for 30 min. The aqueous extract following removal of methanol was exhaustively extracted with ethyl acetate and purified using reversed-phase thin-layer chromatography (TLC). Extraction with boiling ethanol (5 min) followed by aqueous ethanol (1 h) has also been applied [230] and the authors noted that boiling inactivated enzymes and aided in phenol recovery. Phenols in the filtered ethanolic extract were quantified by ultraviolet derivative spectroscopy.

Extraction procedures for other sample types (e.g. vegetables, herbs, peel) [231] show minor variations from those described for fruit. For example, herbs were freeze-dried, extracted with aqueous methanol, filtered and analysed by HPLC [70]. Chocolate was frozen in liquid nitrogen and then ground in a laboratory mill [68]. In recognition of the high lipid content, the ground chocolate was defatted with hexane in an ultrasonic bath before extraction of catechins and procyanidins with an acetone–water acetic acid extractant. The sample was filtered and the organic solvent removed under vacuum. The aqueous residue was analysed by HPLC without further clean-up.

6. Solid-phase extraction

The variation in detector response and levels of phenols in a single sample often requires different recovery procedures. For example, flavanone glycosides were isolated [150] from the juice of blood oranges by simple extraction whereas *trans*-cinnamic acid was concentrated by reversed-phase SPE. Several SPE formats are commercially available ranging from the original cartridges to disks in a range of sorbents. SPE is a rapid and sensitive sample preparation technique [232] that has successfully replaced many tedious conventional methods of isolation and

extraction. Sample preparation and concentration via SPE can be achieved in a one-step extraction.

SPE using mini-cartridges has been employed for juices and wines [27,41,144,149,191,233,234] usually with a C_{18} reversed-phase although recoveries of cinnamic acids, *cis*- and *trans*-resveratrol, flavonoids and flavanols from (grape) wine were higher [15] from C_8 cartridges than from diatomaceous earth or C_{18} cartridges. The presence of ethanol in the wine samples presented problems that were eliminated by distillation although matrix dilution with water was equally effective and a simpler solution. This also reduced matrix interference by other components and improved recoveries of phenolic species. The phenolic compounds were eluted from the SPE cartridge with ethyl acetate, evaporated to dryness by azeotropic distillation and derivatised with BSTFA prior to quantification by GC–mass selective detection using an internal standard.

With reversed-phase cartridges, interfering sugars can be eluted with aqueous methanol prior to elution of phenols with methanol [144,191]. Preliminary fractionation of citrus juice phenols has also been performed [235,236] on polyamide cartridges eluting with methanol. Phenolic acids were determined [237] in four fruit juices after pre-concentration by SPE using a combination of reversed-phase and ion-exchange cartridges in series. Gallic acid was concentrated on the latter but was not retained on the reversed-phase. The acids were eluted with 0.1 *M* HCl and methanol after washing of the cartridges with water. Free and glycosylated resveratrol have been determined [238] in wines by HPLC following pre-concentration by automated SPE.

Fractionation of the phenolic components is readily achieved by SPE as illustrated by the analysis of kiwifruit juice which contains relatively low levels of phenols. Juice was extracted in a hammer mill and treated with pectolytic enzymes and ethanol prior to filtration to remove protein [146]. The extract was fractionated into strongly acidic (derivatives of coumaric, caffeic and 3,4-dihydroxybenzoic acids) and weakly acidic materials (epicatechin, catechin and procyanidins plus flavonols present as the glycosides of quercetin and kaempferol) by processing on Sep-Pak C_{18} cartridges. Further examples are the fractionation of neutral and acidic phenols from cranberry juice [161] and apple must and cider [147]. Similarly, phenolic and cinnamic acids were recovered from sherry wine by an initial clean-up on a C_{18} cartridge followed by fractionation into acidic and neutral phenolic fractions using an anion-exchange cartridge [239] or by an on-line automated robotic system with a polymeric polystyrene divinylbenzene cartridge using tetrahydrofuran as eluent [240]. Recoveries from spiked samples typically exceeded 85% [147,240] although other data [239] suggessts much lower recoveries are typical. Nevertheless, SPE was regarded as superior to liquid–liquid extraction and reduced analysis times by 50%.

Anthocyanins were recovered from diluted fruit juice or wine (after removal of ethanol) [241–243] by elution from a C_{18} cartridge with an aqueous eluent of low pH. Alternatively, a class separation was achieved by elution with an alkaline borate solution [242] in which anthocyanins possessing *o*-dihydroxy groups (cyanidin, delphinidin, petunidin) formed a charged borate complex, resulting in a more hydrophilic species, that was preferentially eluted from the reversed-phase cartridge. Those anthocyanins not containing *o*-dihydroxy groups (pelargonidin, peonidin, malvidin) were enriched on the cartridge. On the other hand, elution with hydrochloric acid (0.01%) in methanol produced no fractionation. A more exhaustive clean-up on polyvinylpolypyrrolidone was also examined. The relative proportions of the anthocyanins was different for the two procedures demonstrating the need to thoroughly examine extraction and/or clean-up procedures [244].

Condensed polymeric anthocyanins formed during the winemaking process by interactions between anthocyanins and other phenols such as flavanols (e.g. catechin) were recovered [245] from red wine or apple cider on an ODS column by elution with methanol. The concentrated lyophilised extracts were then fractionated by gel permeation chromatography using a mixture of acetone and acidified aqueous urea as eluent. Anthocyanins and other phenolic compounds were recovered from the GPC fractions by sorption on a C_{18} Sep-Pak cartridge that was washed with water to remove urea. The sorbed phenolic compounds were eluted with methanol.

The versatility of SPE has been exploited [246] for the recovery of phenolics from olive oil. Mannino et al. [207] reported gallic acid in olive oil and attributed its appearance to their extraction procedure involving SPE which eliminated oxidation prevalent

in other procedures. Suitable sorbents are alkylsilicas (C_8 or C_{18}) [203,207], amino phases [247] and anion-exchanger [248] using one of two experimental approaches. In one procedure, a solution of the oil in hexane was applied [207,249] to a pre-conditioned (typically reversed-phase) cartridge which was washed with hexane–ethoxyethane or hexane–cyclohexane [250] mixtures to remove the non-polar lipid fraction. Phenols were then eluted with acetonitrile or methanol, filtered and stored overnight at reduced temperature to precipitate oil droplets prior to HPLC [250]. Consistent recoveries over 95% were achieved [207,249] from spiked samples in contrast to the variable results with solvent extraction. In other cases, differences between results obtained by SPE and solvent extraction were not statistically significant [247] and data comparing the two techniques remain controversial [251]. Alternatively, the polar fraction of olive oil has been partitioned into aqueous methanol from a hexane solution [110] and fractionated into two parts (A and B) by SPE. Analysis of the two fractions showed that Part A (eluted from Sep Pak C_{18} with methanol–water, 20:80 v/v) contained only simple phenols and phenolic acids while Part B (eluted with mixtures of methanol–chloroform) had a complex nature. The two parts tested for their antioxidant activity showed relatively high protection factors in safflower oil although Part B was found to contribute more than part A to the stability of the oil in agreement with the findings of Montedoro et al. [8,9]. The antioxidant activity of both fractions [110] was related to their content of total phenols and *o*-diphenols. Significant changes in the HPLC profiles were observed following acid and alkaline hydrolysis which yielded valuable information indicating the presence of ether and ester bonds in the various components.

Solvent extracts from solid samples that have been processed by SPE include quince jams [32] and plant materials [252] such as leaf tissue of *Myrtus communis* L. [253]. Leaf tissue contained small amounts of phenolic acids (caffeic, ellagic and gallic acids) and quercetin derivatives (quercetin 3-*O*-galactoside and quercetin 3-*O*-rhamnoside), whereas catechin and myricetin derivatives were present in large amounts. Flavonoids were identified [254] in aqueous extracts of dry spinach after removal of lipophilic compounds by SPE. Epicarp, mesocarp, endocarp and leaf tissue of *Citrus* were lyophilised, ground and extracted [149] at ambient temperature for 12 h using methanol–dimethyl sulfoxide. The extracts were centrifuged and subjected to clean-up by SPE using C_{18} cartridges to remove polar components. The retained flavonoids were eluted with methanol–dimethyl sulfoxide which enhanced solubility of hesperidin, diosmin and diosmetin. Recoveries of eriocitrin, naringin, hesperidin and tangeretin from spiked samples of mesocarp tissue exceeded 96%. Flavones were relatively abundant in leaves. Servili et al. [92] achieved higher recoveries of phenolic compounds from olive drupes by SPE than by liquid–liquid extraction and developed [255] a comprehensive scheme for the extraction of phenolic compounds from olive pulp that introduced several precautions aimed at inhibiting enzyme activity and hence phenolic modification or destruction. Olives were peeled and destoned, and the olive pulp was placed in liquid nitrogen and subsequently freeze dried. The freeze dried material was stored at −30 °C prior to analysis. Phenolic compounds were recovered from the olive matrix by extraction with aqueous methanol containing sodium diethyldithiocarbamate. This mixture was homogenised for 30 s and filtered using a Buchner funnel. The methanolic extracts were evaporated under vacuum and nitrogen flow at 35 °C and purified by SPE using a High-load C_{18} cartridge, the phenolic compounds being eluted with methanol. Alternatively, the extracts have been further processed on a diatomaceous earth Extrelut cartridge [151] which was sequentially eluted with hexane, ethyl acetate (non-anthocyanic phenols) and acidic methanol (anthocyanins).

7. Supercritical fluid extraction

The industrial applications of SFE to isolation of plant products generally preceded analytical uses of this technique. As high pressure systems became available in the laboratory the transition to laboratory scale separations occurred [256] and SFE has been applied to several groups of non-polar compounds, including essential oils, other flavour and fragrance compounds, medicinal compounds, lipids, carotenes and alkaloids. Efficiency comparisons between SFE and solvent extraction would be useful but are often unavailable. Nevertheless, conventional methods often have limited application to solid and semi-solid

samples because of the long extraction times and precautions needed to protect the highly reactive phenolic species from degradation processes. In these instances, supercritical fluid extraction offers a number of advantages for the recovery and the extraction behaviour of phenolic compounds has been modelled using supercritical carbon dioxide and either sand [257] or an inert support as a sample matrix [258]. Phenolic compounds were selected to cover a range of polarities (including benzoic and cinnamic acids, hydroxybenzaldehydes and catechin). Extraction and collection variables were optimised and revealed [258] that the use of methanol as modifier was mandatory. Only the less hydroxylated compounds such as *p*-coumaric acid, *tert*.-resveratrol and salicylic acid could be quantitatively recovered from spiked diatomaceous earth while mean recoveries of more polar phenolic acids and flavonoids were between 30 and 70%.

SFE [259,260] facilitates off-line collection of extracts and on-line coupling with other analytical methods such as GC and supercritical fluid chromatography [261]. It has been used in a two-step fractionation of leaves of rosemary and sage into an essential oil and antioxidant fraction. Phenols have also been isolated from dried (100 °C), ground and sieved ($\leq$500 μm) olive leaf using supercritical carbon dioxide modified with methanol [262]. The influence of extraction variables such as modifier content, pressure, temperature, flow-rate, extraction time, and collection/elution variables, was studied. The dynamic SFE method produced clean extracts with higher phenol recoveries (measured as total phenols by Folin Ciocalteu) than sonication in liquid solvents such as *n*-hexane, ethoxyethane and ethyl acetate. However, the extraction yield obtained was only 45% of that obtained with liquid methanol. Extracts were screened for acid compounds such as carboxylic acids and phenols using electrospray ionisation mass spectrometry (ESI-MS) in the negative ionisation mode.

8. Newer developments

The increasing demand for new extraction techniques, amenable to automation with reduced solvent consumption and analysis times has seen the introduction of SFE, solid-phase microextraction (SPME), microwave assisted extraction (MAE) [263], pressurised liquid or fluid extraction [264] (accelerated solvent extraction), membrane extraction and surfactant cloud point extraction [265]. The relative merits of many of these developments have been summarised [263].

SPME is a versatile technique finding wide acceptance as a sampling method in GC and, more recently, HPLC and capillary electrophoresis. The commercially available SPME fibres can be divided into adsorbent- and absorbent-type fibres [266]. The latter extract by partitioning of analytes into a ''liquid-like'' phase and hence are more akin in their action to traditional liquid extractions. The recent trend in sample handling involves miniaturisation and single drop microextraction has evolved from this approach. According to this technique a microdrop of a water-immiscible solvent is left suspended on the tip of a conventional syringe immersed in an aqueous solution of the sample. The application of these techniques to plant phenols has not been reported. Nevertheless, the application of SPME and HPLC to analysis of hydroxyaromatic compounds in water [267] suggests that the technique warrants closer examination for analysis of plant phenols. General aspects of the technique as applied in food analysis have been reviewed [268].

In a comprehensive review of the applications of MAE [263] there were no papers relating to plant phenols. However, a 1994 paper [269] described the application of MAE with aqueous sodium hydroxide for the release of esterified and etherified hydroxycinnamic acids from cell walls of maize, wheat, barley and oilseed rape stems. The beta-ether linked phenolic acids were obtained by subtraction of values for saponifiable phenolic acids obtained after treatment with aqueous sodium hydroxide (1 *M*) at room temperature from digest results. MAE was shown to be an order of magnitude more effective than dioxane–HCl at liberating beta-ether bound phenolic acids. MAE in closed vessels offers the opportunity for elevated temperature and pressure operation that should enhance recoveries and reduce extraction times. For thermolabile compounds, reduced extraction times should minimise analyte degradation although the effects of temperature are not always intuitive [263]. It is in the extraction of

proanthocyanidins [54] that SFE and MAE may be particularly suited.

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