The Analyst

Critical Review

Phenolic compounds in olives

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Keywords: *Phenolic compounds; olives; mass spectrometry; chromatography; fruit*

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Introduction

Archaeological evidence traces olive trees back to 6000BC and frequent Biblical references appear throughout the New Testament. The Mediterranean region nowadays serves as the major international olive growing area, accounting for almost 98% of the world's olive tree plantation.[1] Olives are rarely consumed as a natural fruit due to their extreme bitterness, but rather are consumed in either one of two forms; as oil or table olives. The significance of the olive oil market in the olive industry is apparent as it consumes approximately 90% of the annual production of olives. Annual world consumption of olive oil in 1995/96 was 1777 thousand tonnes and this increased from 1508 thousand tonnes in 1970/71 but most striking has been the rapid growth in consumption[2,3] in high-income countries like Japan, the United States, Canada and Australia.

The increasing health consciousness of today's more cosmopolitan society explains the rising consumption of olive oil around the world and hence the rapid growth of the olive industry. The beneficial health properties of olive oil have been known for centuries, particularly in the Mediterranean region. Olives and olive oil are an inherent part of Mediterranean culture and diet, and hence the decreased incidence of cardiovascular disease in this area (being one of the lowest in the Western Hemisphere) has been attributed to their consumption.[4] These effects have been attributed[5] to the high content of oleic acid in olive oil, which serves to slow down the penetration of fatty acids into the arterial walls. The preventative superiority of olive oil is also attributed to its antioxidant composition; namely, tocopherols and phenolic compounds. The latter are highly diverse,[6–8] both in their chemical structure and proposed biological functions. Their metabolic pathways are particularly complex with, in many cases, multiple alternative metabolic fates. Profiling of the components of a pathway over time provides a dynamic view of the metabolic events occurring in the plant. As stated by Amiot *et al.*:[9] 'Knowledge of the variations in phenolic compounds should make it possible first to obtain better understanding of the relationships that may exist between these substances and the physiology and organoleptic qualities of the fruit and second to provide a more solid basis for processing techniques, thus leading to improved quality.'

The intense interest in plant phenolics which has been manifested over several decades, accounts for the many reviews and monographs[10–12] devoted to various aspects of these compounds. For example, the role of plant phenolics in the prevention and treatment of disease has been examined.[13] Food sources of plant phenolics have been reviewed[14] and the same authors discussed the biological activities and functions of phenolic compounds, especially as they relate to their mechanisms of anticarcinogenicity.

This review critically examines the analytical chemistry of the phenolics in olives. Methods used for the analysis of samples other than olives will be discussed where these illustrate current applications which can be extended to include olives or which may emerge as important advances over existing methods. The review also addresses the factors that impact upon the phenolic composition of olive fruits and oils.

Structure of plant phenolics

The plant phenols are aromatic secondary metabolites that embrace a considerable range of substances possessing an

aromatic ring bearing one or more hydroxy substituents.[15] In the present context, this definition is not entirely satisfactory since it inevitably includes compounds such as oestrone, the female sex hormone (which is principally terpenoid in origin). For this reason, a definition based on metabolic origin is preferable, the plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoid metabolism (Fig. 1). Phenolic compounds present in olives are conventionally characterised as 'polyphenols', an unfortunate term since not all are polyhydroxy derivatives. In particular, a number of compounds, namely, cinnamic acid, elenolic acid, shikimic acid and quinic acid, are treated in the present discussion as phenolics because of metabolic considerations although they lack a phenolic group or even an aromatic ring. Plant phenols have been classified[15] into 15 major groupings distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton. The range of known phenolics is thus vast but of the various groups only the benzoic acids, cinnamic acids, flavonoids and iridoids (Table 1) are of major significance in olives.

Additional structural complexity is introduced by the common occurrence of certain phenolics as the O-glycosides in which one or more of the phenolic hydroxy groups is bound to a sugar or sugars by an acid-labile hemiacetal bond. Glucose is the most commonly encountered sugar with rhamnose and the disaccharide, rutinose (6-O-α-L-rhamnosyl-D-glucose) also encountered. Acylation of the glycosides in which one or more of the sugar hydroxys is derivatised with an acid, such as acetic or ferulic acid, is occasionally observed. Phenolic compounds associated with olives are listed in Table 2 with some representative structures shown in Fig. 2.

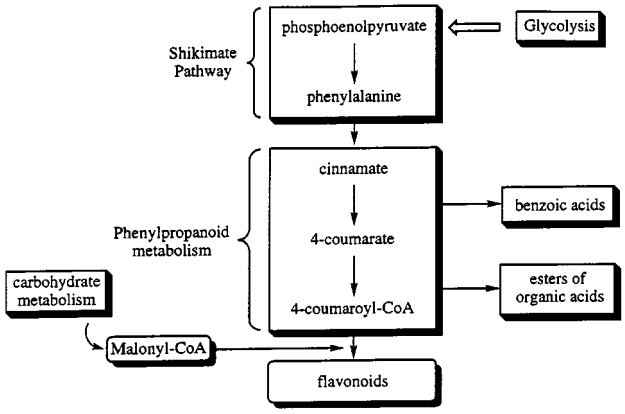

Fig. 1 Metabolic pathways leading to the formation of phenolic compounds.

Table 1 Major classes of fruit phenolic compounds in olives

Number of C atoms	Basic skeleton	Class	Example
7	C_6-C_1	Benzoic acids	*p*-Hydroxybenzoic acid
			Vanillic acid
			Protocatechuic acid
9	C_6-C_3	Hydroxycinnamic acids	Caffeic acid
15	C_6-C_3-C_6	Flavonoids	Cyanidin
		Anthocyanins	
		Flavonoid glycosides	Rutin
—		Iridoids	Oleuropein
			Ligstroside
n		Lignins	
		Tannins	

Role of phenolics in olives

Metabolic pathways involving phenolics are complex with, in many cases, multiple alternative metabolic fates for a given metabolite which may vary markedly from tissue to tissue, from one growth condition to another, and in response to environmental stimuli. Hence, establishing a biological function for such compounds is often very difficult. Nevertheless, almost all of the phenolic compounds possess several common biological and chemical properties; namely, antioxidant activity, the ability to scavenge both active oxygen species and electrophiles, the ability to inhibit nitrosation and to chelate metal ions, the potential for autoxidation, and the capability to modulate certain cellular enzyme activities.

Properties and function

In some cases, phenolic function may well be related to primary metabolism. Some phenolics have an effect on olive plant growth while others protect the more vulnerable cell constituents against photooxidation by UV light[15] by virtue of their strong ultraviolet absorption.[15] Hence, phenolics play a key role in fruit preservation. In general, however, the search for a function for these compounds has focused on the interaction that may take place between the plant and other living organisms and phenolics in olives are now recognized for their antimicrobial activity[47], molluscicidal properties[34], their preventative role in *Dacus oleae* infestations[32] and resistance to other parasite invasions.[15] Their role in disease resistance is well established. Muller[48] defined phytoalexins as 'compounds produced after infection under the influence of two metabolic systems, that of the host and that of the parasite, and inhibitory to the parasite'. Most literature reports are concerned with the role of phytoalexins in resistance to disease caused by fungi whilst fewer relate phytoalexin accumulation to resistance to disease caused by bacteria. However, Chowdhury *et al*.[49] have reported the minimum inhibitory concentration of several simple and complex phenolics found in olive fruits against four pathogenic bacteria. Caffeic acid was the most effective agent although oleuropein, the major phenolic constituent of olives also exhibited[50] bactericidal action. By the mid-1960s, it became apparent that phytoalexins were produced not only in response to infection but also in response to various forms of physiological stress. A common response of plant cells to stress, such as wounding, infection or elicitation, is the induced incorporation of phenylpropanoids into the cell wall. However, as stated by Matern and Grimmig:[51] 'The precise role of the polyphenol cell wall reinforcement for the protection of plants has . . . remained ill-defined due to *limited analytical knowledge* and the complexity of the cell wall architecture.' The discovery of new analytical methods underpins scientific progress and nowhere is this more evident than in the study of the phenolics.

Phenolics as antioxidants

As a consequence of their fundamental chemical properties, the phenolics inhibit lipid peroxidation[52] and exhibit various physiological activities.[53] The antioxidant properties of the phenolics are well known[54–57] and continue to attract considerable research effort. Thus, plants such as the herb rosemary are highly acclaimed for their antioxidant properties,[58] which have largely been attributed to the phenolic compounds carnosol, rosmanol and rosmadial.[54] Similarly, the phenolics in olives have attracted attention as antioxidants.[9,33] Total hydrophilic phenols and the oleosidic forms of 3,4-dihydroxyphenylethanol (hydroxytyrosol) were correlated ($r = 0.97$) with the oxidative stability of virgin olive oil[59] whereas tocopherols showed low correlation ($r = 0.05$). More specifically, antioxidant activity in refined olive oil decreased[60] in the series hydroxytyrosol,

caffeic acid > butylated hydroxytoluene (BHT) > protocatechuic acid, syringic acid. Tyrosol, *p*-hydroxyphenylacetic acid, *o*-coumaric acid, *p*-coumaric acid, *p*-hydroxybenzoic acid and vanillic acid had very little or no antioxidant activity, and their contribution to the stability of the oil was negligible.

A variety of methods are used to assess the antioxidant activity of crude olive extracts and purified phenolics. One approach involves measurement of the inhibition of oxidative deterioration of an oil or model substance, such as methyl linoleate.[33] This is conveniently performed in the Rancimat apparatus,[59] which has been used to demonstrate[61] that the activity of tyrosol (in refined tallow) was lower than that of the synthetic BHT whereas oleuropein showed a stronger activity although the best protective effect was obtained with gallic acid esters and hydroxytyrosol. Care must be exercised in the interpretation of data relating to antioxidant activity as the substrate[62] and also the analytical technique influences the results. The effect of substrate can be attributed[62] to the strong influence of the unsaturation type and degree of the lipid system on the kinetics and mechanism of the antioxidative action of the phenols. For example, when tested in another accelerated oven-test on refined sunflower oil thin films,[61] the activity of hydroxytyrosol was lower than that of gallic acid esters. Similarly, the trends in antioxidant activity of phenolics differed[63] according to whether hydroperoxide formation (peroxide value) or decomposition (hexanal and volatiles) was measured in accelerated stability tests on olive oil. These results emphasise the need to measure at least two oxidation parameters to better evaluate antioxidants and the oxidative stability of olive oils.

Alternative techniques for measuring antioxidant activity include the electrochemical measurement of oxygen consumption and electron spin resonance (ESR) spin trapping.[64] The latter involves generation of hydroxyl radicals by the Fenton reaction which are then trapped by 5,5-dimethyl- 1-pyrroline-*N*-oxide in competition with the test sample. The electrochemical technique involves measurement of the oxygen depletion rate in a heterogeneous lipid/water emulsion with lipid oxidation initiated by metmyoglobin. The data relate to the effect of antioxidant on the propagation of oxidation while the ESR free radical method relates to the effect of antioxidant on the initiation step. The two techniques have not been applied to

Table 2 Literature survey of phenolic compounds found in olives

Phenolic compound	Leaves	Seed	Pulp	Oil	Molecular mass
Apigenin	16		16 (absent)		270
Apigenin-7-glycosides	26, 32		26, 44		
Caffeic acid	26		(18), 26	27, 28, 37, 38, 40, 41	180
Chlorogenic acid	21				343
Cinnamic acid				27, 28, 29, 37, 41	148
Cornoside			39		316
o-Coumaric acid			17	40, 41	164
p-Coumaric acid	26		17, 18, 26, 35	20, 27, 28, 29, 31, 32, 37, 40, 41	164
Cyanidin-3-glycosides	26		26, 44		
Demethyloleuropein	26		24, 26, 43		526
Elenolic acid	26		26	30	242
Elenolic acid glucoside			(35), 43	36	
Ferulic acid			17	20, 28, 37, 41	194
Gallic acid			17	36	170
(Halleridone)			39		154
Hesperidin	26		26		610
Homovanillic acid				38	182
p-Hydroxybenzoic acid				39, 41	138
p-Hydroxyphenylacetic acid			22	38, 39, 40	152
(*p*-Hydroxyphenyl)ethanol				30, 41	138
Hydroxytyrosol	7, 26		18, 26	28, 30, 31, 32, 36, 38, 41, 42, 45	154
Ligstroside	6, 7, 39, 46		34		524
Luteolin	16, 21		16		286
Luteolin-7-glucoside	21, 26, 32		9, 19, 26, 35, 44		448
Luteolin-7-rutinoside	32				
Nuezhenide		23			
Nuezhenide oleoside		23			
Oleuropein	6, 7, 16, 26, 32, 46		9, 16, 18, 19, 25, 26, 33, 34, 35, 39, 43	28, 30, 41	540
Oleoside and oleuroside	6, 7				
Protocatechuic acid				40, 41	154
Quercitin	16		16		302
Quercitin-3-rutinoside (rutin)	21		9, 35, 44		610
Salidroside		23, 39			
Sinapic acid				28, 29	224
Syringic acid			17	20, 27, 28, 29, 40, 41, 45	198
Tyrosol			17, 39	27, 28, 29, 31, 36, 38, 42, 45	138
Tyrosol glucoside			39		300
Vanillic acid			17, 18	20, 27, 28, 29, 31, 32, 36, 41	168
Veratric acid			22		
Verbascoside			9, 18, 19, 35		624

olives but they were compared for measuring the antioxidant activity of various spices.

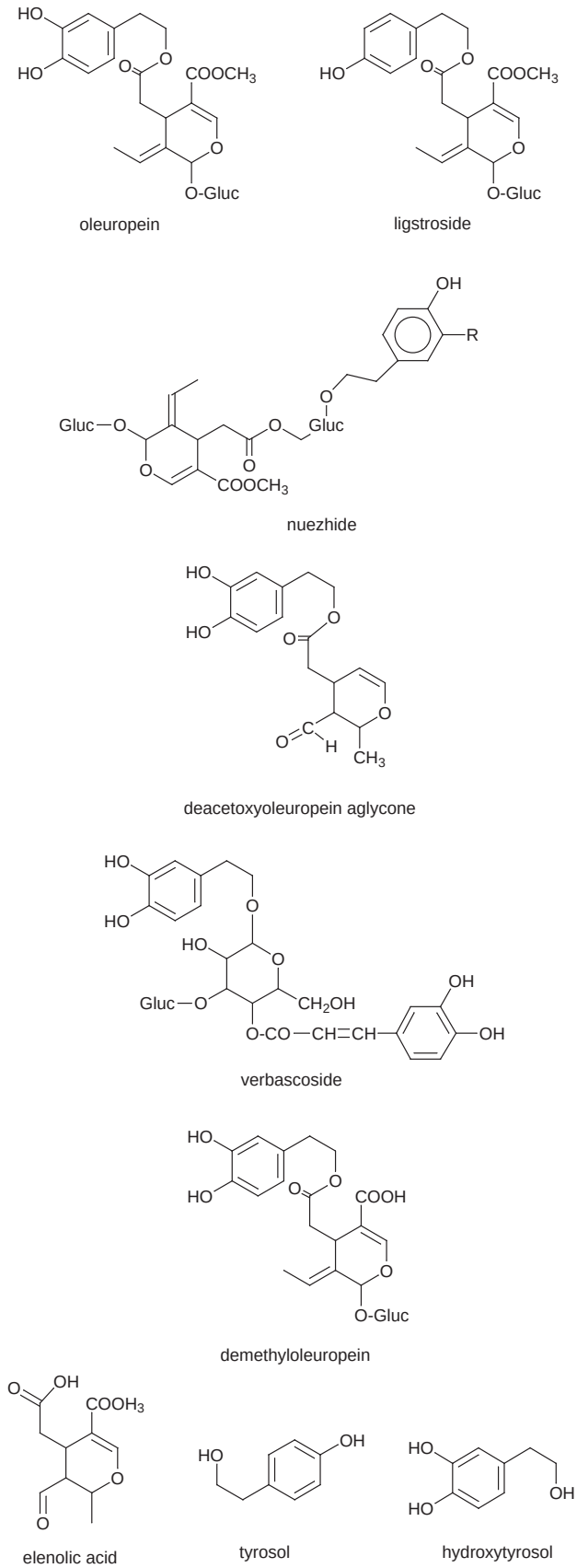

Fig. 2 Chemical structure of representative phenolic compounds found in olives.

In olive oil, the phenolic content serves as an important qualitative parameter due to its correlation with the peroxide number, free fatty acidity, and sensorial quality.[30,41] Free fatty acids (FFA) provide an index of the degree of lipase activity and when present at high concentrations, produce undesirable aromas in the oil.[65] Because phenolics function as antioxidant constituents of olive oil, a high FFA content invariably indicates a high degree of lipase activity and hence a reduced antioxidant content. Similarly, peroxide number, or peroxide value (PV) monitors the initial products of oxidation; that is, the hydroperoxides. The PV therefore offers one of the most direct measures of lipid peroxidation.[66] The amount of peroxides that must be formed to produce noticeable rancidity is dependent upon the composition of the oil and, in particular, the degree of unsaturation and the presence of antioxidants, notably, the phenolics.

Phenolics and fruit quality

Phenolic compounds may contribute to fruit quality in a number of ways; for example, by contributing to sensory attributes, such as colour and flavour, and through the contribution of some specific phenolics, in particular oleuropein,[9] to the intense bitterness of the olive fruit. Other bitter phenolics occurring in the fruit include the glucosides, salidroside, nuezhenide, and nuezhenide oleoside, together with two secoiridoid glucosides of uncertain structure containing tyrosol, elenolic acid and glucose moieties, which have been identified[23] in the seeds of *Olea europaea*. Cimato *et al.*[42] attribute the organoleptic value and the preservability of olive oil to the phenolics and tocopherols although the phenolic compounds may also contribute to flavour in a negative sense. Thus, the ethyl ester of cinnamic acid and 4-vinylphenol was identified[67] by gas chromatography–mass spectrometry (GC–MS) in the steam distillate from unacceptable olive oils. The source of the 4-vinylphenol was attributed to decarboxylation of *p*-coumaric acid.

Phenolics can also contribute to fruit quality *via* their role in browning reactions. Thus, oxidation products of oleuropein, in conjunction with those of other native phenolics are known to be responsible for the characteristic black colour of mature olive fruits.[1,35] Enzymatic oxidation of endogenous *o*-diphenols into *o*-quinones, which can then polymerise into brown products, results in the discolouration and softening of olive fruits, and the ultimate destruction of the product's commercial value. The reaction is catalysed by polyphenol oxidases, which comprise a large group of enzymes all of which are characterised[68] by their ability to utilise molecular oxygen during the oxidation of phenolic substrates. The browning reactions are mediated by various metal ions such as iron(III)[69] and manganese.[70] The susceptibility of olives to browning can be examined using model solutions[71] and illustrates the complex interactions between polyphenol oxidase activity and phenolic content. In some instances, the rate of browning has been positively correlated with both the content of oleuropein, the major substrate for the reaction, and the polyphenol oxidase activity.[72] In most instances, however, the rate of reaction has been substrate limited[18,73,74] with no correlation to the enzyme activity.

Factors affecting the phenolic profile of olives

Phenolics are characteristic constituents of green plants occurring in virtually all parts of the plant but with quantitative distributions that vary between different organs of the plant and within different populations of the same plant species. Phenolics in olive pulp and oil constitute a complex mixture, the complete chemical nature of which has not, as yet, been elucidated.[66] For example, there are many phenolics present in

low concentrations which remain unidentified but whose significance may far outweigh their concentration level. Isolation and structure elucidation of these compounds are the initial steps to understanding their significance and action. Information on their biosynthesis is essential to understand the interaction between plants and the environment. Methods of characterisation and identification follow those in general use for natural substances. Hence, preparation of an extract, biological screening, bioguided fractionation, isolation and structure elucidation is the usual approach. For the latter, physical methods based on spectral characteristics feature prominently although older chemical and biochemical approaches should be considered particularly as adjuncts to spectral analysis.

Factors contributing to the variability in phenolic distribution include the cultivar and genetics, maturity, climate, position on the tree, rootstock and agricultural practices. In the case of processed products, technological processes to which olive fruits are exposed may also impact significantly on the phenolic content.

Varietal influences

The olive fruit is characterised by the epicarp (skin), with a soft, pulpy flesh (mesocarp), and the endocarp (stone), which contains the seed or kernel. In ripe olives, the seed makes up some 2–3% of the total mass, the stone 13–23% and the flesh or mesocarp some 84–90% but occasionally as low as 65%. The composition of the flesh, stone and seed components is given in Table 3[75] but clearly the components of the flesh are quantitatively the more important.

There are approximately 2500 known varieties of olives, 250 of which are classified as commercial cultivars by the International Olive Oil Council (IOOC). These commercial cultivars are used for the production of either olive oil or table olives. The particular use of a given cultivar is determined by its oil content and size, with larger fruits (> 4 g) being favoured for table olive consumption. Olive varieties with an oil content of less than 12% such as Ascolano, Calamata and Manzanillo, are almost exclusively used for table olive production.[75] Similarly, olive varieties with a high oil content are exploited for the purposes of olive oil production.

The flesh components pass either as is or transformed, to the oil, which is mainly composed of triacylglycerols with small quantities of free fatty acids, glycerols, phosphatides, pigments, carbohydrates, proteins, flavour compounds, phenols, sterols and unidentified resinous substances (Table 4). The amount of these constituents varies[17,27,77–79] with cultivar and environmental conditions. For example, the effects of cultivar, growth locality and extraction technology on the phenolic content (tyrosol, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, and cinnamic acid) of Sardinian olive oils has been examined by multivariate statistics.[27] The variety Bosana is particularly rich in phenols and within this variety there was evidence that the extraction technology exerted a considerable effect on phenolic composition. Oil components from the seed, though a minor component, still become part of the olive oil but do not have the same composition[80] as that from the flesh.

Other factors

Although few studies have examined the effects of agronomic and climatic factors on fresh fruit, the effects particularly of rainfall and growing temperature on the characteristics of olive oil have been extensively reported.[27,77,78,81] For instance, oil produced from orchards at 800 m was of better quality than the oil from an altitude of 100 m.[17] The enhanced quality of oil obtained from higher elevation was largely explained by the oil's higher oxidative stability compared to that from lower altitudes. This can be attributed to the higher tocopherol and total phenolic content of fruit harvested from the higher altitude. In another study of climatic effects, the compounds mainly affected were aliphatic alcohols, phenolics and headspace constituents,[79] which are of particular importance in the sensory characterisation and quality of olive oil. Tous and Romera[78] concluded that the oxidative stability of olive oil varies according to cultivar and location.

Variation in phenolic content with harvesting period and its subsequent effect upon oil quality has been investigated.[42] During the first harvesting period, phenolic levels were higher irrespective of environment and cultivar and gradually declined as the olives ripened. Deidda *et al.*[81] and Alessandri *et al.*[82] have identified a relationship between early harvesting and the production of high quality olive oil. Similarly, Garcia and co-workers [70,83] studied the oil obtained from different olive varieties, and found that most of the oils exhibited an increase in titratable acidity, responsible for the production of rancid flavours, and a decrease in total phenols as ripening progressed. The selection of an optimal harvesting date should therefore be ascertained to preserve the organoleptic properties of the oil and to prevent the production of inferior quality olive oil due to delayed harvesting.

Olive development and maturation

The flowering of the olive tree marks the beginning of fruit development. In the following six–eight months the olive attains its maximum fruit weight.[75] This is followed by fruit colour change and associated physiological modifications, with the appearance of the purplish-black olive fruit indicating the end of olive morphology. Two degrees of maturation are recognised[84] in olive fruits, namely green maturation and black maturation. Amiot *et al.*[43] have included a third phase in olive development, aptly named the growth phase, which occurs prior to that of green and black maturation, during which the accumulation of oleuropein occurs. In contrast, four stages of maturation have been identified by Garcia and co-workers,[70,83] which quite simply correspond to the apparent changes in fruit

Table 3 Olive fruit composition[75]

Constituent	Flesh	Stone	Seed
Water	50–60	9.3	30.0
Oil	15–30	0.7	27.3
N matter	2–5	3.4	10.2
Sugars	3–75	41.0	26.6
Cellulose	3– 6	38.0	1.9
Ash	1–2	4.1	1.5
Phenolics	2–2.5	0.1	0.5–1.0
Intermediate		3.4	2.4

Table 4 Minor components (ppm) of virgin and refined olive oil (data from ref. 76, p. 30)

Component	Virgin olive oils	Refined olive oils
Phenolics and related substances	350	80
Hydrocarbons	2000	120
Squalene	1500	150
β-Carotene	300	120
Tocopherols	150	100
Esters	100	30
Aldehydes and ketones	40	10
Fatty alcohols	200	100
Terpene alcohols	3500	2500
Sterol alcohols	2500	1500

colour and anthocyanin content at the green, spotted, purple and black stage of olive maturation.

One of the difficulties associated with maturation studies is the precise identification of the various physiological stages. Some authors[43] in recognition of this difficulty have plotted harvest date *versus* change in phenolic content. This approach makes no allowance for the vastly different rates of maturation of fruit on the same tree unless data for a given harvest date are selected also for the stage of development by fruit colour, for example. Fruit development can fortunately be characterised[15] using one or more of the following criteria:

(1) the appearance of new compounds, (2) the disappearance of certain compounds, (3) the occurrence of various characteristic ratios between certain compounds, and/or (4) the evolution of the activity of numerous enzymes leading to the biosynthesis or degradation of phenolic compounds.

For example, green maturation is characterised by a reduction in chlorophyll content in conjunction with fruit softening and an increase in oil content. A further reduction in chlorophyll content is apparent in the black maturation phase, along with a significant increase in CO_2 accumulation, ethylene secretion and anthocyanin content. Anthocyanins are responsible for black fruit colouring, and are classified as phenolics. The notion of phenolic compounds serving as biological markers of the physiological stages of growth and fruit maturation is well known.[15] Cimato[42] showed that with fruit ripening, hydrolysis of components with 'higher molecular weight' occurred, with the formation of tyrosol and hydroxytyrosol. Thus, the concentration of tyrosol and hydroxytyrosol was also shown to increase with the harvesting period, which has been correlated with an evident reduction in four unidentified, but presumably phenolic components.

The majority of research on the relationship between phenolics and olive development concerns oleuropein, which is known to be the most prominent and significant individual phenolic component of olive pulp, reaching concentrations of up to 14% on a dry weight basis in young Picholine olives.[9] The concentration of oleuropein declines with fruit maturity in accordance with the second criteria used to characterise fruit maturation; that is, the decrease in concentration of certain compounds. Amiot *et al*.[43] have also shown that oleuropein degradation in olives is accompanied by the accumulation of two compounds, namely demethyloleuropein and elenolic acid glycoside, of which, only the former is phenolic. The fact that neither of these compounds was present prior to green maturation, in conjunction with the difficulty associated with identifying this stage, enabled the characterisation of the green maturation phase of olive development. It should also be noted that out of eleven cultivars examined, demethyloleuropein was only present in two cultivars.[43] The idea of demethyloleuropein serving as a varietal marker has therefore been suggested. Similarly, Vlahov[44] has suggested the exploitation of distinct flavonoid compositions of olive cultivars as a tool for biochemical characterisation of fruit varieties.

Amiot *et al*.,[9] have established an inverse relationship between oleuropein content in olive fruit and other phenols, such as certain flavonoids and verbascoside (a heterosidic ester of caffeic acid and hydroxytyrosol). Research conducted by Vlahov[44] supports the findings of Amiot *et al*.,[9] who observed increases in given flavonoid compounds in three olive fruit varieties with the onset of maturation. It is interesting to note that part of the verbascoside and oleuropein molecule are the same. It could therefore be hypothesised that partial degradation of the oleuropein molecule is responsible for the formation of verbascoside, since verbascoside cannot be detected in very young fruits.[9] This is in agreement with the hypothesis proposed by Amiot *et al*.[9] that 'the successive evolution of oleuropein and verbascoside and their biochemical relationship may suggest the existence of a metabolic relationship between these two compounds'.

Olive trees are known to be alternate bearers,[85] providing high fruit yields one year and low yields the next. Alternate bearing causes a major problem in the olive industry, particularly in warmer climates,[85] since climatic conditions are known to impact significantly on olive production. Alternate bearing is an overall response of cropping due to yearly overlapping between two biennial cycles.[86] This irregularity can actually cause fruit produced in the high yielding year to be coarse or even valueless.[87] The involvement of intermediates of the cinnamic acid–lignin pathway on flower bud differentiation, rooting and callus development has been studied.[85,88] The endogenous level of chlorogenic acid in olive leaves of fruit bearing trees was 3–4 times higher than the non-bearing ones. Application of chlorogenic acid decreased the amount of differentiating buds when injected prior to flower bud induction but had no effect when applied thereafter.

Processing and storage

The major uses of olives, namely olive oil and table olives involve extraction and/or chemical treatment of the fruit which impacts on the phenolic content of the resulting product and hence product stability and quality.[89] Incorrect storage can also result in a reduction in 'total phenols' and other quality parameters as shown by the data of Table 5.

The composition of olive fruit and olive oil exhibits some notable differences which are attributed to a series of chemical and enzymatic alterations of some substances during oil extraction. These modifications include hydrolysis of glycerides by lipases, with the formation of free fatty acids, hydrolysis of glycosides and oligosaccharides by glucosidases, oxidation of phenolic compounds by phenoloxidases and polymerisation of free phenols.[90] The major phenolic compounds identified in

Table 5 Variation in the quality of oils obtained from two varieties of olives stored in jute sacks (data from ref. 66, p. 19).

Olive varieties	Oil extraction date	Organoleptic evaluation	Free acidity (%) (as oleic acid)	Peroxide value/mequiv. O_2 kg^{-1}	*trans*-Hex-2-enal (ppm)	Isoamyl alcohol (ppm)	Total alcohols (ppm)	Total phenols (ppm)
DRITTA	Nov. 16	7.1	0.45	7.0	279.5	4.5	45.8	578.6
	Nov. 20	6.2	0.73	12.0	175.3	33.1	57.2	172.1
	Nov. 23	5.4	1.21	10.5	62.4	56.0	115.2	130.8
	Nov. 28	3.8	3.25	10.5	3.3	85.6	187.6	32.2
	Dec. 07	3.6	7.27	17.1	1.0	96.8	256.0	—
LECCINO	Nov. 17	7.0	0.33	4.2	924.2	1.7	10.8	703.7
	Nov. 20	6.5	0.36	11.1	450.9	7.4	24.9	484.5
	Nov. 23	6.0	0.36	11.2	345.3	9.1	22.9	142.5
	Nov. 28	5.1	1.24	19.1	11.8	45.0	68.5	137.9
	Dec. 07	4.5	4.79	15.0	8.4	66.7	73.6	—

both olive fruit and virgin olive oil (see Table 2) include tyrosol, hydroxytyrosol, caffeic, *p*-coumaric and vanillic acids,[18,91] whilst the glycosides oleuropein and verbascoside[18,92] in conjunction with ligstroside, demethyloleuropein[9] and the flavonoids luteolin-7-glycoside and rutin[66] have been isolated from olive pulp. Ferulic, homovanillic, *p*-hydroxybenzoic, protocatechuic and syringic acids have also been isolated from virgin olive oil.[91] Duran[90] has extended the number of known phenolic compounds in virgin olive oil and has characterised some of them according to their specified role in the oil. The apparent reduction in glycosidic and flavonoid compounds in olive oil compared to olive pulp may be attributed to glycosidic modification or degradation as a result of oil extraction, which may arise due to the addition of water to the olive paste. The relative contribution of partition phenomena to the reduction has not been examined. Nevertheless, it is likely to contribute significantly, particularly in the case of the more hydrophilic phenolics.

Oil production

Oil production commences with the grinding of the fruit to form olive paste, which is then used for oil extraction by, for example, centrifugation, pressure or percolation. The method of oil extraction has a significant effect[89] on the content of both total phenols and 1,2-diphenols. The various extraction systems differ in two important aspects, namely, the physical forces used to recover the oil, and the amount of water added to the olive paste during extraction. Oil extraction is more effective with olives of a lower water content[93] Furthermore, phenolic compounds, which are critical to the organoleptic quality of olive oil, are water soluble, and so addition of water to the olive paste effectively reduces the phenolic content and quality of the oil produced. This conclusion is supported by the findings of Di Giovacchino[94] who investigated the effect of the three different extraction systems on olive oil quality.Table 6 shows selected results from this investigation which found that the total phenol and *o*-diphenol content of oils obtained by pressing and percolation were significantly greater than that of the centrifugally extracted oils. Nevertheless, the organoleptic rating of oils obtained by the three processes was the same and hence the system of choice to ensure the highest quality oil remains a controversial issue. This can largely be attributed[94] to the natural variability in the chemical composition of olive fruits.

Table olives

The focus in the production of table olives is the reduction of the characteristic bitterness of olive fruits. This is achieved by lye treatment, which hydrolyses the phenolic glycoside, oleuropein[35], the main contributor to fruit bitterness. Brenes-Balbuena *et al*.[18] demonstrated that the concentration of tyrosol, *p*-coumaric acid and vanillic acid in an experimental set of olives remained constant throughout processing whereas the concentration of caffeic acid and hydroxytyrosol declined markedly after lye treatment. This behaviour was therefore attributed to differences in chemical structure and the fact that caffeic acid and hydroxytyrosol possess an *o*-diphenol group.

Table 6 Selected quality characteristics of olive oils obtained by pressing, percolation and centrifugation[94]

Determination	System	Average*	Minimum	Maximum
Total phenols (gallic acid, mg l^{-1})	Pressing	158 a	111	197
	Percolation	157 a	103	185
	Centrifugation	121 b	87	158
o-Diphenols (caffeic acid, mg l^{-1})	Pressing	100 a	66	154
	Percolation	99 a	62	149
	Centrifugation	61 b	32	92
Organoleptic rating	Pressing	6.9 a	6.2	7.4
	Percolation	7.0 a	6.7	7.4
	Centrifugation	7.0 a	6.7	7.2

* Different letters indicate significant differences at $P < 0.05$.

Analysis

The structural diversity of the phenolics and its effect on physicochemical behaviour, such as solubility and analyte recovery, presents a challenging analytical problem. Moreover, a number of phenolic compounds are easily hydrolysed and all are relatively easily oxidised which further complicates sample handling.[30,41]

Sample preparation

Sample preparation encompasses a series of steps ranging from exhaustive solvent extraction, filtration and concentration procedures to simple liquid–liquid extraction. Isolation of phenolic compounds from the sample matrix is a necessary prerequisite to any comprehensive analysis scheme, but it is a difficult task because the olives constitute a 'natural' matrix, and hence extreme care must be taken to ensure correct extraction, devoid of chemical modification, which will invariably result in artefacts.[15]

The precise procedure will depend on the nature of the sample (olives fruit or leaves, oil) and the desired class of phenols to be extracted.[15] This accounts for the different techniques employed by Amiot *et al*.[9] and Vlahov[44] who examined total phenols and flavonoids, respectively. Similarly, Montedoro and co-workers[30,37,41] have concentrated only on the simple and hydrolysable phenolic compounds present in virgin olive oil and hence have adopted an appropriate extraction procedure.

Extraction of phenolics from olive oils is generally achieved by dissolution of the oil in hexane, followed by liquid–liquid extraction using various mixtures of water and alcohol in order to isolate the desired analytes from unsaturated, interferring species.[32,40,95] Of the solvents examined, a methanol–water (80 + 20 v/v) mixture provided the highest recoveries of phenolics[41] measured as Folin-Ciocalteu total phenols. The addition of specific lipid solvents (hexane, light petroleum, chloroform) to the oils did not enhance the phenolic concentration of the extracts. Hexane provided best selectivity in clean-up of the methanolic extract prior to HPLC.[30,37,41]

More recently, the versatility of solid phase extraction (SPE) has been exploited[96] for the recovery of phenolics from olives. Suitable sorbents are alkylsilicas (C8 or C18)[36,38] and anion exchangers.[97] The oil sample was typically applied[36,98] to a preconditioned Sep Pak C18 cartridge, which was then washed with a hexane–ethoxyethane mixture to remove the non-polar fraction. Phenols were then eluted with methanol, filtered, evaporated to dryness and reconstituted in water for analysis by reversed-phase chromatography (RPC). Consistent recoveries over 95% were achieved from spiked samples in contrast to the variable results with solvent extraction.

The polar fraction of virgin olive oil obtained by extraction with aqueous methanol[45] was fractionated into two parts (A and B) by SPE. Analysis of the two fractions showed that part A (eluted from Sep Pak C18 with methanol–water, 20 + 80) contained only simple phenols and phenolic acids whereas part B (eluted with mixtures of methanol–chloroform) had a complex nature. The two parts tested for their antioxidant

activity showed relatively high protection factors in safflower oil although part B was found to contribute more than part A to the stability of the oil. This agrees with the findings of Montedoro *et al*.[30,41] The antioxidant activity of both fractions was related to their content of total phenols and *o*-diphenols although very little is known about the composition and nature of the Part B fraction. A tentative structure was assigned to one of the components in Part B using electron impact ionisation mass spectrometry EPI-MS. This paper demonstrates the potential of SPE in such studies. The versatility of SPE for pre-concentration in on-line methods, coupled for example to an HPLC has not been exploited.

Isolation of phenolic compounds from olive fruit is more exacting than that from olive oil. This can be attributed to the greater homogeneity and reduced enzyme content of the oil compared with the fruit. Hence, extraction of phenolics from olive fruit requires more sample handling, such as filtration to remove solid components, which therefore increases the chance of modification of the phenolics and the relative degree of error in the particular analysis. This, however, is unavoidable when dealing with natural samples. The extraction method employed by Amiot *et al*.[9] has been used with minor modifications in several investigations.[18,73] Amiot *et al*.[9,43] concentrated their efforts on the profiling of phenolic compounds as a function of physiological development. Sample preparation entailed freeze drying and powdering the olives with the aid of liquid nitrogen. The powder was extracted twice with 80% ethanol in the presence of metabisulfite (2%) and concentrated under vacuum. Four successive light petroleum extractions of the ethanolic extract were then performed to ensure lipid and pigment removal, followed by three successive ethyl acetate washes in the presence of ammonium sulfite (20%), metaphosphoric acids (2%) and methanol (20%). The final extracts were then evaporated, and the residue dissolved in methanol for subsequent HPLC analysis. As an alternative to liquid nitrogen, the ethanol extraction step has been performed[35] at −30 °C or following freeze drying.[25] Nevertheless, the inclusion of an aqueous alcohol extraction and liquid–liquid fractionation typically involving ethyl acetate was universally adopted. Vlahov[44] adopted a simpler approach for flavonoid analysis in which olive pulp was subjected to three successive methanol–water (80 + 20 v/v) extractions. The combined extracts were evaporated to dryness, reconstituted in glacial acetic acid–water (5 + 95 v/v) followed by centrifugation, filtration and finally HPLC analysis.

The paper by Vàzquez Roncero *et al*.[99] although somewhat dated is notable for the extensive nature of the work. In this study,[99] the main phenolic compounds in olive pulp were identified after extraction using acetone and methanol, and fractionation with bidimensional paper chromatography (PC). Characterisation was achieved by spectrophotometric and chromatographic methods, primarily thin-layer chromatography (TLC), using an extensive range of solvents and colorimetric reagents to distinguish between different classes of phenolics. Commonly used methods of that era involving precipitation of phenolic compounds with lead acetate, along with the use of sodium hydrogencarbonate, sodium carbonate and sodium hydroxide were avoided[99] due to presumed phenolic modification and the occurrence of artefacts.

Extraction procedures have generally not been subjected to rigorous quality checks and warrant closer examination to eliminate the possibility of qualitative and quantitative changes induced by the recovery procedure.

Quantification

Traditional methods for the determination of the phenolic component relied on measurement of total phenols or, in some instances, 1,2-diphenols[89] because of their association with browning reactions. The usual approach[100,101] is slow and tedious typically requiring 1 h per analysis. It involves a liquid–liquid extraction of the analytes from either the olives or, more usually, the oil into an aqueous alcohol mixture intended to isolate them from unsaturated interferents. The extraction time is an important consideration as longer times increase the possibility of oxidation of phenolic compounds unless reducing agents are added to the solvent system. An aliquot of the aqueous phase is mixed with one of a number of reagents of varying selectivity. Folin-Ciocalteu reagent is the classic reagent recommended for total phenols. An aliquot of the aqueous extract of the oil is reacted with Folin-Ciocalteau reagent in sodium carbonate solution and the blue colour formed after 15–60 min is measured[100] at 725 nm. Results are expressed in terms of molar equivalents of a commonly occurring phenolic, for example, gallic acid. Folin-Ciocalteu reagent is widely used but is not specific and detects all phenolic groups in the sample extract including those found in the extractable proteins. A further disadvantage is the interference of reducing substances, such as ascorbic acid. The concentration of 1,2-diphenols, on the other hand, is determined[101,102] with molybdate by measurement at 350 nm.

The problem of lengthy analysis times has been overcome by the application of flow injection procedures,[95] which facilitate rapid analysis with high sample throughput. Similarly, Wang *et al*.[103] have developed a rapid procedure for determining total phenols in olive oil based on an organic-phase enzyme electrode. The method uses continuous liquid–liquid extraction to obviate the need for sample extraction and to facilitate high speed flow injection determinations of phenols in olive oil. The procedure developed by Cañizares *et al*.[104] for the determination of phenols in oil used on-line coupling of a liquid–liquid extraction flow reversal system to a spectrophotometric flow-through sensor. There are several difficulties associated with direct spectrophotometric measurement whether in flow injection or batch mode. 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 phenolics predominates.

All phenols absorb radiation in the ultraviolet (Table 7) and this provides the basis for an alternative measurement of total phenols. The limited use of direct spectrophotometric measurements whether in the ultraviolet or visible region can be attributed in part to the lack of specificity of such methods. In general, they lead to an overestimation of 'phenolic' content. Specificity can be enhanced in direct spectrophotometric methods by derivative spectrometry. For instance, measurement based on the second derivative of the absorbance at 278 nm provided[105] a rapid, direct method for determination of total

Table 7 Spectral properties of various phenolic compounds (λ_{max}) in methanol, except for anthocyanic pigments where the solvent was methanolic HCl 0.01% (data from ref. 15, p. 14).

Class of compounds	UV band B	UV band A	Visible
Benzoic acids	270–280		
Hydroxycinnamic acids	(290–300)*	305–330	
Anthocyanic pigments	270–280	(315–325)†	500–550
Flavonols	250–270	(300)* 350–380	
Flavan-3-ols	270–280		
Coumarins	220–230	310–350	
Flavones	250–270	330–350	
Flavanones, flavanonols	270–295	(300–330)*	
Chalcones	220–270	(300–320)* 340–390	
Aurones	240–270		370–340
Isoflavones	245–270	300–340	

* Shoulder. † In the case of acylation by hydroxycinnamic acids.

phenols. Catechol was the most appropriate reference standard.

Chromatographic methods

The need for profiling and identifying individual phenolic compounds has seen traditional methods based on colorimetry replaced by chromatographic analyses. Bate-Smith[106] pioneered the identification of plant phenolics through the application of PC,[107] and in the 1950s and 1960s, many paper chromatographic methods were developed for such purposes. PC was subsequently superseded by TLC. The usual advantages of TLC, namely speed and an open-bed technique are realised in phenolic analyses. Similarly, the versatility of the technique is evidenced in the extensive array of solvent systems available which can be exploited for specific class analyses. Detection of phenolics may be achieved by viewing the chromatogram under UV light both before and after exposure to ammonia fumes, which often changes the colour[107] of their fluorescence. Many phenolic compounds also give characteristic colours when treated with diazotised *p*-nitroaniline, sulfanilic acid, *p*-toluenesulfonic acid plus vanillin and heating[30] or iron(III) chloride.[108]

Contrary to the relative ease and adaptability of TLC, the use of this technique for phenolic characterisation in olive matrices is limited. Ragazzi and Veronese[109] have developed a method for phenolic quantification using UV spectrophotometry after analyte separation using either silica gel or cellulose thin-layers. Quantification was achieved using Folin-Ciocalteau reagent. The main phenols with the exception of oleuropein occurring in olive vegetation water, namely catechol, 4-methylcatechol, tyrosol and hydroxytyrosol have been detected[108] by reversed-phase TLC (RP-TLC) whereas high-performance TLC on silica layers (Si-HPTLC) yielded the detection of only tyrosol and hydroxytyrosol. Confirmation of the identities of the phenolic compounds was obtained by RP-TLC and Si-HPTLC analysis of the acetylated organic extracts of the water using the more stable acetyl derivatives of the phenols as standards. The flavonoid content of olive leaves was studied[21] by TLC on silica gel and SIL layers and recording UV spectra of the isolated spots directly on the layer. SIL is a hydrophobic layer and a reversed-phase mechanism operates while silica gel gives normal phase behaviour. From comparison of the chromatographic behaviour and UV spectra three flavonoid glycosides (quercitrin, rutin and luteolin-7-glycoside), one flavonoid (aglycone), luteolin, and chlorogenic acid were identified in the leaves. Flavonoid glycosides were distinguished from the flavonoid aglycone by two-dimensional TLC. Montedoro *et al.*[30,41] have used silica gel TLC for the preliminary isolation of phenolic compounds from virgin olive oil. Four different mobile phases were used covering a range of polarities and selectivities. After separation the phenolics were extracted from the silica layers for analysis by HPLC and UV spectroscopy.

The limited application of gas chromatography to the separation of olive phenolics (see Table 8) can be attributed[114] to their polarity and limited volatility. Hence, a derivatisation step is usually mandatory, thermal decomposition may occur during their elution and the higher molecular mass phenolics cannot be chromatographed. Nevertheless, the excellent resolving power and detection capabilities of GC warrant consideration. Indeed, many useful separations have been achieved and this has been facilitated by the availability of inert open tubular columns. This is seen in the elution of phenolic acids[110] from olive leaves and roots using a 30 m SPB-1 column programmed from 138 to 150 °C. Acid hydrolysis of the plant tissues was employed to obtain free phenolic acids from conjugated forms such as the O-glycosides. The free phenolic acids were recovered from the leaves and roots by extraction with ethyl acetate and converted to the corresponding trimethylsilyl derivatives prior to GC. Experimental conditions were chosen to eliminate interference by sugars and flavonoids. The most abundant phenolic acids were salicylic, cinnamic, *o*-coumaric and ferulic acids. Qualitative differences in the distribution of the phenolic acids were demonstrated between cultivars and within different tissues of the same cultivar notably for shikimic and syringic acids.

GC–MS is now well established as a routine technique carried out with either electron impact ionisation (EI) or chemical ionisation (CI) sources, since these are appropriate for the introduction of volatile compounds. However, because of limited volatility, analysis of phenolic compounds and their glycosides, in particular, by GC and thus GC-MS has not generally found favour. Nevertheless, Angerosa *et al.*[92] have shown GC–MS to be an effective tool for phenol identification after extraction from olive oil with methanol and derivatisation with bis(trimethylsilyl)trifluoracetamide. Peaks in the mass spectra at *m/z* 192 or at *m/z* 280, related only to tyrosol and hydroxytyrosol, were attributed to a McLafferty rearrangement of linked phenols, and were useful for assigning the phenolic nature to minor components. Proposed structures for these linked phenolic compounds and their hypothesised interconversion are shown in Fig. 3. In a later paper, the advantages of chemical ionisation with ammonia for providing molecular masses of the aglycones from the glycosides, ligstroside, decarbomethoxyoleuropein and oleuropein were demonstrated.[111] The phenolic components of wine have been extracted and separated[115] as trimethylsilyl derivatives on a DB-5HT capillary column using MS detection with one target and two qualifying ions for each compound in a total run time of 26 min. Resolution of all 15 phenolic compounds was excellent and the method should be appropriate for phenolics in olives following suitable extraction.

Liquid chromatography. Phenolic extracts from olive oil have been fractionated by classical low-pressure column chromatography on, for example, Sephadex LH20.[30] Alternatively, preliminary fractionation can be achieved on ion exchange columns as demonstrated[116] for carboxylic acid phenolics and non-carboxylic acid phenolics of maize. The poor efficiency of such separations has favoured development of RPC, which currently represents the most popular and reliable technique for phenolic analysis. Compound elution is typical of RPC, that is, polar compounds (*e.g.*, phenolic acids) elute first, followed by those of decreasing polarity. Thus, the typical elution pattern[19,35,113,117] is hydroxytyrosol < tyrosol < vanillic acid, caffeic acid < *p*-coumaric acid < elenolic acid < verbascoside < rutin < luteolin-7-glucoside < oleuropein < ligstroside. In one of the early reports[117] on the RPC of phenolic compounds, different mixtures of acetic acid, water and methanol were used to separate members of several classes of phenols, and the effects of organic modifier on selectivity were deduced. Since then, numerous mobile phases have been employed (Table 8) with different modifiers (usually methanol, acetonitrile or tetrahydrofuran), acids (acetic or formic acid) and/or salts (ammonium phosphate).

Gradient elution has usually been mandatory in recognition of the complexity of the phenolic profile although isocratic elution has been successful for particular applications.[38] In some instances, the success of isocratic elution can be attributed to selectivity effects of one or more components (*e.g.*, acetonitrile) of the mobile phase.[38] The most popular stationary phases have involved C18[32,40] chemistry. In a typical application, phenolics were recovered from olive fruit[35] by extraction in the presence of metabisulfite. After suitable clean-up, the extract was chromatographed on a Spherisorb ODS-2 column using gradient elution with acetonitrile–water (containing phosphoric acid). Eluted species were identified from their retention times and absorption spectra in the 280–380 nm range.

Table 8 Conditions used for the analysis of phenolic compounds in olives

Sample	Method	Column	Mobile phase	Detection	Comment	Ref.
Fruit	Counter-current chromatography	300 columns; 400 × 2 mm	Chloroform, methanol, water	Fraction collection	Isolation of oleuropein and ligstroside	34
Fruit	RPC	300 × 4 mm Micropak MCH-5	Gradient; acetonitrile, water, phosphoric acid	280 nm, 340 nm	Oleuropein; verbascoside, rutin, luteolin-7-glucoside	9
Fruit	RPC	300 × 4 mm Micropak MCH-5	Gradient; acetonitrile, water, phosphoric acid	280 nm, 340 nm	Effect of black maturation on oleuropein, demethyloleuropein and elenolic acid glucoside	43
Oil	RPC	Reverse phase (no further details provided)	Ternary gradient	280 nm	Effect of ripening on formation of tyrosol and hydroxytyrosol by hydrolysis of higher molecular mass phenols	42
Leaves	TLC	Silica gel; SIL C18-50	Various	Densitometer	Determination of flavonoids and flavonoid glycosides	21
Oil	RPC	150 × 4.6 mm C18	Gradient; 2% acetic acid in water, methanol	239 nm, 278 nm	Elenolic acid; phenolic acids	41
Oil	TLC	Silica gel	Various	Various spray reagents 239 nm, 278 nm	Characterisation of hydrolysable phenolic fraction of oil (oleuropein aglycone, elenolic acid)	30
	RPC	150 × 4.6 mm Erbasil C18	Gradient; 2% acetic acid in water, methanol			
Oil	RPC	250 × 4.6 mm Spherisorb ODS2	Gradient: acetic acid, methanol, water	280 nm	Effect of hydroxytyrosol and tyrosol on stability of oil	100
Oil	RPC	250 × 4.6 mm Spherisorb ODS2	Gradient: methanol, water, acetic acid	280 nm	Use of UV detection	40
Fruit	RPC	250 × 4.0 mm Spherisorb ODS2	Gradient: water, acetonitrile, phosphoric acid	DAD	Effect of lye treatment on hydroxytyrosol, verbascoside, tyrosol, vanillic acid, *p*-coumaric acid, oleuropein	18
Leaves	RPC	250 × 4.6 mm Ultrasphere ODS	Gradient; acetonitrile, water, tetrahydrofuran, phosphoric acid	280 nm, 340 nm	Antioxidant activities of oleuropein and flavonoids	33
Fruit	RPC	100 × 4.6 mm Microspher C18	Gradient; methanol, water, acetic acid	520 nm	Effect of maturation on flavonoid content	44
Oil	RPC	250 × 4.6 mm μ Bondapak C18	Isocratic; acetonitrile, water, acetic acid	Amperometric	Use of electrochemical detection	38
Oil	RPC	500 × 9.4 mm Partisil 10 ODS2	Gradient; water, methanol, acetic acid	278 nm	Isolation of four new phenolic compounds	37
Fruit	RPC	250 × 4 mm Spherisorb ODS2	Gradient; water, acetonitrile, phosphoric acid	280 nm	Effect of cultivar and processing on levels of oleuropein, verbascoside, and luteolin-7-glucoside; hydroxytyrosol increased due to hydrolysis of major phenolic compounds in brines	19
Leaves, roots	GC	30 m × 0.32 mm SPB-1	Helium	FID	Phenolic acid composition	110
Oil	GC–MS	25 m × 0.32 mm SE-54	Helium	MS	Phenol identification	92
Oil	RPC	250 × 4.6 mm Spherical Resolve C18	Gradient; water, methanol, acetic acid	Amperometric	Use of amperometric detection	36
Oil	RPC	250 × 4.0 mm Lichrosorb RP18	Gradient; water, acetic acid, methanol, acetonitrile	280 nm	Effect of anti-Dacus treatment on hydroxytyrosol, tyrosol, vanillic acid and *p*-coumaric acid	32
Fruit	RPC	250 × 4 mm Spherisorb ODS2	Gradient; water, acetonitrile, phosphoric acid	280 nm	Changes in phenolic compounds during olive processing	35
Fruit	RPC	250 × 4 mm Lichrospher RP18	Gradient; acetic acid, methanol	DAD	Effect of altitude on phenolic acid content of olives	17
Oil	GC–MS	30 m × 0.25 mm DB5	Helium	CI–MS	Characterisation of phenolic compounds	111
Oil	RPC	250 × 4.6 mm Spherisorb ODS	Gradient; methanol, water	280 nm	Antioxidant activity of various fractions; some mass spectral data	45

continued next page

Table 8 *Continued*—

Leaves	LC–API–MS	300 × 4.6 mm C18	Gradient; acetonitrile, water, formic acid	API-MS	Characterisation of phenolic glucosides	46
Oil	RPC	250 × 4.0 mm Lichrosorb C18	Gradient; water, acetic acid, methanol, acetonitrile	DAD	Chemometric analysis	31
Water	LC–APCI–MS	150 × 2 mm Supelcosil LC-18	Gradient; methanol, water, formic acid	280 nm; APCI–MS	APCI mass spectra	112
Oil	RPC	Various including ODS-1, ODS-2, phenyl and C-8	Stepwise gradient; sulfuric acid, acetonitrile	225 nm	Low wavelength detection	113

The limited availability of suitable reference standards is a problem which has been overcome, in part, by synthesis[92,111,113] of the relevant compounds. Vlahov[44] has reported the only detailed examination of the change in flavonoids during olive fruit maturation. Based on RPC with detection at 520 nm (anthocyanins) or 350 nm (flavones and flavonols), different olive varieties were characterised by their flavonoid profiles.

Numerous papers demonstrate the power of RPC for analysis of the phenolic fraction of olives. For example, with RPC and NMR Montedoro *et al.*[30,37,41] separated and identified some aglycone derivatives present as the dialdehydic forms of elenolic acid linked to both hydroxytyrosol and tyrosol in the olive oil. In an atypical study[113] involving low wavelength detection at 225 nm, phenolics were separated by RPC using a stepwise gradient of sulfuric acid and acetonitrile. The response of a range of simple and complex phenols was 3–14 fold higher at 225 nm than at the more usual detection wavelength of 280 nm. Apart from tyrosol, hydroxytyrosol, oleuropein aglycone and elenolic acid, dialdehydic derivatives of oleuropein and ligstroside were identified in the chromatograms. Further signals of unknown, but possibly phenolic substances, were also detected at the lower wavelength. Data for one of these peaks were consistent with the elution of an oleuropein derivative previously assigned[118] as deacetoxyoleuropein aglycone. Nevertheless, problems associated with high background absorption of typical mobile phases in RPC have limited the use of low wavelength detection. This study is also interesting for its use of SPE on C8 cartridges for recovery of phenolics and for the systematic investigation of stationary phases for the analytical separation. Phases examined for this purpose were ODS2, ODS1, C8 and phenyl phases and whilst all showed similar retention behaviour the best separation was achieved on ODS2 columns.

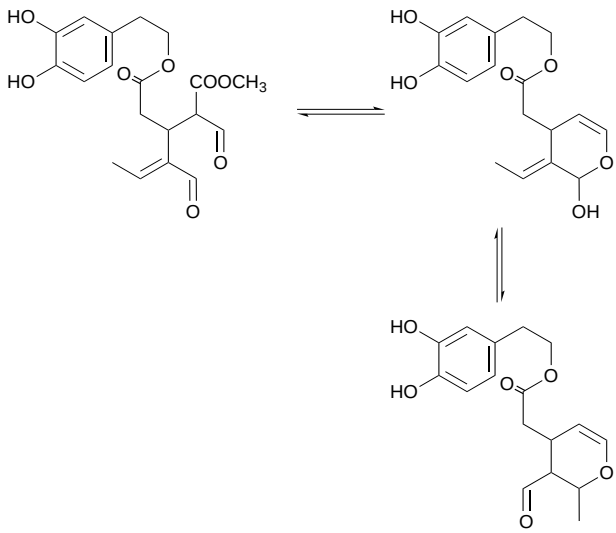

Fig. 3 Chemical structure of linked phenolic compounds and their hypothesised interconversion.[92]

Phenolics were extracted[31] from oil samples obtained from olives that had reached different degrees of ripeness and that had been affected by *Dacus oleae* infestation differently. RPC of the extracts using a quaternary mobile phase showed 23 significant peaks in the chromatograms. Such data are ideally suited to chemometric analysis and partial least squares regression produced models that showed a significant correlation between the phenolic composition of the oil and conditions of the olives sampled. In particular, the first principal component reflected the *o*-diphenol content of the oil and was directly linked with the state of health of the olives. Moreover, prediction of the shelf life of the oil was possible.

Detection. Detection in RPC is typically based on measurement of UV absorption. No single wavelength is ideal for all classes of phenolics since they display absorbance maxima at distinctly different wavelengths (Table 7). Indeed, there are significant differences in absorption maxima and molar absorptivities[40] of even the major phenolics identified in olives. This creates problems in quantification as discussed by Tsimidou *et al.*[40] who classified the various phenolics into four groups and used a single calibration standard for the members of each group. The results suggest that in cases of unidentified phenols, it is preferable to report data as peak areas rather than to assign concentrations[42] using an arbitrary reference. On the other hand, these different spectral characteristics can be exploited (Fig. 4) to provide useful qualitative information about an eluted species and in the ideal case enable selective detection. The most commonly used wavelength has been 280 nm which represents a suitable compromise,[32,40,116] although detection at other wavelengths including 340 nm[9] has been applied. The choice of detection wavelength will invariably depend on the desired class of phenolics to be investigated. Hence, the absorption maximum around 340–350 nm has been used,[33,44] for example, for flavonoid analysis whereas elenolic acid glucoside from the hydrolysis of oleuropein was detected[35] at 240 nm. Dual wavelength measurement at 278 and 239 nm provided some interesting differences in the resulting chromatograms as might be anticipated.[41] Elenolic acid was identified in the samples from additional information in the chromatogram at 239 nm. Such measurements are conveniently performed with a photodiode array detector. The extensive use of photodiode array detection can be attributed to the ability to collect on-line spectra without using stop–flow techniques. The UV spectra of phenolic compounds are particularly informative (Table 7) providing considerable structural information. 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. Nevertheless, in most instances, diode array detection (DAD) has been employed for fixed wavelength or, at most, dual wavelength detection. Hence, the outstanding capabilities of this mode of detection have not been realised.

RPC using amperometric detection has also been used successfully[36,98] for the quantitative determination of phenolic

compounds in virgin olive oils. The detector system employed a dual electrode detector in the parallel configuration, operating at +0.5 and 1.0 V *vs.* Ag/AgCl. The voltammetric behaviour was useful in assigning peak identity. For example, *o*- and *p*-diphenols were easily recognised by their facile oxidation to *o*- and *p*-quinones, respectively, in the range from +0.5 to +0.6 V. On the reverse scan, a cathodic wave due to the reduction of the quinone species formed was also observed for compounds having the catechol moiety. The authors assigned an antioxidant activity to the phenolic species in olive oil based on their measured oxidation potential. Other investigators have conducted studies using cyclic voltammetry to optimise amperometric detection conditions[38] and to provide selective as well as sensitive detection of the major phenolic compounds in olive oil.

A number of techniques which have the potential to improve sensitivity and/or selectivity of phenolic analyses appear to have been ignored. Thus, post-column derivatisation offers a number of advantages including enhanced selectivity. Fluorescence detection is an obvious means of improving both sensitivity and selectivity. It is interesting that one of the earliest papers on HPLC of phenolic compounds[119] employed this means of detection as an adjunct to conventional UV detection. Stop–flow scans were used to obtain excitation and emission spectra of the eluted species. This early paper demonstrated the complementary nature of the two methods of detection. The limited stability and light sensitivity of several phenolics were noted and should serve as a warning.

The on-line coupling of RPC and MS is of enormous potential because the selectivity can then be tuned in an optimal way. Classical mass spectrometric gas phase ionisation techniques, such as EI and CI, are generally less suitable for polar, non-volatile compounds such as the phenolics. The power of atmospheric pressure ionisation (API) methods, such as electrospray (ES), as alternative, highly sensitive soft ionisation techniques for investigation of polar, non-volatile and thermolabile molecules has been demonstrated (Fig. 5). API procedures overcome the lack of analyte volatility by direct formation or emission of ions from the surface of a condensed phase. Hence, they eliminate the need for neutral molecule volatilisation prior to ionisation and generally minimise thermal degradation of the molecular species. Atmospheric pressure chemical ionisation (APCI) is a development of ES in which a combination of a heated capillary and a corona discharge is used to promote the formation of ions from the nebulised sample. As the name implies, APCI involves gas phase ion–molecule reactions which cause the chemical ionisation of analyte molecules under atmospheric pressure conditions. Aramendía *et al.*[112] reported the LC–APCI-MS of phenolics in olive mill wastewater. Analytes were separated on a C18 phase by gradient elution with methanol–water containing formic acid. Mass spectral conditions were optimised by direct infusion of standards in flow injection mode into the APCI source. The study was restricted to negative-ion mode with detection limits in total ion current mode ranging from 0.5 to 500 ng. These detection limits were about 20 times better when working in selected ion monitoring mode and monitoring the $[M–H]^-$ ion. Mass spectra were recorded with soft (−15 V) and strong (−50 V) voltages applied at the ion source of the mass spectrometer. With the smaller voltages, deprotonated molecular species $[M–H]^-$ were the major ions observed in the mass spectra with the appearance of very few fragment ions which

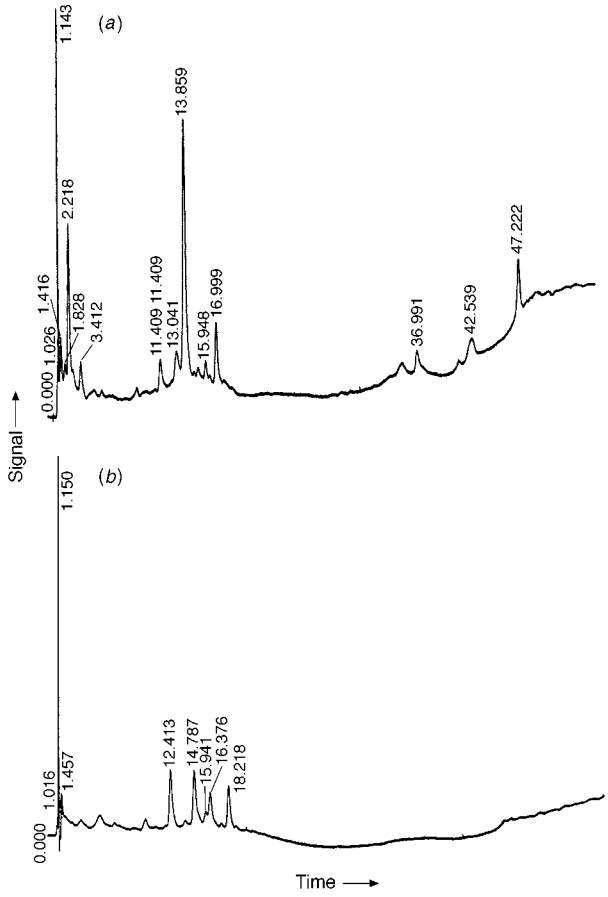

Fig. 4 Chromatograms showing the effect of detection wavelength in RPC. Phenolics were extracted from freeze-dried green olives by SPE and separated on a Varian C18 column (150 mm) using gradient elution. Detection wavelength (a) 280 nm or (b) 340 nm.

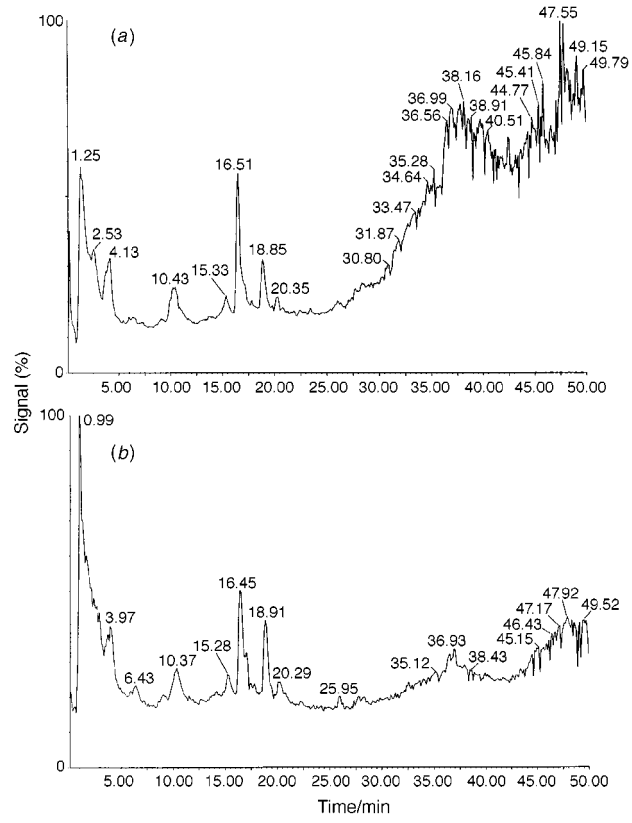

Fig. 5 Total ion chromatograms in (a) positive- and (b) negative-ion mode obtained by ESI-LC–MS using gradient elution and RPC. The peak eluting at 16.5 min is confirmed as oleuropein. Samples were obtained as for Fig. 4.

were all of low intensity. The presence of substantial fragmentation from collisionally induced dissociation processes, which became evident on increasing the voltage applied at the source (extraction and cone) voltages, gave structural information about the molecules.

Structures were assigned to major eluent cluster ions from methanol–water–formic acid mixtures occurring at *m/z* 91, 113, 137, 159, 181 and 183. Ionspray (or pneumatically assisted electrospray) has been applied[46] to the identification of the phenolic glucoside content of olive leaves following extraction with methanol and partitioning in acetonitrile–hexane. Structural data on oleuropein and ligstroside were obtained from the positive-ion spectra. Moreover, the presence of a disaccharide containing the hydroxytyrosol moiety was confirmed.

The bioactivity of the phenolics (P) is exerted by supramolecular formation, for example, between the phenol and sensorial receptors (SR) on the tongue or globular and proline-rich mucoproteins (MP) or other food components (FC). The specific interaction among P, SR, MP and FC may involve absorption and desorption equilibria with formation of charge transfer host–guest aggregates. FAB-MS has been used[120] to study such supramolecular formations between hydroxytyrosol and caffeine or the dipeptide, Asp–Phe, as protein models. The data demonstrated a preferential molecular recognition site provided by caffeine, the biomimetic model of proline-rich mucoproteins.

The complexity of the biochemical processes controlling phenolic metabolism have been shown[39] in a ^{1}H NMR study of phenolics in three cultivars and their changes with fruit development. The major phenolic compounds, common to all three cultivars, were identified from chemical shifts, peak multiplicities and scalar correlations in two-dimensional and selective excitation experiments on aqueous extracts (olive vegetation water) as tyrosol, 4-hydroxyphenylethanol glucoside, and oleuropein. Considerable differences in the content of these compounds occurred in the fruits during growth and maturation of the drupe. In contrast, the glucoside, cornoside was detected in only two of the cultivars. Possible metabolic pathways leading to cornoside and halleridone (not a phenol) were discussed. Of most interest, is the discrepancy noted by Limiroli *et al.*[39] between the limited number of substances found in the olive vegetation waters and the large number of phenolic compounds cited in the literature as occurring in olives.

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Paper 7/08920H
Received December 11, 1997
Accepted January 27, 1998