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Review

Strategies for the determination of bioactive phenols in plants, fruit and vegetables

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Abstract

Analytical strategies dealing with bioactive phenols in plants and foods are reviewed. These depend on the purpose of the analysis which may be classified as studies where the principal purpose is biological screening, phytochemical and/or chemical screening. Nevertheless, extraction of the phenol from the sample matrix is common and methods of achieving a suitable extract are assessed. Advances in the separation sciences and spectrometry are exploited for identification and quantification of isolated phenols. The various procedures are summarized and some typical ''case studies'' are presented. Two important areas are introduced briefly. Thus, plant phenols are reactive species and their ultimate fate has been relatively neglected. Studies of bioactive compounds generate a considerable volume of data making data handling and informatics important topics that warrant a separate review.

Keywords: Reviews; Plants; Fruit; Bioactivity; Phenols

Contents

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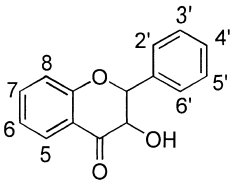
1. Introduction

Epidemiological studies over the last three decades have consistently correlated certain diets, specific foods and disease expression. At the same time, the number of bioactive compounds has increased dramatically and a new diet-health paradigm has evolved that emphasizes the positive aspects of diet. The terms ''phytochemical'', ''nutraceutical'' and ''functional food'' have been introduced [1] to describe various aspects of this development. Databases detailing the presence and amount of bioactive compounds in foods have recently been reviewed [2]. Bioactive compounds included a range of compounds with diverse chemical structures such as plant sterols, carotenoids, ω-3-fatty acids, indoles (benzopyrroles) and phenols. The number and diversity of these compounds preclude an exhaustive coverage of their determination and analytical strategies are illustrated in this review with specific reference to plant phenols.

Plant phenols (Fig. 1) embrace a wide range of secondary metabolites that are synthesized from carbohydrates via the shikimate pathway. This is the biosynthetic route to the aromatic amino acids and is restricted to microorganisms and plants. Thus, phenolic compounds are ubiquitous in the plant kingdom being found in all fruits and vegetables in virtually all parts of the plant but with quantitative distributions that vary between different tissues of the plant and within different populations of the same plant species [3]. The phenolic component of plants constitutes a complex mixture, and only a small number of plants have been examined systematically for their phenolic content. Thus, the data on phenolic content of plants, fruits and vegetables are incomplete. Nevertheless, both qualitative and quantitative data have been summarized in various reviews [4–11] although quantitative data are not reliable [8] due to the wide diversity of extraction and quantification procedures.

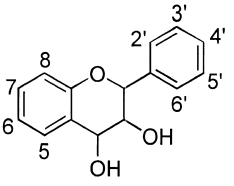
The determination of phenols encompasses a number of distinct aspects and the analytical strategy will depend on the sample, analyte and nature of the problem. Given the diversity of analytes and sample types and the number of permutations of the three, there is no global strategy that will suffice for a given phenol in all situations although a number of generalisations can be made. Thus, the general analytical strategy involves recovery of the phenol from the sample matrix followed by separation, identification and measurement. For most phenols, the recovery step typically involves solvent extraction using a range of solvents. Special considerations apply to some phenols such as the anthocyanins and oligomeric species. Polymeric phenols introduce a new range of considerations and are not considered in this review. Separation is commonly achieved by HPLC although GC is used in some instances. The most common mode of separation exploits reversed-phase systems typically with a C_{18} column and various mobile phases. Detection is routinely achieved by ultraviolet absorption often involving a photodiode array detector although the versatility of the latter often appears to have been neglected. Coupled techniques particularly various mass spectral methods are being used increasingly for routine work although analyte collection using preparative-scale HPLC and off-line identification are often still needed for non-routine samples.

Considering the nature of the analytical problem, several roles can be identified although there is no rigid distinction and an investigation may encompass aspects of each one. In the first role, screening of bioextracts for biologically active natural products played a strategic role in the phytochemical investigation of crude plant extracts [12,13]. The primary strategy for the discovery of bioactive natural products through the 19th century and into the 20th century was the structure elucidation of active ingredients of plants with reported biological properties. Methods of characterization and identifi-

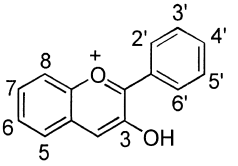


Flavanonols

Taxifolin 5=7=3'=4'=OH
(dihydroquercetin)



Flavan-3,4-diols
(leucoanthocyanidins)

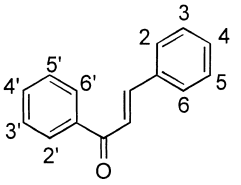


Anthocyanidins

Pelargonidin 5=7=4'=OH
Cyanidin 5=7=3'=4'=OH
Peonidin 5=7=4'=OH, 3'=OCH_3
Delphinidin 5=7=3'=4'=5'=OH
Petunidin 5=7=4'=5'=OH, 3'=OCH_3
Malvidin 5=7=4'=OH, 3'=5'=OCH_3

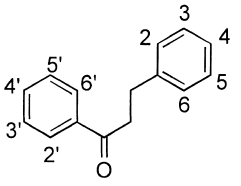
Anthocyanins

Cyanidin 3-glucoside
Cyanidin 3-galactoside
Cyanidin 3-arabinoside
Cyanidin 3-rutinoside
Malvidin 3-glucoside
Malvidin 3,5-diglucoside



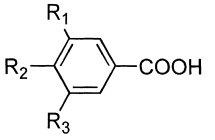
Chalcones

Butein 2'=4'=3=4=OH
Licochalcone B 4'=3=4=OH, 2=OCH_3
Okanin 2'=3'=4'=3=4=OH
Chalconarigenin 2'=4'=6'=4=OH



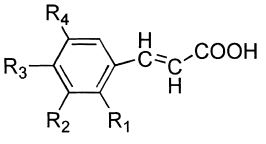
Dihydrochalcones

Phloridzin 4'=6'=4=OH, 2'= *O*-glucosyl



Benzoic Acids

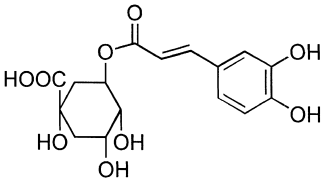
Gallic acid R_1=R_2=R_3=OH
Protocatechuic acid R_1=H, R_2=R_3=OH
Vanillic acid R_1=H, R_2=OH, R_3=OCH_3
Syringic acid R_2=OH, R_1=R_3=OCH_3



Cinnamic Acids

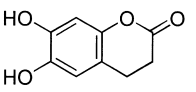
Ferulic acid R_1=R_2=H, R_3=OH, R_4=OCH_3
p-Coumaric acid R_1=R_2=R_4=H, R_3=OH
o-Coumaric acid R_2=R_3=R_4=H, R_1=OH
Caffeic acid R_1=R_2=H, R_3=R_4=OH
Sinapic acid R_1=H, R_3=OH, R_2=R_4=OCH_3

Cinnamic Acid Esters

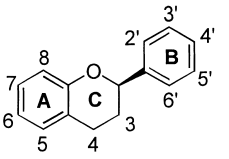


Chlorogenic acid (5-caffeoylquinic acid)
Coutaric acid (*p*-coumaroyltartaric acid)
Caftaric acid (caffeoyltartaric acid)

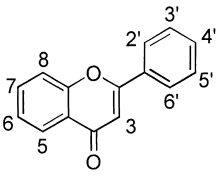
Fig. 1. Structure of typical phenols.



Coumarins (aesculetin)

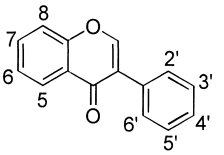


Flavan



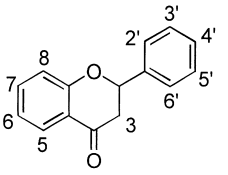
Flavones

Apigenin 5=7=4'=OH
Luteolin 5=7=3'=4'=OH
Diosmetin 5=7=3'=OH, 4'=OCH_3
Tricin 5=7=4'=OH, 3'=5'=OCH_3
Sinensetin 5=6=7=3'=4'=OCH_3
Tangeretin 5=6=7=8=4'=OCH_3
Nobiletin 5=6=7=8=3'=4'=OCH_3
Isovitexin 5=7=4'=OH, 6 = Glucose



Isoflavones

Daidzein 7=4'=OH
Genistein 5=7=4'=OH

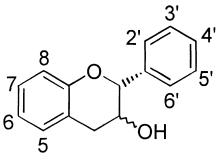


Flavanones

Naringenin 5=7=4'=OH
Isosakuranetin 5=7=OH, 4'=OCH_3
Eriodictyol 5=7=3'=4'=OH
Hesperitin 5,7,3'=OH, 4'=OCH_3

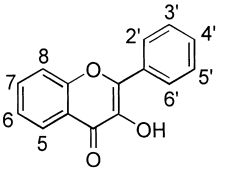
Flavanone glycosides

Prunin narigenin 7-glucoside
Naringin narigenin 7-neohesperidoside
Narirutin narigenin 7-rutinoside
Hesperidin hesperitin 7-rutinoside



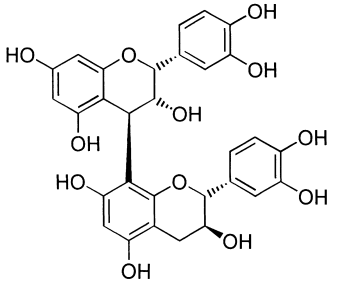
Flavanols

Catechin (2R, 3S) 5=7=3'=4'=OH
Epicatechin (2R, 3R) 5=7=3'=4'=OH
Epigallocatechin (2R, 3R) 5=7=3'=4'=5'=OH
Epicatechin gallate (2R, 3R) 5=7=3'=4'=OH, 3-gallic acid ester
Epigallocatechin gallate (2R, 3R) 5=7=3'=4'=5'=OH, 3-gallic acid ester



Flavonols

Fisetin 7=3'=4'=OH
Kaempferol 5=7=4'=OH
Morin 5=7=2'=4'=OH
Herbacetin 5=7=8=4'=OH
Quercetin 5=7=3'=4'=OH
Robinetin 7=3'=4'=5'=OH
Isorhamnetin 5=7=4'=OH, 3'=OCH_3
Myricetin 5=7=3'=4'=5'=OH
Gossypetin 5=7=8=3'=4'=OH



Procyanidin B-1

Flavonol glycosides

Rutin quercetin 3-*O*-rhamnosylglucoside
Hyperin quercetin 3-*O*-β-D-galactopyranoside

Fig. 1. (*continued*)

cation of plant phenols followed those in general use for natural products [14]. Hence, preparation of an extract, biological screening, bioguided fractionation, isolation and structure elucidation was the usual approach where complete characterization was required. However, the number of biological assays that are available in a given laboratory is often limited and the range of activities screened is thus restricted. As a complementary approach, the second role employs ''chemical screening'' using coupled techniques at the earliest stage of separation on crude extracts. With this approach, extract preparation is followed by isolation of the phenol(s) and structure elucidation. This efficient and targetted isolation of compounds permits an optimization of an investigation and avoids the time-consuming and costly isolation of ''trivial'' natural products. A third application area involves the determination of previously identified substances as in the quality control of the active principles in herbal and other products [15–18]. In this role, the analysis is simplified to the preparation of an extract and quantification of the analyte. A separate clean-up step may be mandatory in some cases. In the first three application areas, there is a clearly defined desired outcome. However, in some instances, analysis is carried out simply to profile the phenolic content of the plant or food. Such investigations are conveniently termed phytochemical studies and involve diverse approaches.

This review examines each of these application areas and covers the period from approximately 1990. Sample collection and storage is relevant to all application areas and is treated briefly. A further application area that is not treated in this review involves use of quantitative structure–activity relationships (QSAR) [19] and molecular modeling of bioactive species [20] to assess structure–activity relationships. The ultimate goal of such studies is the identification of a compound with a high level of bioactivity and a low degree of toxicity plus optimal pharmacokinetic properties. For instance, the antioxidant activity of flavonoids is closely related to the position and degree of hydroxylation of the molecule [11,21]. Structure–antiviral activity effects [22] have been assessed using three series of 3-methoxyflavones. Such studies demonstrate the importance of isomerism as a feature of bioactivity. For instance, cinnamic acid derivatives generally occur as the trans isomers but verbascoside and lantanaside, both phenylpropanoid glycosides, were isolated [23] from the polar fraction of a methanolic extract of the leaves of *Lantana camara*. Lantanaside contains a *cis*-caffeoyl moiety instead of *trans*-caffeoyl as in verbascoside.

2. Sample collection and storage

In many instances, definitive procedures for collection and storage have not been established although the limited data clearly indicate the importance of this step. Three extraction and hydrolysis procedures were examined for the recovery of flavonols (kaempferol, quercetin, myricetin) and phenolic acids (*p*-coumaric, caffeic, ferulic, *p*-hydroxybenzoic, gallic and ellagic acids) from frozen non-grape berries [24–27]. The thawing method (refrigerator, room temperature or microwave) showed differential effects on the level of various phenols. Microwave thawing produced the most reliable results and was also the most practical approach for routine analyses. This is clearly an area which requires closer examination to establish guidelines and definitive procedures.

3. Screening of bioextracts

Current strategies for choosing candidate plant species or tissues for isolation of bioactive components are based on ethnobotany, chemical ecology and plant anatomy [28]. Phytochemicals such as flavonoids have been used historically to identify plants in chemotaxonomy [29,30]. This role is being reversed and chemotaxonomy is generating bioactive compounds of the same or related molecular structures. The isolation of new bioactive compounds from plants can be directed by bioassays [31]. Alternatively, new uses of compounds can be identified when known compounds are tested in new bioassays. The availability of specific in vitro bioassays has facilitated the screening of numerous bioactivities of natural products. Screening has uncovered new pharmaceuticals and structure–activity relationships which has provided leads for design of new drugs.

Bioassays can range from molecular assays to whole-organism assays. Each has its advantages depending on the objectives and these have been discussed by Duke et al. [28]. Many factors can complicate results when using bioassays or bioassay-guided fractionation. Solvent is important as many bioextracts have limited solubility [32]. The selection of solvent must be considered carefully in relation to the nature of the bioassay in order to avoid false results. Other factors include synergistic effects, chemical changes during extraction and cancellation of activity by certain concentrations of substances. For example, in the isolation of leurosine, the crude alkaloid fraction exhibited no in vitro activity whilst the pure alkaloid showed pronounced cytotoxicity in the same test [33]. Caution is necessary to avoid artefacts ranging from the obvious such as antioxidants added to certain solvents used in measurement of antioxidant activity to the less obvious. However, solvents often appear as the culprits. In the isolation of an oleoside derivative from olive, the active ingredient was isolated as a hemiacetal from alcoholic solvents [34] rather than the naturally occurring dialdehydic species. Plasticizers are ubiquitous and their exclusion from extracts is a challenging problem [35,36]. Triacontanol is bioactive and is a known contaminant of some filter papers. Middleditch [37] has compiled a list of commonly encountered artefacts.

The most studied bioactivity of the phenols is their antioxidant status. Their antioxidant activity means that phenolic compounds (e.g. caffeic esters, catechins) also function as good browning substrates in fruit and vegetables [11]. They are functional as antioxidants at relatively low concentrations while at higher concentrations, since they themselves are susceptible to oxidation, they can behave as pro-oxidants due to their involvement in initiation reactions. The action of phenols as antioxidants is viewed as beneficial in both foods and the body where phenols are oxidized in preference to other food constituents or cellular components and tissues. Thus, measurement of antioxidant activity of a phenol or mixture of phenols (as in a juice extract) has been applied in two situations; the determination of antioxidant potential (to determine level of food protection) or physiological activity using in vitro [38] or in vivo tests [39,40].

Methods of measuring antioxidant activity have been reviewed elsewhere [41] and are not re-examined here. It is sufficient to state that methods show extreme diversity [42,43] and that activity depends on the analytical technique [44] and substrate [45]. For example, the trends in antioxidant activity of phenols differed [46] according to whether hydroperoxide formation (peroxide value) or decomposition (hexanal and volatiles) was measured in accelerated stability tests on olive oil. These results emphasize the need to measure at least two oxidation parameters to better evaluate antioxidant activity. Three methods widely employed in the evaluation of antioxidant activity, namely 2,2′-diphenyl-1-picrylhydrazyl radical scavenging method, static headspace gas chromatography and β-carotene bleaching test, have been compared with regard to their application in the screening of plant extracts of differing polarity [47]. Activity in each assay was affected by the complex composition of the extracts and partition phenomena.

The problem with bioassays is that they provide no data on individual compounds. Moreover, they do not distinguish between members of a class of bioactives and provide only semi-quantitative measurements of substances detected. Nevertheless, they continue in use because of their simplicity, low cost and the potential to generate activity data of direct relevance.

4. Phytochemical studies

4.1. Sample preparation

Sample handling strategies have been reviewed recently [48]. The following presents an overview of this area but with an emphasis on the sample type. The nature of both the sample and analyte (e.g. total phenols, *o*-diphenols vs. other phenols, specific phenolic classes such as flavonone glycosides or individual compounds; bound vs. free phenols; monomeric, oligomeric or polymeric species) impact on the choice of a method. However, other factors must also be considered in designing an optimal sample handling strategy that will ensure a sample extract uniformly enriched in all components of interest and free from interfering matrix components.

Thus, structural diversity of the phenols affects physicochemical behaviour such as solubility and partitioning behaviour and makes optimisation of the recovery system difficult in all but the simplest cases. 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 artefactual changes involving hydrolysis, oxidation [49,50] and/or isomerization [51].

Isolation of the phenolic compound(s) from the sample matrix is generally a pre-requisite to any comprehensive analysis scheme although enhanced selectivity in the subsequent quantification step may reduce the need for sample manipulation. Traditional procedures include homogenization, filtration/centrifugation, distillation, solvent and Soxhlet extraction, SPE and headspace analysis. The introduction of SFE, solid-phase microextraction, pressurized liquid or fluid extraction [52], microwave-assisted extraction [53], membrane extraction and surfactant cloud point extraction [54] have met the increasing demand for new extraction techniques, amenable to automation with reduced solvent consumption. Nevertheless, conventional techniques continue to dominate this application area. Some procedures have limited application such as headspace analysis whereas others are more broadly applicable (e.g. solvent extraction). Derivatization of the analyte may also be incorporated in the recovery step.

In a number of instances, an hydrolysis step has been included to minimize interferences in subsequent chromatography [55] and to simplify chromatographic data [56–59] particularly in instances where appropriate standards are commercially unavailable [60]. Hydrolysis has also been used as an aid to structural elucidation and characterization of phenolic glycosides [61] and phenolic choline esters [62]. Care is necessary as structural rearrangements can occur as seen in the case of flavanones with appropriate hydroxyl-substitution that can be easily converted to isomeric chalcones in alkaline media (or vice versa in acidic media) [63]. There is considerable variation in the lability of the glycosidic bond under hydrolytic conditions and this was exploited in the HPLC analysis of a flower extract (Fig. 2). On-line spectra (Fig. 3) were used to identify the components in the crude extract as shown in Fig. 2a. Acyl groups were removed by alkaline hydrolysis and confirmed the identity of late eluting peaks as acyl derivatives of compound **3**. Luteolin and kaempferol were obtained by acid hydrolysis (Fig. 2c) which cleaved *O*-glycosides but peaks assigned to apigenin glycosides remained, indicating that these were C-glycosides.

Chemical treatment dominates applications (Table 1) because it is more exhaustive and less selective. Acid hydrolysis has been the traditional approach to measurement of aglycones and phenolic acids from flavonoid glycosides and phenolic acid esters, respectively [59,67–74]. 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 are complex and that knowledge of these is still very limited.

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 [75]. For instance, the major characteristic phenols of olive are secoiridoids and their reactivity in alkali has been examined [77]. Secondly, many phenols 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 have been employed in the isolation of phenolic acids from samples such as citrus (juices) [79], grape and cherry juices [80], coffee [81], cereals [82], oilseeds [83] and medicinal plants [85] in order to determine ''bound'' phenols. The loss of *o*-diphenols by oxidation via the corresponding quinones [84] is a concern under alkaline conditions. In many instances, an inert atmosphere and addition of an antioxidant stabilizer was used as a routine precaution (Table 1) whereas the use of an inert atmosphere was an essential precaution in other cases [80] due to poor stability of some phenolic acids in alkaline ambient conditions.

4.2. Analyte recovery

Some liquid samples are amenable to direct analysis requiring no treatment other than centrifugation, filtration and/or dilution as summarized in Table 2. Clear fruit juices [89] and wines [86] often fall into

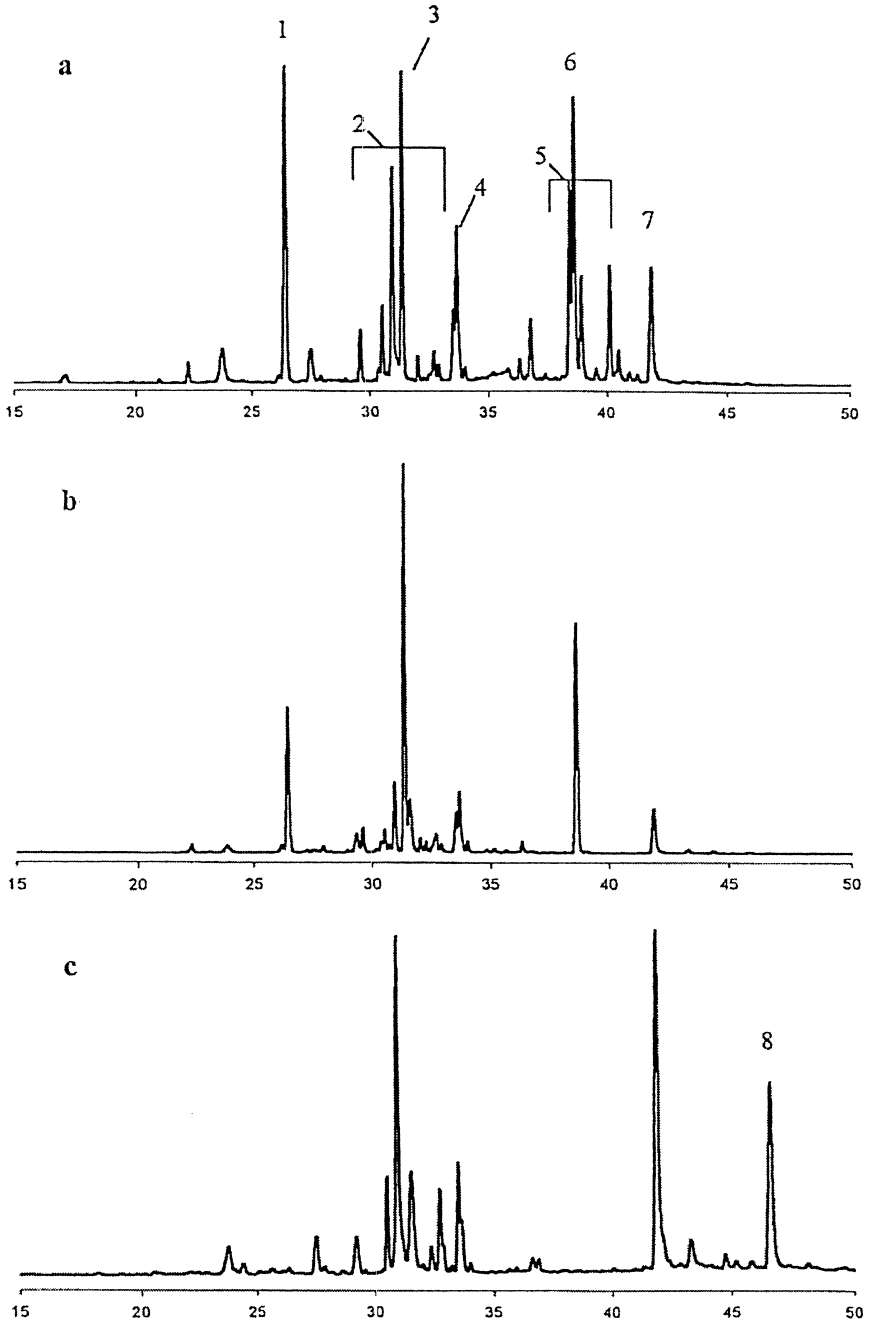


Fig. 2. HPLC chromatograms [absorbance at 352 nm vs. time (min)] of (a) a flavonoid mixture showing seven different groups of compounds: a kaempferol triglycoside (**1**), a set of apigenin glycosides (**2**), a kaempferol diglycoside (**3**), a luteolin glycoside (**4**), a set of acylated kaempferol glycosides (**5**), a chalcone (**6**), and luteolin (**7**); (b) the alkaline hydrolysis product of the same mixture shows a large relative increase in peak 3 and loss of the acylated kaempferol glycoside peaks; and (c) the acid-hydrolyzed mixture showing luteolin and kaempferol [8]. Peaks due to apigenin glycosides are still present, showing these are apigenin C-glycosides. Source: S.J. Bloor, Overview of methods for analysis and identification of flavonoids. In: L. Packer (Ed.), Methods in Enzymology, Vol. 335, Flavonoids and Other Polyphenols. Academic Press, London, 2000, p. 10 [64].

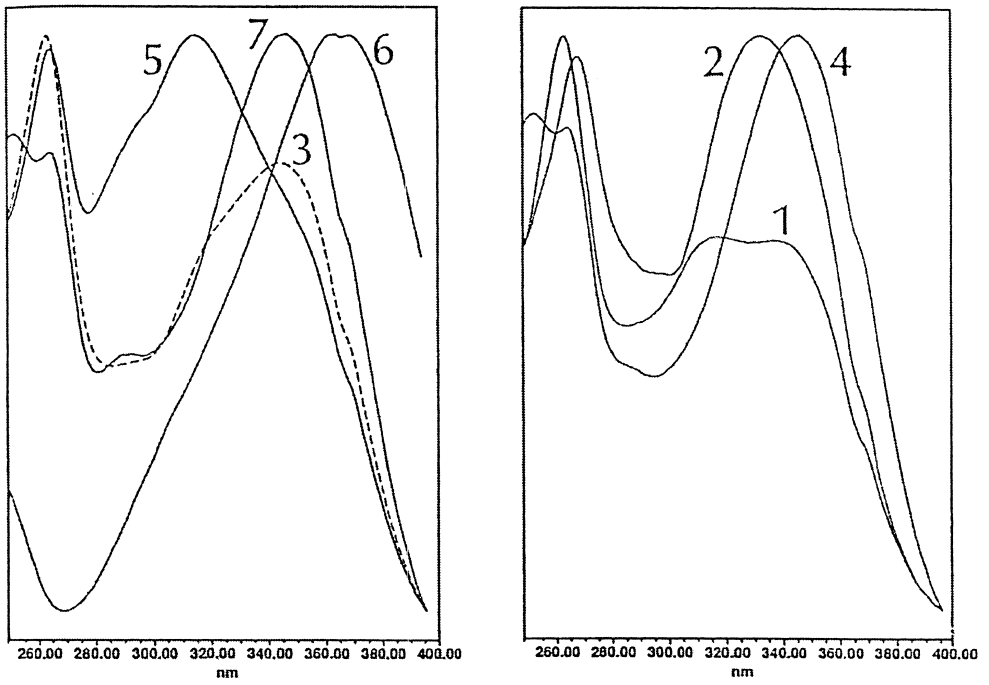


Fig. 3. On-line UV spectra of selected peaks from Fig. 2A. Peak numbers as in Fig. 2A. Source: S.J. Bloor, Overview of methods for analysis and identification of flavonoids. In: L. Packer (Ed.), Methods in Enzymology, Vol. 335, Flavonoids and Other Polyphenols, Academic Press, London, 2000, p. 11 [64].

this category. Cloudy juices such as citrus juices are also amenable to direct analysis following filtration and centrifugation [35,90–92] although poor recoveries have been attributed to low solubility of certain phenolics and/or to sorptive losses on the filtration medium [48]. Aqueous percolation or infusion is useful for samples such as coffee and tea, respectively where the extracts provide data on probable dietary intakes [96,97]. However, caution is necessary as some flavan-3-ols (catechins) are unstable [98] in neutral or alkaline solutions but were precipitated with aluminium chloride [99] which reduced pH and stabilized the extract.

In many instances, simple filtration is ineffective in recovering a broad range of phenols and alternative strategies are necessary (Table 3). Liquid extraction represents a simple and convenient alternative that has been widely used (Table 3). The advantages of liquid extraction versus direct injection have been demonstrated [100] for HPLC analysis of a wine sample. Fresh, freeze-dried [130,132] or air-dried [133,135] samples are typically extracted with a variety of solvents, and sometimes sequentially with solvents of increasing polarity. Freezing of the sample with liquid nitrogen is often a convenient first step in sample preparation [143,144]. The frozen sample can be ground and then freeze-dried or extracted directly. In either case, endocellular material is also extracted. For example, vanadocytes were recovered [141] from the tunicate *Ascidia nigra* after treatment with liquid nitrogen and freeze drying. Further treatment of the extracts was carried out as shown in Fig. 4 to recover tunichrome B, the reducing blood pigment. Tunichromes are air- and water-sensitive and thus sampling was carried out under a current of dry, oxygen-free argon in the presence of an antioxidant stabilizer.

Extraction method and solvent choice are generally critical [107] as are extraction time and temperature [120] reflecting the conflicting actions of solubilization and analyte degradation by oxidation for example. No single solvent will provide optimum recovery of all phenols or even a limited range of phenols. Plant phenols are ionizable with typical pK_a

Table 1
Hydrolysis conditions used in preparation of various samples for analysis of phenols

Sample	Analyte	Hydrolysis conditions	Quantification	Detection	Ref
Berries	Flavonols, hydroxybenzoic and hydroxycinnamic acids	Three extraction and hydrolysis procedures using freeze dried berries (e.g. acidic hydrolysis in ascorbic acid stabilized aqueous methanol	RPLC using C_{18} and acetonitrile/phosphate buffer (gradient)	260 nm (ellagic and *p*-hydroxybenzoic acids), 280 nm (catechins), 320 nm (hydroxycinnamic acids), 360 nm (flavonols)	[24,26]
Berries	Flavonols	Hydrolysis in acidified aqueous methanol containing TBHQ	RPLC using formic acid/acetonitrile (gradient)	UV at 360 nm; PDA and LC–MS (ion trap)	[27]
Vegetables (28), fruits (9)	Flavonols, flavones	Acidic hydrolysis of flavonoid glycosides	RPLC using C_{18} and acetonitrile/phosphate buffer (isocratic)	UV at 370 nm (PDA used to confirm peak identity)	[58]
Rapeseed	Phenolic choline ester fragments	SPE of methanolic extract of defatted rapeseed followed by alkaline hydrolysis	RPLC on C_{18} using aqueous methanol/phosphate buffer (gradient)	UV at 210 nm	[62]
Persimmon fruit	Phenolic acids	Petroleum ether extraction of powdered mesocarp; alkaline hydrolysis and ethyl acetate extraction	GC–MS of oxime TMS derivatives	FID and EI-MS	[65]
Berries	Flavonoids	(Enzymatic pectinase extraction), followed by aqueous methanol or aqueous acetone extraction	Colorimetry; HPLC using ternary mobile phase	Flavan-3-ols and benzoic acid derivatives, 280 nm; hydroxycinnamates, 316 nm; flavonols, 365 nm; anthocyanins, 520 nm	[66]
Various foods	Flavonoid aglycones representing five major sub-classes	Acid hydrolysis	RPLC		[67]
Fruits	Flavonols (quercetin, kaempferol, myricetin), flavones (luteolin, apigenin)	Freeze-dried, acidic hydrolysis in TBHQ stabilized solution and liquid extraction	RPLC	UV detection	[68]
Cranberry juice	Flavonoids and phenolic acids	Acidic hydrolysis under nitrogen of ascorbic acid stabilized methanolic extract. Free flavonoids and phenolic acids fractionated by SPE into neutral and acidic groups	RPLC on C_{18} using water/methanol (containing acetic acid) (gradient)	UV at 280 and 360 nm	[69]
Berries, vegetables, tea, wine, fruits	Catechins, flavonols	Acid hydrolysis in BHA stabilized aqueous methanol; ethyl acetate extraction of catechins	RPLC using ODS3 and acetonitrile/phosphate buffer (gradient: flavonols or isocratic: catechins)	UV at 270, 280, 329 and 370 nm (flavonols) and electrochemical array detector (catechins)	[70]
Tomato	Quercetin, kaempferol, naringenin and hydroxycinnamic acids	Acid hydrolysis in sodium diethyldithiocarbamate stabilized aqueous methanol	RPLC on C_{18} using water/methanol (containing trifluoroacetic acid) (gradient)	UV at 365 and 280 nm	[71]
Blueberries and blackberries	Phenolic acids, catechins, kaempferol, myricetin, quercetin	Acid hydrolysis in ascorbic acid stabilized aqueous methanol under nitrogen and reflux	RPLC on C_{18} using water/methanol (containing formic acid) (gradient)	UV using PDA	[72]

Onion, spinach	Flavonoid (glycosides)	Plant material freeze-dried and mixed with ascorbic acid stabilized aqueous methanol. Acid hydrolysis under nitrogen and reflux	RPLC using C_{18} column (gradient)	UV at 280 and 340 nm	[73]
Soybean and soybean products	Isoflavones	Acidic hydrolysis using HCl at reflux versus phosphoric acid at lower temperatures	RPLC using a phenyl column with acetonitrile/water (isocratic)	UV at 249, 259 and 343 nm. LC–MS–MS with heated nebulizer interface in positive mode	[74]
(Apple juice and cider)	Phenolic acids, catechins, rutin	Stability of various phenols in base and when added to apple juices	UV spectrophotometry	UV spectra from 190–690 nm	[75]
Blood orange juice	Hydroxycinnamic acids	Free acids: acid hydrolysis and, ethyl acetate extraction; Total acids: alkaline hydrolysis in dark and ethyl acetate extraction	RPLC on C_{18} using tetrahydrofuran/water/acetic acid (gradient)	UV at 280 nm	[76]
Olive fruit and brines	Oleuropein and derivatives	Ethanol and sodium metabisulfite added to sample; filtered, washed with hexane and extracted with ethyl acetate	RPLC on C_{18} using water/acetonitrile/phosphoric acid (gradient)	UV at 280 nm and 330 nm. Spectra recorded from 200 to 380 nm	[77]
Fruits, vegetables, beverages	Flavonols, flavones (glycosides)	Acid hydrolysis in aqueous methanol	RPLC on C_{18} and acetonitrile/phosphate buffer plus methanol/phosphate buffer (isocratic)	UV at 370 nm	[78]
Orange	Phenolic and hydroxycinnamic acids, flavonoids	Effect of sample treatment including alkaline hydrolysis	RPLC on C_{18} using methanol/phosphate buffer (gradient)	UV at 252 and 284 nm; LC–MS with APCI and ESI in positive and negative ion modes	[79]
Juices	Phenolic acids		RPLC using isocratic elution	UV absorption	[80]
Green coffee	Phenolic acids	Alkaline hydrolysis and solvent extraction	RPLC on C_{18} using water/methanol (containing formic acid) (gradient)	UV at 320 nm using PDA	[81]
Rye	Phenolic acids plus ferulic acid dehydrodimers	Enzymatic hydrolysis of starch followed by alkaline hydrolysis under nitrogen and ethyl acetate extraction	RPLC on C_{18} using methanol/phosphate buffer (gradient)	UV at 280 nm	[82]
Rapeseed and canola meals	Total phenolic acids	Refluxing with acidic acetone, alkaline hydrolysis of esterified phenolic acids followed by acidification, extraction with ethyl acetate/ethyl ether	Colorimetry	Folin-Denis reagent	[83]
Cherry laurel fruits	Phenolic acids	Petroleum ether extraction of powdered mesocarp, followed by alkaline hydrolysis of residue under nitrogen and ethyl acetate recovery	GC of TMS derivatives	FID and GC–MS	[84]

Table 2
Representative examples of the use of simple dilution/filtration for the recovery of phenols from plants and foods

Sample	Analyte	Sample preparation	Quantification	Ref
Wines	*Cis*- and *trans*-resveratrol	Nil	RPLC, 288 nm (cis-isomer), 308 nm (trans-isomer)	[86]
Red wines	Rutin, gallic acid, quercetin, t-resveratrol	Nil	RPLC-PDA	[87]
Fortified wines	Phenolic acids, coumarins, flavan-3-ols, flavonol aglycones	Nil	RPLC-PDA	[88]
Apple juice	Phloretin glucosides	Centrifuge and adjust sugar content	RPLC, 285 nm	[89]
Red wine, beer, apple cider, and sour cherry and blackthorn fruit liqueurs	Flavan-3-ols	Filtration	RPLC, 280 nm and post-column reactor with absorption at 640 nm	[90]
Wine	Flavan-3-ols	Filtration	HPLC-PDA, 280 nm	[91]
Orange juice	Flavanone glycosides	Heat and centrifuge	RPLC, 280 nm	[92]
Orange juice	Flavanones, flavones and hydroxycinnamic acids	Soluble fraction centrifuged; insoluble fraction extracted with dimethyl sulfoxide	RPLC, 290 nm (flavanones); 340 nm (flavones and hydroxycinnamic acids)	[93]
Pomegranate juice	Anthocyanins	Filtration	RPLC, 520 nm	[94]
Grape juice		Filtration except for procyanidins (isolation on Sephadex LH-20 column)	Colorimetry; HPLC-PDA, 280 and 320 nm	[95]
Black and green teas	Catechins, theaflavins		RPLC and capillary electrophoresis	[96]
Coffee, tea	Flavanols, flavonol glycosides	Percolation or infusion	RPLC	[97]

values ranging from 8 to 12 and oil/water partition coefficients ranging from 6×10^{-4} to 1.5 [121]. Thus, they exhibit considerable diversity in terms of acidity as well as polarity ranging from hydrophobic to hydrophilic in character. The range of physicochemical behaviours must be considered when determining sample handling strategies as, for example, in pH control to ensure favourable partitioning behaviour during extraction [121]. The situation with respect to pH-dependent equilibria is especially complex in the case of anthocyanin extraction [122]. Some practical illustrations of this diversity are seen in the need to optimize the alcohol content of aqueous alcoholic extractants for phenols of diverse structures [24–27]. In many instances, different recovery procedures may be required for the range of phenols encountered in a single sample [124].

Ethyl acetate [110] and dimethyl sulfoxide [133] have been used as extractants but aqueous mixtures of methanol [135], ethanol [136] or acetone [144] are often the solvent(s) of choice for recovery of a wide range of phenols from diverse sample types including oats [111], fruits and vegetables [126,134], oil [104,119], soy [117] and spices [123]. There are some important distinctions between fresh and dried samples. Thus, in the case of extractants using aqueous mixtures, the required proportion of water in the extractant is lower with fresh than with dried samples. Furthermore, with dried materials, low polarity solvents and ethyl acetate will simply leach the sample whereas alcoholic solvents presumably rupture cell membranes and enhance the extraction of endocellular materials. Thus, the relative proportion of endocellular and exocellular components may be determined by solvent choice.

Many extraction procedures incorporate the use of an antioxidant as a stabilizer and compounds that have been used for this purpose include BHA [70], TBHQ [142] and ascorbic acid [24,26]. The choice of stabilizer can be influenced by the subsequent procedure as co-elution with plant phenols can occur [26]. The effectiveness of added stabilizer will inevitably depend on its concentration and activity relative to indigenous phenols. Moreover, analyte recovery can be reduced at higher concentrations of stabilizer due to the pro-oxidant action of the latter [73].

Supercritical fluid extraction offers some advantages relative to conventional extraction particularly for chemically or thermally labile compounds. Moreover, solvent strength can be controlled by varying the pressure thus facilitating sequential extraction of phenols of increasing polarity. The extraction behaviour of phenolic compounds covering a range of polarities has been modelled using supercritical carbon dioxide and an inert support as a sample matrix [128]. Extraction and collection variables were optimized and revealed that the use of methanol as modifier was mandatory. However, quantitative recovery was limited to the less hydroxylated compounds such as *p*-coumaric acid, *trans*-resveratrol and salicylic acid while mean recoveries of more polar phenolic acids and flavonoids were between 30 and 70%. Dynamic SFE produced clean extracts with higher recoveries of total phenols from dried olive leaf [129] 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.

4.2.1. Recovery of flavan-3-ols and anthocyanins

Extraction of some phenolic groups warrants special consideration. The monomeric flavan-3-ols (or catechins) predominantly (+)-catechin and (−)-epicatechin are found mainly in brewed tea and red wine. Their levels in three model foods: apples, black grapes, and canned kidney beans were not affected [145] by sample drying processes 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. In the case of the oligomeric and polymeric proanthocyanidins and procyanidins which are based on the flavan-3-ols, various extractants have been used [146–148]. Aqueous acetone [144] generally gives the best yields although a variable proportion of proanthocyanidins resist extraction particularly in aged or oxidized tissues [149]. Thus, extraction yield varies with the solvent system used and also the polymerization degree of the analyte. Aqueous methanol was chosen for extraction from diverse food samples [146] because

Table 3
Examples of procedures used for the recovery of phenols from plants and foods

Sample	Analyte	Recovery	Quantification	Ref
Wines	Phenolic acids and aldehydes, flavonoids	Addition of acid to lower pH and extraction with diethyl ether	RPLC, UV at 280 nm, fluorescence at exc. 278 nm, em. 360 nm and exc. 330 nm, em. 374 nm	[100]
Soy-based foods	Isoflavones	Extraction of powdered samples with 80% aqueous ethanol at low temperature followed by SPE	RPLC; PDA	[101]
Dried plums	Hydroxybenzoic and hydroxycinnamic acids, rutin, chlorogenic acids, anthocyanins	Dried plums homogenized in 80% aqueous methanol at low temperature; followed by SPE	RPLC, 280 and 316 nm; LC–MS–MS ion trap in positive and negative ion modes	[102]
Orange	Polymethoxylated flavones	Extraction with benzene, evaporation and dissolution in methanol	RPLC, 240 nm	[103]
Olive oil	Phenols	Extraction with methanol and isopropanol/methanol	RPLC, 280 nm	[104]
Pear	Chlorogenic acid, epicatechin	Aqueous ethanol extraction of powdered fruit; clean-up by liquid liquid extraction	RPLC-PDA, 325 nm (hydroxycinnamic acids); 280 nm (flavanols); 360 nm (flavonols)	[105]
Cider apple tissues	Procyanidins	Freeze dried, successive extractions with methanol and aqueous acetone, thiolysis. Butanol/hydrochloric acid hydrolysis for procyanidins	Colorimetry; HPLC-PDA, 540 nm (procyanidins), 280 nm (other phenols); LC–ESI-MS negative ion mode	[106]
Red beetroot peel	*p*-Coumaric acid, ferulic acid, flavonoids	Extraction with aqueous methanol	RPLC-PDA; LC–ESI-MS	[107]
Chinese medicine	Isoflavones	Soxhlet extraction of ground powder with aqueous methanol, evaporation and ethyl acetate extraction	RPLC, 280 nm	[108]
Oat groats	Hydroxycinnamic acids	Extraction with methanol	RPLC, 280 and 340 nm	[109]
Pollen	Flavonoid aglycones	Extraction with ethyl acetate	RPLC, 280 and 350 nm	[110]
Oats	Phenolic acids	Extraction with aqueous ethanol	RPLC	[111]
Lentils and beans	Benzoic and cinnamic acids, flavonols, flavones	Extraction in an ultrasonic bath in the dark with acidified aqueous methanol containing BHT	RPLC, 280 nm. Spectra recorded 210–350 nm	[112]
Olive oil	Phenolic acids, tyrosol, hydroxytyrosol, oleuropein and derivatives, ligstroside	Extraction of oil with aqueous methanol; evaporation to dryness, dissolution in acetonitrile and hexane washing	RPLC, 280 nm	[113]
Rose hip	Flavanols, flavones, flavanones, flavonols and glycosides	Homogenized with methanol (containing formic acid), sonicated at low temperature, dried and redissolved	RPLC-PDA full scan mode; LC–ESI-MS–MS negative (and positive) ion modes	[114]
Grapevine leaf	Caffeic acid, flavonoids	Petroleum ether wash followed by aqueous methanol extraction and fractionation by column chromatography	HPLC, 340 nm	[115]
Wine	Anthocyanins, hydroxycinnamic acids, flavonols esculin	Dilution with aqueous methanol	FT-ion cyclotron resonance-ESI-MS	[116]
Soy	Isoflavone	Extraction with 80% ethanol	RPLC-PDA; LC–ESI-MS negative (and positive) ion modes	[117]
Peach and apple purees and concentrates	Benzoic and cinnamic acids, flavonols, dihydrochalcones, flavan-3-ols	Homogenized in aqueous methanol, dried and extracted with ethyl acetate	HPLC–DAD, 210–360 nm	[118]
Olive oil	Derivatives of tyrosol and oleuropein	Methanol extraction	GC and GC–MS of TMS derivatives; HPLC, 232 and 278 nm	[119]
Fruit juices (apple, pineapple, orange, grape, peach, pear, apricot)	Benzoic and hydroxycinnamic acids, flavan-3-ols, flavonols, chalcones, flavonol glycosides	Juice concentrated at controlled temperature in under 40 min. Extraction with diethyl ether and then ethyl acetate; residue dissolved in aqueous methanol	RPLC, 254, 280, 340 and 365 nm	[120]
Olive oil	Tyrosol, gallic acid, oleuropein and derivatives	Examined partitioning behaviour after removal of phenolics with aqueous methanol	Not applicable	[121]
Red wine	Anthocyanins	Examined pH-dependent equilibria	Not applicable	[122]
Sage	Caffeic acid, luteolin-7-*O*-glucoside, rosmarinic acid, apigenin, hispidulin, cirsimaritin	Extraction of ground samples with ethanol	RPLC, 280, 320 and 350 nm	[123]
Olive fruit	Nine phenols	Two extraction methods used	RPLC-PDA	[124]

Table olives, olive oil	Phenolic acids, tyrosol, oleuropein derivatives	Different procedures depending on analyte; e.g. extraction of oil with aqueous hydrogen carbonate and SPE for (hydroxy)tyrosol	LC–APCI-MS–MS	[125]
Yellow onion, green tea	Flavonol glycosides	Infusion (tea) or aqueous methanol extraction (onion) of freeze-dried sample followed by SPE	MALDI–TOF-MS positive (and negative) ion modes	[126]
White grape seeds	Gallic acid, catechins	Sequential SFE using carbon dioxide and adding methanol as a polar modifier	RPLC, 280 nm	[127]
Spiked diatomaceous earth	Phenolic acids, catechins, flavonoids	SFE using carbon dioxide with methanol modifier	RPLC	[128]
Olive leaves	Phenols	SFE using carbon dioxide with methanol modifier of dried, ground leaves	Colorimetry using Folin Ciocalteu reagent and ESI-MS negative ion mode screening	[129]
Apple	Flavonols, catechin, phloridzin, chlorogenic acid	Frozen in liquid nitrogen, freeze-dried and extracted with methanol	RPLC, 280, 350, 525 nm	[130]
Blueberries	Anthocyanins	Ground, frozen berries extracted with acetone/methanol/water/formic acid followed by SPE	RPLC, 520 nm versus MALDI–TOF-MS (complementary role)	[131]
Tart cherries	Flavonoids	Freeze-dried and sequentially extracted with hexane, ethyl acetate, methanol	RPLC, UV	[132]
Grapefruit and pummelo	Flavanones	Extraction of dried, ground fruit with dimethyl sulfoxide	RPLC-PDA	[133]
Apple skin	Flavonols, anthocyanins, proanthocyanidins, phenolic acids	Extraction of ground apple peel with acidified methanol	HPLC, 350 nm (flavonols), 530 nm (anthocyanins), 280 nm (proanthocyanidins), 313 nm (phenolic acids)	[134]
Eucalyptus	Phenolic acids, flavonol glycosides, flavones and naringenin	Extraction of dried, ground leaves with aqueous methanol; methanol evaporated and partitioning into diethyl ether	RPLC, 325 nm	[135]
Sour orange	Flavonoids and glycosides	Extraction with aqueous ethanol of dried, ground fruit	RPLC, 290 nm; LC–ESI-MS positive ion mode	[136]
Orange juice	Flavanones and flavanone glycosides	Extraction with methanol (glycosides) or acid hydrolysis (aglycones)	LC–MS, ionspray with heated turboprobe (negative ion mode); RPLC, 280, 370 nm	[137]
Lemon verbena leaves	Flavonoids	Room temperature extraction of dried, ground leaves with aqueous ethanol	RPLC, 350 nm	[138]
Olive pulp	Oleuropein (derivatives), flavonoids	Pulp frozen in liquid nitrogen, ground and aqueous ethanol extraction followed by liquid–liquid partitioning	RPLC, 280, 340 nm	[139]
Grape skin	Anthocyanins, flavonols	Aqueous methanol extraction	HPLC, 520 nm; spectrophotometry, 280 nm, 355 nm, 535 nm	[140]
Ascidia nigra	Tunichrome B	Refer to Fig. 4		[141]
Onions, parsley, blackberries	Anthocyanidins, flavonols, flavones	Freeze-dried samples refluxed under nitrogen in acidified aqueous methanol containing TBHQ	Kinetics method (RPLC, 520 nm)	[142]
Chocolate	Procyanidins	Sample freeze-dried, and extracted sequentially with hexane then acetone/water acetic acid	LC–ESI-MS(-MS), ion trap negative ion mode	[143]
Cider apple tissues	Hydroxycinnamic acid derivatives, flavan-3-ols, flavonols, dihydrochalcones	Tissues isolated while spraying with aqueous formic acid, frozen, freeze-dried and extracted sequentially with hexane, methanol and acetone	RPLC, 280 nm, 320 nm	[144]
Apple and grape	Flavan-3-ols	Extraction with aqueous methanol	RPLC, UV (270 nm) or fluorescence (280/310 nm excitation/emission)	[145]

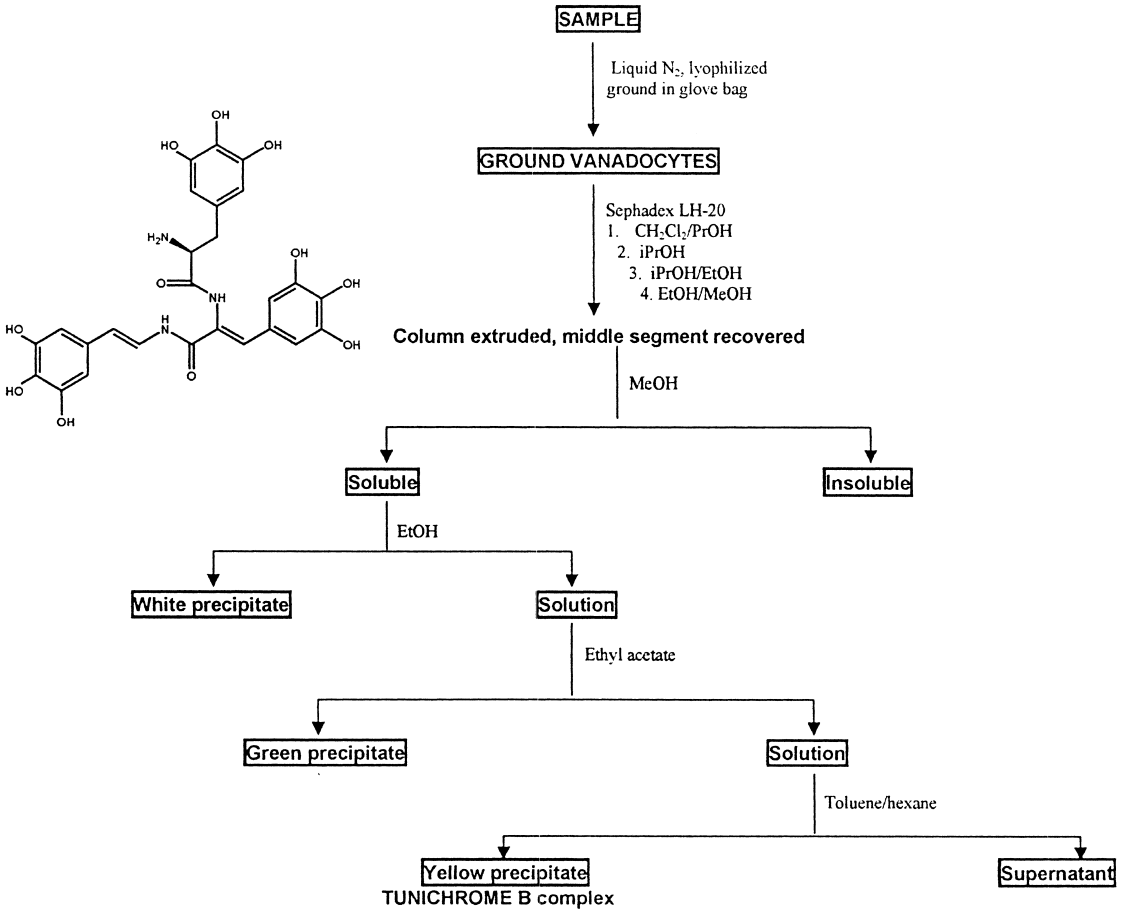


Fig. 4. Isolation of tunichrome B complex from *Ascidia nigra* showing the structure of one of the components. Source: Adapted from Ref. [141].

of its efficiency in recovering flavanols of low polymerization degree.

Anthocyanins are widely distributed and comprise a significant portion of the phenolic content of many fruits (e.g. dark-coloured berries) [51,150–153]. They are acylglycosides and glycosides of the anthocyanidins of which six are commonly encountered. Their chemistry is complicated by various pH-dependent equilibria and this was exploited in traditional strategies for their recovery as the flavylium cation form by extraction with cold methanol containing hydrochloric acid [154,155]. However, the acylated anthocyanins are frequently labile under such conditions [67]. For instance, Merken and Beecher [67] developed an 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 TBHQ-stabilized aqueous methanol under which conditions anthocyanidins and catechins are unstable thus limiting the applicability of the method. The replacement of hydrochloric acid with weaker acids, either formic or acetic acid [156] will overcome this problem and both grape and cherry anthocyanins were extracted [51,157] at room temperature using a mixture of formic acid in aqueous methanol. With the most labile anthocyanins, the use of nonacidified solvents is probably a sensible precaution.

4.2.2. Fresh versus processed samples

Recovery methods for use with processed products generally exploit the same principles as for fresh and/or dried materials but allowing for any differ-

ences in moisture content and/or enzyme activity. Enzymatic activity can produce qualitative and quantitative changes in the phenolic content of fresh samples [12,130]. Thus, enzymatic activity in green olive drupes [158] 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 TLC. Extraction with boiling ethanol (5 min) followed by aqueous ethanol (1 h) has also been applied [159] and the authors noted that boiling inactivated enzymes and aided in phenolic recovery. Phenols in the filtered ethanolic extract were quantified by ultraviolet derivative spectrometry. It follows that the phenolic profile of processed products typically differs from that of the fresh material [160] arising from the effects of enzymatic activity during processing.

4.3. Clean-up of extracts

Many extracts contain significant quantities of carbohydrates and/or lipoidal material that potentially interfere with subsequent quantification. Various strategies have been designed to cope with this situation, namely, sequential extraction or liquid liquid partitioning and/or SPE. An example of sequential extraction (Table 3) is provided by the analysis of chocolate which was freeze dried, ground and defatted with hexane in an ultrasonic bath [143] 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. A similar approach was used for tissues of mature cider apples [144]. The tissues were isolated while spraying with aqueous formic acid to avoid oxidation, 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 polymerized structures.

In the case of aqueous alcoholic extracts, the alcohol content can be reduced and the phenols partitioned into a non-polar solvent [135]. Samples such as olive oil are typically dissolved in hexane [161] or ethoxyethane [119] followed by liquid–liquid extraction using various mixtures of water and alcohol in order to isolate the desired analytes from unsaturated, interfering species. Residual oil is removed by overnight storage at subambient temperature [119], by centrifugation [115] or by further extraction with hexane although Sephadex column chromatography has also been used [49,50] to effect further clean-up.

Simultaneous sample clean-up and pre-concentration of juices and wines [162–171], oils [172–178] and extracts [179–183] can be achieved by SPE or traditional polyamide columns [184]. Representative examples of the approaches used for clean-up are summarized in Table 4. Several SPE formats are commercially available ranging from the original cartridges to disks in a range of sorbents although C_{18} and other reversed-phase materials (e.g. C_8) [185] remain the most popular. With reversed-phase cartridges, interfering sugars can be eluted with aqueous methanol prior to elution of phenols with methanol [167]. Phenolic acids were determined [186] 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. Fractionation of the phenolic components is readily achieved by SPE [69,187–191] as illustrated by the analysis of kiwifruit juice [192] which 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. In some cases, preparative-scale HPLC has been used [193] in sample preparation.

4.4. Quantification

The number, type and concentrations of phenols in plants [24,194] exhibit extreme diversity. For example, flavonoids were not detected in cultivated mushrooms [195] whilst they were present in orange juice at levels up to 500 mg l^{-1} [196]. In orange

Table 4
Techniques used for extraction of phenols and clean-up of extracts

Sample	Analyte	Sample extraction/clean-up	Quantification	Ref.
Olive oil	Hydroxytyrosol derivatives	Various procedures compared. Extraction with various solvents direct from oil or from oil dissolved in various solvents	RPLC, 239 nm, 278 nm	[49,50]
Eucalyptus	Phenolic acids, flavonol glycosides, flavones and naringenin	Extraction of dried, ground leaves with aqueous methanol; methanol evaporated and phenols partitioned into diethyl ether	RPLC, 325 nm	[135]
Olive oil	Phenols (ca. 15)	Oil dissolved in diethyl ether and extracted with methanol or aqueous methanol; overnight refrigeration	GC–MS after derivatization; RPLC, 332 nm, 278 nm	[119]
Olive oil	Total phenols, *o*-diphenols	Oil dissolved in hexane and phenols partitioned into aqueous methanol	Colorimetry, 725 nm (total phenols), 370 nm (*o*-diphenols)	[161]
Raspberry juice	Hydroxybenzoic and hydroxycinnamic acids, catechins, flavonols	Acid and base hydrolysis followed by SPE on C_{18} cartridge eluting with acidified methanol	RPLC, 260 nm, 280 nm, 320 nm, 360 nm	[60]
Fruit juices and syrups	Anthocyanins	SPE	RPLC	[162]
Purple passion fruit	Anthocyanins	Solvent extraction and two SPEs	RPLC	[163]
Grapes and wine	Proanthocyanidins	SPE	RPLC	[164]
Red wine	Phenols	Bond Elute SPE eluting with acidified methanol	FT-IR (mid-infrared) and UV–Vis spectra	[165]
Red wine, vegetables	Flavones, flavonols	SPE using phenyl boric cartridges	RPLC	[166]
Berry and fruit wines and liquors	Phenols	Dealcoholized (wines) and SPE to remove sugars	Colorimetry	[167]
Citrus tissues	Flavonoids (25)	Extraction with methanol/dimethyl sulfoxide, SPE on C_{18} cartridge	HPLC-PDA, 285 nm	[168]
Citrus juices	Flavanones, flavones, flavonols	SPE	RPLC	[169]
Fruit of *Libanotis*	Phenolic acids	Refluxed with methanol followed by SPE on C_{18} microcolumn and then SPE on quaternary amine microcolumn	RPLC, 254 nm	[170]
Olive oil	Phenols (ca. 15)	SPE using amino phase and diol phase	RPLC, 24, nm, 280 nm, 336 nm	[174]
Olive oil	Oleuropein, ligstroside	Comparison of SPE on C_{18} cartridge and liquid liquid extraction	GC	[178]
Spinach	Flavonoid aglycones and glycosides	Various extractants and methods (including Soxhlet) compared. ''SPE'' on C_{18}	FT-IR, EI-MS, RPLC at 260 and 315 nm	[180]
Blood orange	Flavanone glycosides and t-cinnamic acid	Dilution in dimethylformamide/ammonium oxalate solution and centrifugation; SPE concentration for *tert*.-cinnamic acid	HPLC, 280 nm	[182]

juice, flavanone glycosides (e.g. hesperidin, 100–500 mg l^{-1}) were present in much higher concentrations than polymethoxylated flavones with typical values of 1 mg l^{-1} [196,197]. In another study, total flavanol contents varied from non-detectable in most vegetables to 1840 mg kg^{-1} in a broad bean sample [146]. Moreover, there are significant quantitative differences between cultivars of a single species and between tissues of a single plant. Post-harvest and processing-induced changes further modify the phenol contents. This variability in the quantitative distribution of phenols coupled with the wide variation in relative sensitivity of detectors often limits the ability to measure more than a restricted range of phenols in a single analysis. On a practical level, it requires that the sample details are fully specified and characterized [198].

The limited availability of suitable reference standards for quantification is a problem that has been overcome, in part, by synthesis [119,177,199] of the relevant compounds. Alternatively, the relevant compounds isolated by preparative scale chromatography can serve as reference standards. In many instances, quantification is carried out by reference to one or more appropriately selected reference compounds. For instance, an unidentified substance in pineapple juice was quantified [200] as *p*-coumaric acid whilst kaempferol and quercetin glycosides were calculated as the corresponding aglycones [135]. The contents of phenolic acids and flavonoids in flowerheads were quantified by HPLC using quercetin and cynarin as internal standards [201]. The availability and use of standard reference materials and certified methods of analysis will greatly enhance the confidence in analytical data.

Traditional methods for the determination of the phenolic component relied on colorimetric measurement of total phenols using one of a number of reagents of varying selectivity. The diversity of phenolic compounds means that selection of a reagent and/or absorbing wavelength will be a compromise although this is less of a problem where a single class of phenols predominates. For instance, the Davis Test [202,203] is based on the reaction of dilute alkali with flavanones to form the corresponding yellow chalcones that are measured at 470 nm. The concentration of 1,2-diphenols is determined [204] with molybdate by measurement at 350 nm whilst Folin-Ciocalteu reagent is the classic reagent recommended for total phenols [135,205]. The blue colour formed after 15–60 min is measured at 725–735 nm [206] and results are expressed in terms of molar equivalents of a commonly occurring phenol, for example, gallic acid [205] or quercetin [135]. A disadvantage is the interference of reducing substances such as ascorbic acid. All phenols absorb radiation in the ultraviolet and this provides the basis for an alternative measurement of total phenols [207]. However, all spectrophotometric measurements lack specificity and give an over-estimation of ''phenolic'' content. Specificity can be enhanced by derivative spectrometry or by preliminary separation. For instance, measurement based on the second derivative of the absorbance at 278 nm [208] provided a rapid, direct method for determination of total phenols using catechol as the reference standard. TLC methods have been devised [135,209] and exploited [49,50] for the preliminary separation/clean-up of sample extracts. They are generally semi-quantitative at best and, somewhat surprisingly, high-performance TLC [210] has not been widely used for phenols.

4.4.1. Chromatographic methods

The need for profiling and identifying individual phenolic compounds has seen traditional methods replaced by high-performance chromatographic analyses. The limited volatility of many phenols has restricted the application of GC to their separation. However, with suitable derivatization (e.g. trimethylsilylation) they are amenable to GC and GC–MS [119,185,193,199,211–216]. Nevertheless, HPLC currently represents the most popular and reliable technique for analysis of phenols [71,82,114, 148,217–233].

Merken and Beecher [234] have presented a comprehensive review on the analytical chemistry of food flavonoids in which they present detailed tabulations of columns and mobile phases used in HPLC. The typical system involves RPLC comprising a C_{18} stationary phase [71,114,135,193,231] or other alkyl [205,232] chemistry. Columns are commonly 100 to 300 mm in length with 10 μm or increasingly 5 μm packings thus favouring the shorter columns. In some instances, isocratic elution [193] has provided adequate resolution due to selec-

tivity effects of one or more components (e.g. acetonitrile) of the mobile phase [118] although gradient elution has usually been mandatory [71,77,114,135,205,235] in recognition of the complexity of the phenolic profile of most samples. Numerous mobile phases have been employed but binary systems comprising an aqueous component and a less polar organic solvent such as acetonitrile or methanol remain common. Acid (acetic, formic or phosphoric acid) is usually added to both components to maintain constant acid concentration during gradient runs. Ternary phases offer greater flexibility and will likely increase in popularity [236].

Compound elution is typical of reversed-phase LC, that is, polar compounds (e.g. phenolic acids) elute first, followed by those of decreasing polarity. Hence, an elution order can be developed as phenolic acids<cinnamic acids<flavonoids [80,168,237] although overlap of the individual members of different classes is inevitable because of the diversity of compounds. The elution pattern for flavonoids containing equivalent substitution patterns [238] is flavanone glycoside followed by flavonol and flavone glycosides and then the free aglycones in the same order. In cinnamic and phenolic acids, polarity is increased most by hydroxy groups at the 4-position, followed by those at the 3- and 2-positions. Methoxy and acrylic groups reduce polarity and hence increase retention times.

4.4.1.1. Detection

Routine detection in HPLC is typically based on measurement of UV absorption [193,205] or, less commonly, visible radiation [239,240] in the case of anthocyanins. No single wavelength is ideal for all classes of phenols since they display absorbance maxima at distinctly different wavelengths [241]. Indeed, there are significant differences in absorption maxima and molar absorptivities [242] of even the major phenols identified in a single fruit. This creates problems in quantification as discussed by Tsimidou et al. [242] who classified the various phenols into four groups and used a single calibration standard for the members of each group. The most commonly used wavelength for routine detection has been 280 nm which represents a suitable compromise [71,205,231] although detection at other wavelengths [77,238,243] and dual wavelength detection [49] has been applied. The advantages of low wavelength detection [177,193] at 225 nm have been demonstrated but problems associated with high background absorption of typical mobile phases in RPLC have limited its use.

The complementary nature of fluorescence detection [35,244] has been demonstrated and used in series with a UV detector for catechin detection [145]. Chemical reaction detection of catechins [90,245] was based on reaction with *p*-dimethylaminocinnamaldehyde to form coloured derivatives absorbing at 640 nm. Evaporative light scattering detection [15] offers the opportunity for non-selective ''universal'' detection. In contrast, electrochemical array detection [59,118,172, 176,246–248] is a powerful tool for the selective as well as sensitive detection of phenolic compounds. The linearity, precision and limits of detection have been compared for HPLC of benzoic and cinnamic acids using UV, electrochemical and mass spectrometric detection [247].

Identification of the eluted phenols in GC and HPLC is usually based on correspondence of retention data with an appropriate standard. Alternatively, fraction collection and characterization off-line have been used particularly in preparative scale separations. All phenols possess a strong chromophore system. Their UV spectra are particularly informative providing considerable structural information that can distinguish the type of phenol (e.g. xanthone, simple phenol, flavone) and the oxidation pattern [249]. Furthermore, spectra of eluting peaks obtained at, for example, the apex and both inflexion points of the peak can be compared and used as an indicator of purity. The popularity of PDA in HPLC attests to the value of UV spectra and can be combined with the use of post-column shift reagents [250].

An optimization and validation strategy has been developed [112,251] for the HPLC analysis of representative phenols from different food sources. The important feature of this strategy was the selection of the representative phenols and food extracts and the rapid analysis time. The method provided an alternative provisional identification of unknown phenols prior to their full characterization.

5. Chemical screening

The on-line coupling of chromatography with spectrometry has been the single-most important advance in analysis of bioactive compounds facilitating optimization of an investigation [252,253] by the efficient and targetted isolation of bioactive compounds.

5.1. Structural characterization

5.1.1. Mass spectrometry

Mass spectrometry can be carried out either on-line in combination with chromatographic or electrophoretic techniques or off-line. MS is potentially a powerful tool for elucidating phenolic structures but the number of applications involving direct inlet introduction of analytes to the mass spectrometer was limited (Table 5) until the advent of MALDI–MS. The latter has been used successfully to analyse a number of phenolic classes in various foods (Table 5). However, the preparation of a proper matrix and sample preparation procedure are very important [261]. MALDI–MS and HPLC have been compared [131] for the analysis of blueberries for anthocyanins. Both techniques generally provided comparable quantitative profiles for anthocyanin contents. HPLC distinguished anthocyanin isomers whereas MALDI–MS was more rapid in the identification and quantification of anthocyanins with different masses. Somewhat surprisingly, tandem MS has enjoyed limited popularity in off-line mode.

In coupled mode, the mass spectrometer may function simply as a highly selective detector but it is in qualitative analysis that it excels providing unsurpassed opportunities for compound ''identification''. On-line applications of MS are extensive and growing at an increasing rate with GC–MS now well established (Table 6) as a routine technique carried out with either EI or CI sources for the introduction of volatile compounds such as phenolic acids [213] and flavonoids [211,212] in chamomile flowers [211], propolis [212] and rapeseed leaves [213]. Schmidt et al. [262] analysed 49 flavones, flavonols, flavanones and chalcones without derivatization by GC–MS in EI mode. Compared with direct inlet mass spectra, the GC–MS data exhibited the same typical fragmentation patterns but with slight differences in intensities [254,263,270]. In instances where the classical mass spectrometric gas phase ionization techniques such as EI and CI are unsuitable (e.g. with polar, non-volatile and thermolabile phenols such as glycosides), chemical derivatization usually involving silylation may overcome these limitations (Table 6) but can introduce further difficulties by increasing the molecular mass of the analyte possibly beyond the range of the mass analyzer. This is a major consideration for glycosidic species with numerous hydroxyl groups although permethylation or perdeuteromethylation offer suitable alternatives. Derivatization also often produces mixtures of partially derivatized compounds [199] from a single analyte.

The analyte fragmentations in EI mass spectra may provide sufficient information to determine molecular mass, elemental formula and substitution patterns. For instance, the analysis and identification of the phenolic metabolites of the ascomycete *Eutypa lata* was performed by GC–MS of their trimethylsilyl ether derivatives [193] whilst individual compounds were quantified by analytical HPLC and separated by preparative HPLC. In less favourable situations, the mass spectrum will assist in structural elucidation although other techniques such as NMR are required for a definitive structural assignment. Despite the obvious successes of GC–MS, it is the hyphenation of liquid chromatography with MS that has revolutionized the analysis of non-volatile species as evidenced by the number of reviews (e.g. Refs. [271–277]).

LC–MS interfacing has been achieved in a number of ways. The moving belt method [278] represented an early attempt to provide data under both EI and CI conditions but the first real success was achieved with the thermospray interface that dominated applications in the 1980s and was still in use through part of the 1990s (Table 7). It is interesting to note that in 1989, Games and Martinez [278] predicted that LC-FAB-MS using the moving belt interface might provide improved detection performance for more polar, non-volatile compounds. However, it was with the advent of API techniques that LC–MS came of age.

API is a soft ionization source suitable for the analysis of polar, non-volatile, thermolabile and high molecular mass molecules such as plant phenols

Table 5
Conditions used in the mass spectrometric analysis of plant phenols

System	Ionization	Sample	Analyte	Ref.
MS	ESI (negative ion)	Olive leaf	Phenols (ca. 15)—no derivatization	[129]
MS	EI	Olive oil	''Tyrosol ester''—no derivatization	[191]
MS	EI, CI (positive ion)	Not applied	Flavonoids—no derivatization	[254]
MS	EI	Evening primrose seeds	Gallic acid, catechins—no derivatization	[255]
MS	EI	Tangerine oils	Polymethoxylated flavones—no derivatization	[256]
MS, MS–MS	ESI and FAB (positive and negative ion)	Soybean root nodules	Phenolic acids and glucosides—isolation by HPLC	[257]
MS–MS	EI, CI (positive and negative ion)	Blood/urine following ingestion of citrus products	Naringenin, hesperitin (and glycosides)—no derivatization	[258]
MS–MS	Fast atom bombardment (negative ion)	Echinacea roots	Caffeoyl esters	[259]
FT-ion cyclotron resonance-MS	ESI (positive and negative ion)	Wines	Anthocyanins	[111]
FT-ion cyclotron resonance-MS	ESI (positive and negative ion)	Wine	Anthocyanins, hydroxycinnamic acids, flavonols esculin	[116]
MALDI–TOF-MS	Positive (and negative) ion	Yellow onion, green tea	Flavonol glycosides	[126]
MALDI–TOF-MS	Positive ion	Blueberries	Anthocyanins	[131]
MALDI–TOF-MS	Positive ion	Red wine, fruit juice	Anthocyanins	[260]
MALDI–TOF-MS	Positive ion	Soy products	Isoflavones	[261]

Table 6
Conditions used in the GC–MS analysis of plant phenols

Ionization	Sample	Derivatization	Analyte	Ref.
EI	Persimmon fruit	Oxime/trimethylsilylation	Phenolic acids	[65]
EI	Cherry laurel fruits	Trimethylsilylation	Phenolic acids	[84]
EI	Olive oil	Trimethylsilylation	Phenols (ca. 15)	[119]
EI	*Eutypa lata*	Trimethylsilylation	Acetylenic phenols	[193]
EI	Mushrooms	?	Phenolic acids	[195]
EI, CI (positive ion)	Olive oil	Trimethylsilylation	Phenolic and secoiridoid aglycones	[199]
EI	Soybean root nodules	Trimethylsilylation	Gallic acid, gallic acid methyl ester	[257]
EI	Flowers	Nil	Flavonoid aglycones	[262]
EI	Not applied	Nil	Polymethoxylated flavones	[263]
EI	Citrus and grape juices	Trimethylsilylation	Flavanones following hydrolysis of glycosides	[264]
EI	Wines	Trimethylsilylation	Phenolic acids, resveratrol, flavonoids	[265]
EI, CI (negative ion)	Passionfruit juice or peel	Trifluoroacetylation	Glycosides of methyl salicylate and eugenol	[266]
EI	Olives	Trimethylsilylation	Phenolic acids, flavonoids	[267]
EI	Corn, wheat, rice	Trimethylsilylation	Dehydrodimers of ferulic acid	[268]
	Distilled alcoholic beverages	Silylation	Phenolic acids	[269]

[35,36,136,226,235,287,293,297–299]. API-based interfacing systems which are liquid-based are ESI and ISI. A related gas phase system is the heated nebulizer APCI interface and each of these techniques has been applied to a range of phenols and sample types (Table 7). Although API has revolutionized the application of LC–MS, some problems remain, the major limitation being the strong dependency of the response on the nature of the analyte plus the mobile phase. Thus, generation of mass spectral libraries is difficult. Moreover, it is difficult to optimize conditions for a typical extract containing a broad range of analytes although many instruments now have provision for programmed operation of spectral conditions.

5.1.1.1. Applications of mass spectrometry

API mass spectra typically comprise protonated molecular ions, $[M+H]^+$ or sodium adduct ions in positive ion mode, or deprotonated molecular ions, $[M\text{-}H]^-$ in negative ion mode with few fragment ions and thus have a low structure information content. On rare occasions, LC–MS can provide data sufficient for full structure analysis [201] but more generally it is used to determine molecular mass and to establish the distribution of substituents on the phenolic ring(s). However, structural information about the molecules can be obtained from CID processes [293]. For instance, appropriate choice of interface parameters distinguished structural isomers of flavonoid glycosides differing in the nature of the disaccharide linkage [35,137]. However, the fragmentation behaviour varied between the two papers illustrating the need for additional fundamental studies on fragmentation mechanisms. HPLC with tandem mass spectrometry (LC–MS–MS) and negative and positive ESI was used [114] for analysis of phenols in rose hip extract. Negative ESI was more sensitive for the majority of phenols with the exception of anthocyanins that were more sensitive in positive ion mode. Thus, molecular masses of the separated phenols were obtained through prominent $[M\text{-}H]^-$ ions for most of the compounds and M^+ ions for the anthocyanins whilst CID of the $[M\text{-}H]^-$ (or M^+) precursor ions yielded product ions that determined the molecular mass of the aglycones. Similarly, the unconjugated phenols were identified by in-source fragmentation followed by CID of the resulting deprotonated aglycone [A-H]. UV spectra obtained from PDA detection assisted in confirming the identities of several compounds.

The application of LC–MS, LC–MS–MS [114] and LC–NMR to rapid detection of biologically active natural products has been reviewed [300]. The use of multiple approaches in phytochemical research is illustrated by the identification of an unknown peak in the chromatogram of apple fruit extracts [294]. HPLC with PDA indicated that the unknown peak was an isorhamnetin glycoside having the same retention time as isorhamnetin 3-*O*-gluco-

Table 7
Applications of LC–MS to the determination of plant phenols

System	Ionization	Mode	Sample	Analyte	Ref.
LC–MS–MS	Heated nebulizer interface	Positive ion	Soybean and soybean products	Isoflavones	[74]
LC–MS	EI		Wines	Resveratrol	[279]
LC–MS	Thermospray		Plant extracts	Flavonol glycosides, secoiridoids, xanthones	[280]
LC–MS	Thermospray	Negative and positive ion mode	Malt, beer, hop	Proanthiocyanidins, flavonol glycosides	[281]
LC–MS	Thermospray		Needles of Norway spruce	Flavonol glucosides, chlorogenic acid	[282]
LC–MS	Thermospray	Positive ion (negative ion for one phenol)	Medicinal plants	Flavonol glycosides	[283]
LC–MS–MS	Thermospray	Positive ion	*Camellia sinensis*	Catechins	[284]
LC–MS	Plasmaspray	Positive ion	Tea	Flavanol (glycosides), chlorogenic acids, flavonol glycosides	[285]
LC–MS	Thermospray		Lemon peel	Flavonols, flavanones, flavone glyosides	[286]
LC–MS	Ionspray	Negative ion mode	Orange juice	Flavanones and flavanone glycosides, flavones, flavonols	[137]
LC–MS–MS	Ionspray, FAB	Positive ion	Olive leaf	Oleuropein, ligstroside, hydroxytyrosol derivative	[226]
LC–MS	Ionspray	Positive ion	Grape	Anthocyanins	[287]
LC–MS	ESI	?	Berries	Flavonols	[27]
LC–MS–MS	ESI	Positive and negative ion	Dried plums	Hydroxybenzoic and hydroxycinnamic acids, rutin, chlorogenic acids, anthocyanins	[102]
LC–MS	ESI	Negative ion	Cider apple tissues	Procyanidins	[106]
LC–MS	ESI		Red beetroot peel	*p*-Coumaric acid, ferulic acid, flavonoids	[107]
LC–MS	ESI	Negative (and positive) ion	Rose hip	Flavanols, flavones, flavanones, flavonols and glycosides	[114]
LC–MS	ESI	Negative (and positive) ion	Soy	Isoflavone	[117]
LC–MS	ESI	Positive ion	Sour orange	Flavonoids and glycosides	[136]
LC–MS(-MS)	ESI	Negative ion	Chocolate	Procyanidins	[143]
?	ESI		Extracts of medicinal plants	Phenols	[288]
LC–MS	ESI	Negative ion	Olive fruits	Hydroxytyrosol and hydroxytyrosol glucoside	[289]
LC–MS	ESI	Negative ion	Cornmeal fibre	Phenolic acids	[290]
LC–MS	ESI	Positive and negative ion	Tea	Flavonoid glycosides	[291]
LC–MS	ESI	Negative ion	Apple and pear	Extensive	[292]
LC–MS	APCI, ESI	Positive and negative ion	Orange	Phenolic and hydroxycinnamic acids, flavonoids	[79]
LC–MS–MS	APCI	Negative ion	Table olives, olive oil	Phenolic acids, tyrosol, oleuropein derivatives	[125]
LC–MS	APCI	Negative ion	Olive mill wastewater	Tyrosol, phenolic acids	[293]
LC–MS–MS	APCI	Negative ion	Apple	Isorhamnetin glycosides	[294]
LC–MS	APCI	Positive and negative ion	Rye, wheat	Steryl ferulate	[295]
LC–MS	APCI	Positive and negative ion	Soy foods	Isoflavones	[296]

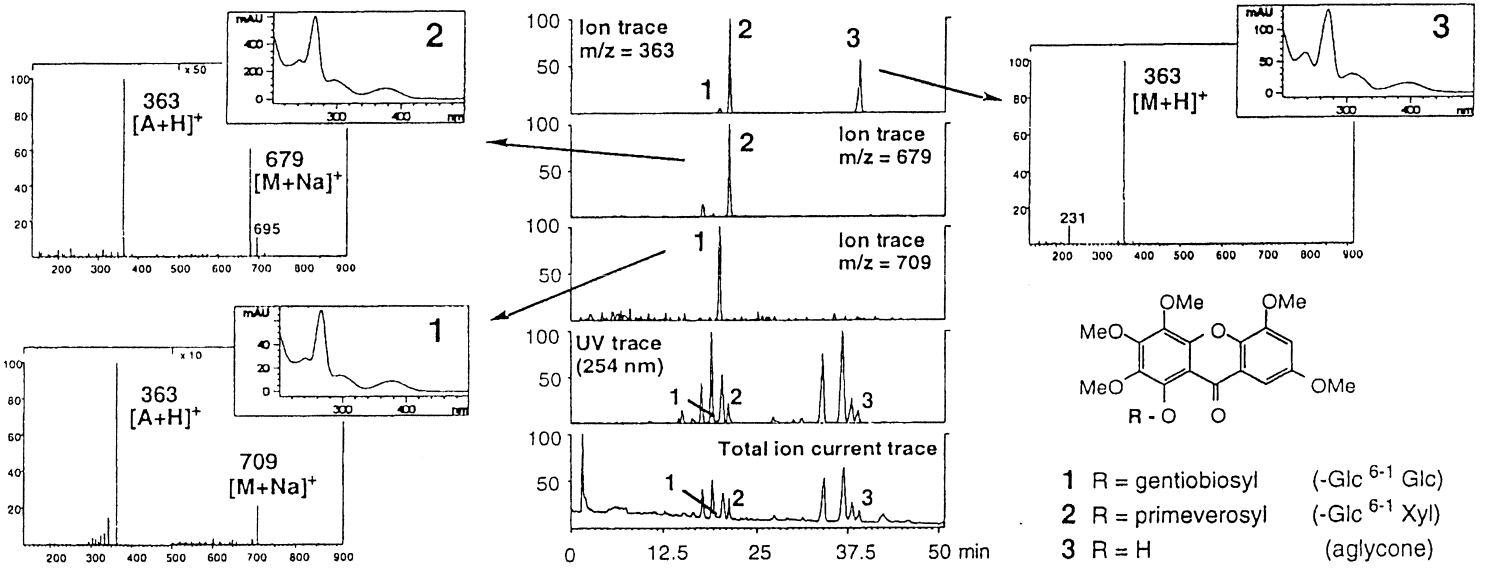


Fig. 5. Total ion current, UV and selected ion chromatograms of an extract of Gentianaceae species, *Halenia corniculata* using a C_{18} reversed-phase and acetonitrile–water gradient. UV and mass spectra of three related xanthones are shown. Reprinted from K. Hostettmann, S. Rodriguez, J.-L. Wolfender, G. Odontuya, and O. Purev, Phytochemistry 40 (1995) 1265. Copyright 1995 [252].

side. HPLC–APCI-MS in the negative ion mode supported the preliminary identification by loss of 162 a.m.u. (corresponding to a hexose unit) from the pseudomolecular ion (m/z 477). CID of the aglycone (m/z 315) in the MS^3 product ion analysis allowed the differentiation of rhamnetin and isorhamnetin, and unambiguous assignment of the peak by comparison with standard compounds as isorhamnetin-3-*O*-glucoside. The use of tandem MS as in this instance offers high selectivity but this paper also illustrates the continued need to consider the chromatographic system. Apart from the requirement for volatile eluents in API techniques, the choice of stationary phase is also significant. In this work and elsewhere [292], a stationary phase with hydrophilic endcapping specifically developed for separation of very polar analytes contributed to the success of the analysis.

HPLC of a dichloromethane extract of a Gentianaceae from Mongolia using PDA showed in excess of 19 peaks having UV spectra characteristic of xanthones [252]. Xanthones with monoamine oxidase inhibitory activity have potential as antidepressive drugs and are useful as chemotaxonomic markers. All peaks recorded in the UV chromatogram at 254 nm were detected in the total ion current trace whereas a selected ion trace at m/z 363 showed three peaks with a common hexasubstituted xanthone aglycone (labelled 1, 2 and 3 in Fig. 5). Similar UV spectra supported this assignment. Peaks 1 and 2 were identified as glycosides detected as sodium adduct ions at m/z 709 and 679, respectively. Final structural assignment was based on known chemotaxonomy of the Gentianaceae family.

In other applications, the enhanced selectivity of LC–MS enabled detection of co-eluting neoeriocitrin and naringin in grapefruit extracts [35] whilst ligstroside was identified in olive extracts [36] by a consideration of elution order in combination with UV and mass spectral data. Mass spectral data also supported the assignment of a number of peaks in the HPLC chromatograms of phenolic extracts as phthalate esters [35,36]. Such artefacts arise due to the use of plastic equipment for sample storage and extraction. Other artefacts have been observed in LC–ESI-MS such as the non-covalent dimer $(2M+H)^+$ of oleuropein [301] with further ions corresponding to the loss of one and two glucose moieties. These ions were generated in the electrospray source, either from the loss of glucose from the dimer or the aggregation of oleuropein molecular ions and fragments within the electrospray source. In the same study, LC–MS(–MS) was used to tentatively assign two peaks as verbascoside isomers whilst NMR spectrometry was used to distinguish between the isomers (vide infra).

5.1.2. Nuclear magnetic resonance spectrometry

In instances where mass spectral data are insufficient to establish a definitive structure, NMR spectrometry is a powerful complementary technique for structural assignment. NMR spectra of phenols are frequently complex and identification of the isolated compounds is complicated by the absence of suitable reference standards which requires time-consuming syntheses of the relevant materials. Although 2D NMR spectrometry can be used for structural analysis without a reference compound, the technique requires relatively large amounts of the compound. Limited sensitivity and the need to isolate relatively large quantities of sample are currently the greatest limitations of NMR spectrometry. The following example illustrates the use of stopped-flow LC–NMR in the characterization of the xanthone and flavone constituents from *Gentiana ottonis* [302]. From the chromatogram of the extract (Fig. 6), peaks with UV spectra characteristic of secoiridoids (**4**), flavones (**6** and **8**) and xanthones (**7** and **9**–**11**) were identified. Molecular masses were assigned to these compounds from LC–MS and fragmentation behaviour indicated compounds **5**, **6** and **8** as C-glycosides. Final characterization of compound **8** required NMR spectrometry and MS–MS whilst the secoiridoid glycoside (**4**) presented NMR signals characteristic for glucose in the 3–4.5 ppm region and typical resonances of the monoterpene moiety in the 5–8 ppm range. These data, together with the molecular mass of 374, enabled unambiguous identification of **4** as swertiamarin. The remaining compounds were characterized using similar approaches.

Extracted ion chromatograms at m/z 623 of an olive extract [301] showed two chromatographic peaks with identical mass spectra, consistent with the structure of verbascoside (Table 8). The base peak in the negative ion mode was the pseudomolecular ion

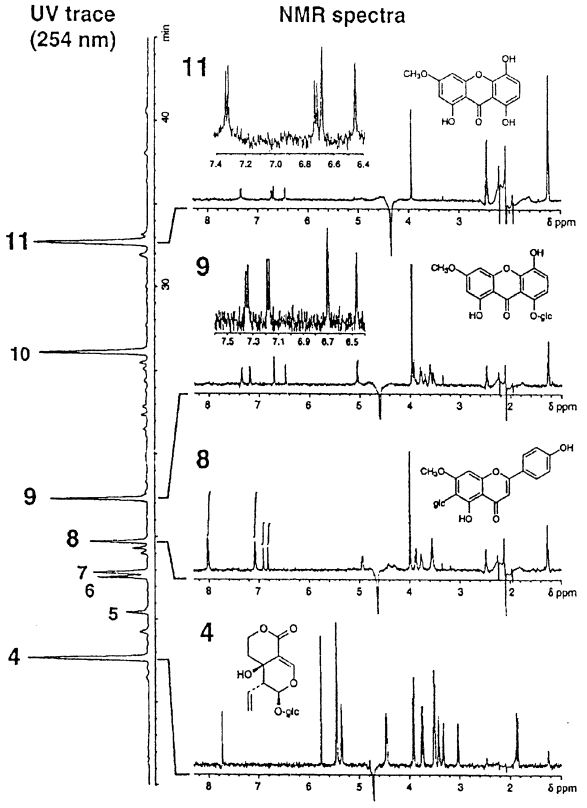


Fig. 6. HPLC chromatogram of a methanol extract of *Gentiana ottonis* showing the stop-flow ^{1}NMR spectra of compounds **4**, **8**, **9** and **11**. Separation was performed on a C_{18} column using an acetonitrile–deuterium oxide gradient. Reprinted from J.-L. Wolfender, S. Rodriguez, W. Hiller, K. Hostettmann, Phytochem. Anal. 8 (1997) 97. Copyright 1997 [302].

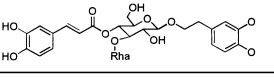
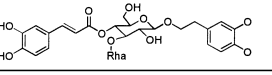
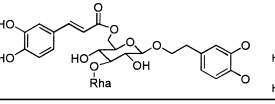
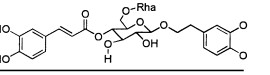
with few other fragment ions, whereas the positive ion spectrum showed some structural information with major peaks at *m*/*z* 479, 471 and 325. These correspond to the loss of the rhamnose sugar, the hydroxytyrosol and the loss of both the rhamnose and the hydroxytyrosol, respectively. As the negative ion mass spectrum showed no structural information, tandem mass spectrometric analysis was carried out on the *m*/*z* 623 ion of both peaks, and the tandem mass spectra of both compounds were very similar with the only ions of significant intensity at *m*/*z* 461 and 161. The similarity of the tandem mass spectra for the two compounds is strong evidence that the two peaks are due to isomers of verbascoside. For full structural assignment the compounds were isolated by preparative-HPLC and examined by ^{1}H NMR whence one of the chromatographic peaks was confirmed as verbascoside by comparison of retention, mass spectral and ^{1}H NMR data (Table 8) with that of a pure verbascoside standard. Based on previous NMR analyses of verbascoside and its isomers, the other was assigned as the ‘‘acteoside isomer’’ identified by Miyase et al. [303].

In many instances, the combination of UV, MS and ^{1}H NMR will provide adequate information for structural elucidation. In other cases, information on the ^{13}C NMR signals is necessary plus 2D correlation experiments involving ^{1}H-^{1}H correlations such as COSY or ^{1}H-^{13}C correlation experiments such as HMBC or HSQC as applied in the structural assignment of a phenol isolated from green olive pulp [34]. Alternatively, TLC screen of a dichloromethane extract of *Monotes engleri* demonstrated antifungal activity that was linked to a compound in the HPLC chromatogram of the extract [253]. The UV spectrum of this compound was characteristic for a flavanone and from mass spectral data it was most probably a flavanone with one *O*-prenyl unit and three hydroxyl substituents. This hypothesis was confirmed by stop-flow LC-^{1}H NMR but the data were insufficient to identify the compound as 2,3-dihydro-5,7-dihydroxy-2-[3-hydroxy-4(3-methyl-2-butenyl)oxyphenyl]-4H-1-benzopyran-4-one. The latter required 2D-NMR correlation experiments and also demonstrated that choice of mobile phase was important not only for the chromatography but also for resolution of the LC–NMR signals. Thus, it was only in methanol/deuterium oxide eluent that complete structural elucidation of the prenylated flavanone was possible whilst with an acetonitrile/deuterium oxide system the substitution pattern of the B-ring of the flavanone was not fully determined.

6. Degradation products of antioxidants

Plant phenols are highly reactive species but little attention has been given to determination of their reaction products. However, by analogy with the synthetic phenolic antioxidants and consistent with their free radical chemistry, dimers and higher oligomers plus, in the case of some phenolics, quinone and hydroquinone derivatives are expected to feature prominently amongst the products. Being

Table 8
^{1}H NMR data for verbascoside and its isomers [301]

Proton	Peak 1	Peak 2	Verbascoside standard	Verbascoside (Acteoside)	Acteoside isomer	Forsythiaside
						
Rhamnose-CH_3	1.18, d, J=7 Hz	1.24, d, J=7 Hz	1.08, d, J=6 Hz	1.10, d, J=6 Hz	1.26, d, J=6 Hz	1.23, d, J=6 Hz
Ar–CH_2	2.79, t, J=12 Hz	2.77, t, J=13 Hz	2.79, t, J=11 Hz	2.78, t, J=7 Hz	2.77, t, J=7 Hz	2.80, t, J=7 Hz
H1 of glucose	4.37, d, J=8 Hz	4.33, d, J=8 Hz	4.37, d, J=8 Hz	4.36, d, J=8 Hz	4.33, d, J=8 Hz	4.35, d, J=8 Hz
Rhamnose-anomeric H	5.18, s	5.17, s	5.18, s	5.17, s	5.18, s	4.63, s
Ar–C=CH	6.27, d, J=16 Hz	6.28, d, J=16 Hz	6.27, d, J=16 Hz	6.28, d, J=15 Hz	6.28, d, J=16 Hz	6.30, d, J=15 Hz
Aromatic H	6.5–7.1	6.5–7.1	6.5–7.1	6.4–7.1	6.4–7.1	6.53–7.10
Ar–CH=C	7.58, d, J=16 Hz	7.55, d, J=16 Hz	7.58, d, J=16 Hz	7.57, d, J=15 Hz	7.54, d, J=16 Hz	7.56, d, J=15 Hz

electron-richer than the A-ring of flavonoids, organic peroxyl radicals selectively attack the B-ring [304]. In the case of dihydroxyflavonoids, a consecutive two-electron oxidation reaction produces the flavonoid phenoxyl radical that subsequently scavenges another peroxyl radical to form a quinone.

Polarography, spectrophotometry and HPLC were used to follow the oxidation of several phenols catalysed by apple PPO [305] between pH 4 and 5. The reactivities of the *o*-quinone products varied greatly from one phenol to another. Two pathways were proposed for the degradation of 4-methylcatechol *o*-quinones. The first, favoured by acid pH, corresponded to an hydroxylation followed by a coupled oxidation of another molecule or *o*-quinone and leading to regeneration of 4-methylcatechol. The second pathway involved polymerization reactions that were favoured by higher pH values. The same pathways were observed with chlorogenic acid although the polymerization reactions seemed to be dominant. The *o*-quinones of (+)-catechin and (−)-epicatechin are much less stable than that of chlorogenic acid [306] and were not examined by Richard-Forget et al. [305]. The reaction products become quite complex with as few as two phenols (*o*-quinones) in admixture [307]. In summary, the *o*-quinones will enter along the different pathways according to their oxidative and electrophilic properties. As oxidants, the *o*-quinones will oxidize any other substances with lower reduction potentials. This will include other phenols, ascorbic acid and sulfur dioxide. In this process the quinones are themselves reduced to the original phenol. They will react as electrophiles with nucleophilic substances including amino derivatives and water. In the absence of other substrates, condensation and polymerization will occur via reaction with the corresponding hydroquinone. The products formed in such reactions are pH-dependent [308]. Mostly colourless products were formed during the PPO-catalysed oxidation of (+)-catechin in aqueous buffers at pH below 4 whereas yellow compounds, less polar than the colourless ones, predominated at higher pH values. The yellow products were identified as dimers and possible mechanisms for their formation were proposed as a Michael-type addition or through a semiquinone radical intermediate. Michael (1→4) addition could be favoured by high pH, which increases the nucleophilic character of (+)-catechin whereas low pH could favour radical mechanisms by increasing the reactivity of semi-quinone radicals.

7. Informatics and data handling

The range of phenols that have been isolated from fruits and vegetables is vast and yet, it is small compared with the total number of known natural products and presumably many phenols remain undiscovered. When the number of potential bioactivities is factored in then the acquisition and storage of chemical and related biological data are crucial aspects of an analysis. The design of chemical libraries [309,310] is an important component of the search for bioactive materials and various commercially available databases exist but there is regrettably no universal data depository. Rediscovery of bioactive compounds from previously unstudied sources is a costly and time-consuming problem that is not completely eliminated by database use. For example, one group found that 72% of compounds that reached the structure determination stage were known compounds [311]. An investigation of phenolic acids and flavonoids in flowerheads of 84 samples of 76 taxa belonging to 66 species resulted in the identification of only three phenolic acids and six flavonoids.

8. Conclusions

There has been a renaissance in the study of bioactive compounds and the interest in plant phenols is intense. Several reasons for this interest can be identified but these are as diverse as the phenols themselves. The adoption of particular analytical strategies is related to the purpose of the analysis and the nature of both the sample and analyte. The classification of sample types as plants and foods is not particularly productive in terms of classifying the analytical strategy nor is the distinction between processed and unprocessed products as similar procedures are used for both groups. The usual procedure now encompasses a high-performance separation technique in combination with diode

array detection or mass spectrometry. Further instrument sophistication in coupling several systems such as multidimensional chromatography with NMR and MS in series is already occurring. The prediction of the future is dangerous but promising approaches involve the application of HPLC with ESI time of flight mass spectrometers and ESI FT ion cyclotron resonance mass spectrometers. An increased emphasis on microcapillary columns with nanotechnology ESI systems driven partly by environmental issues seems inevitable.

9. Nomenclature

API	atmospheric pressure ionization
APCI	atmospheric pressure chemical ionization
BHA	butylated hydroxyanisole
CI	chemical ionization
CID	collisionally-induced dissociation
EI	electron impact ionization
ESI	electrospray ionization
FAB	fast atom bombardment
FT(IR)	Fourier transform (infrared spectrometry)
GC	gas chromatography
GC–MS	gas chromatography–mass spectrometry
HPLC	high-performance liquid chromatography
ISI	ionspray ionization
LC	liquid chromatography
LC–MS	coupled liquid chromatography–mass spectrometry
MALDI–MS	matrix assisted laser desorption ionization-mass spectrometry
MS	mass spectrometry
MS–MS	tandem mass spectrometry
NMR	nuclear magnetic resonance spectrometry
PDA	photodiode array detection
PPO	polyphenoloxidase
RPLC	reversed-phase liquid chromatography
SFE	supercritical fluid extraction
SPE	solid-phase extraction
TBHQ	*tert*.-butylhydroquinone
TOF	time of flight

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