

Review

Phenolic Molecules in Virgin Olive Oils: a Survey of Their Sensory Properties, Health Effects, Antioxidant Activity and Analytical Methods. An Overview of the Last Decade

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Abstract: Among vegetable oils, virgin olive oil (VOO) has nutritional and sensory characteristics that make it unique and a basic component of the Mediterranean diet. The importance of VOO is mainly attributed both to its high content of oleic acid a balanced contribution quantity of polyunsaturated fatty acids and its richness in phenolic compounds, which act as natural antioxidants and may contribute to the prevention of several human diseases. The polar phenolic compounds of VOO belong to different classes: phenolic acids, phenyl ethyl alcohols, hydroxy-isochromans, flavonoids, lignans and secoiridoids. This latter family of compounds is characteristic of Oleaceae plants and secoiridoids are the main compounds of the phenolic fraction. Many agronomical and technological factors can affect the presence of phenols in VOO. Its shelf life is higher than other vegetable oils, mainly due to the presence of phenolic molecules having a catechol group, such as hydroxytyrosol and its secoiridoid derivatives. Several assays have been used to establish the antioxidant activity of these isolated phenolic compounds. Typical sensory gustative properties of VOO, such as bitterness and pungency, have been attributed to secoiridoid molecules. Considering the importance of the phenolic fraction of VOO,

high performance analytical methods have been developed to characterize its complex phenolic pattern. The aim of this review is to realize a survey on phenolic compounds of virgin olive oils bearing in mind their chemical-analytical, healthy and sensory aspects. In particular, starting from the basic studies, the results of researches developed in the last ten years will be focused.

Keywords: Phenols; Virgin olive oil; Sensory properties; Antioxidant activity; Analytical techniques.

Phenolic molecules in virgin olive oil

Oleuropein belongs to a specific group of coumarin-like compounds, the secoiridoids, which are abundant in *Oleaceae*. Secoiridoids are compounds that are usually glycosidically bound and produced from the secondary metabolism of terpenes. The secoiridoids, found only in plants belonging to the family of *Oleaceae* that includes *Olea europaea* L., are characterised by the presence of elenolic acid in its glucosidic or aglyconic form, in their molecular structure. In particular, they are formed from a phenyl ethyl alcohol (hydroxytyrosol and tyrosol), elenolic acid and, eventually, a glucosidic residue. Oleuropein is an ester of hydroxytyrosol (3,4-DHPEA) and the elenolic acid (EA) glucoside (oleosidic skeleton common to the secoiridoid glucosides of *Oleaceae*) [1-5]. Secoiridoids of VOO in aglyconic forms arise from glycosides in olive fruits by hydrolysis of endogenous β -glucosidases during crushing and malaxation. These newly formed substances, having amphiphilic characteristics, are partitioned between the oily layer and the vegetation water, and are more concentrated in the latter fraction because of their polar functional groups. During storage of VOO hydrolytic mechanisms that lead to release of simple phenols, such as hydroxytyrosol and tyrosol, from complex phenols as secoiridoids may be involved [6-8]. The most abundant secoiridoids of VOO, identified for the first time by Montedoro *et al.* [1-3, 9] and confirmed also by other authors [10-13], are the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol (*p*-HPEA) respectively termed 3,4-DHPEA-EDA and *p*-HPEA-EDA, and an isomer of the oleuropein aglycon (3,4-DHPEA-EA) (Table 1). In 1999 another hydroxytyrosol derivative, hydroxytyrosol acetate (3,4-DHPEA-AC) was found in virgin olive oil [14].

Phenolic acids are secondary aromatic plant metabolites that are widely spread throughout the plant kingdom [15-17]. These naturally occurring phenolic acids contains two distinguishing constitutive carbon frameworks, namely the hydroxycinnamic and hydroxybenzoic structures. Elucidation of their roles in plant life is only one of the many ongoing investigations regarding phenolic acids: one vast area of interest lies in food quality [18-20]. Phenolic acids have been associated with color and sensory qualities, as well as with the health-related and antioxidant properties of foods [21-22]. One impetus for analytical investigations has been the role of phenolics in the organoleptic properties (flavor, astringency, and hardness) of foods [23-24]. Additionally, the content and profile of phenolic acids, their effect on fruit maturation, prevention of enzymatic browning, and their roles as food preservatives has been evaluated [25]. Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruit and vegetables, against diseases that may be related to oxidative damage (coronary heart disease, stroke, and cancers) [26-28].

In particular, several phenolic acids such as gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*- and *o*-coumaric, ferulic and cinnamic acid have been identified and quantified in VOO (in quantities lower than 1 mg of analyte kg⁻¹ of olive oil). In this regard two research groups have extensively analyzed samples of VOO for these types of compounds [29-32]. In one of these mentioned articles, for instance, the authors found that *trans*-cinnamic acid, sinapinic acid, caffeic acid and 3,4-dihydroxyphenylacetic acid were present in several monovarietal VOO of the six Spanish olive cultivars analyzed [31]; therefore, these compounds might be potential markers of geographical origin or the olive fruit variety.

(+)-Pinoresinol is a common component of the lignan fraction of several plants such as *Forsythia* species [33] and *Sesamum indicum* seeds, whereas (+)-1-acetoxypinoresinol and (+)-1-hydroxypinoresinol and their respective glucosides have been detected in the bark of the olive tree (*Olea europaea* L.). According to Owen *et al.* [34], the quantity of lignans in VOO may be up to 100 mg kg⁻¹, but as with the simple phenols and SIDs, considerable inter-oil variation exists. As suggested by Brenes *et al.* [35], the amount of lignans may be used as varietal marker, and they reported a method to authenticate VOO produced by Picual olives based on the very low content of the lignan (+)-1-acetoxypinoresinol in these oils.

A few years ago, Bianco *et al.* [36] investigated the presence of hydroxy-isochromans in VOO. In fact, during the malaxation step of VOO extraction, hydrolytic processes through the activity of glycosidases and esterases augment the quantity of hydroxytyrosol and carbonylic compounds, thus favouring the presence of all compounds necessary for the formation of isochroman derivatives. Two hydroxy-isochromans, formed by the reaction between hydroxytyrosol and benzaldehyde or vanillin, have been identified by HPLC-MS/MS technique and quantified in commercial VOOs.

Flavonoids are widespread secondary plant metabolites. During the past decade, an increasing number of publications on the health beneficial effects of flavonoids have appeared, such those related to cancer and coronary heart diseases [37-40]. Flavonoids are largely planar molecules and their structural variation comes in part from the pattern of modification by hydroxylation, methoxylation, prenylation, or glycosylation. Flavonoid aglycones are subdivided into flavones, flavonols, flavanones, and flavanols depending upon the presence of a carbonyl carbon at C-4, an OH group at C-3, a saturated single bond between C-2 and C-3, and a combination of no carbonyl at C-4 with an OH group at C-3, respectively. Several authors have reported that flavonoids such as luteolin and apigenin are also phenolic components of VOO [41-46]. Luteolin may originate from rutin or luteolin-7-glucoside, and apigenin from apigenin glucosides. There are also several interesting studies in which several flavonoids have been found in olive leaves and fruits [47-50].

Table 1. Phenolic compounds in virgin olive oil: compounds name, general chemical structure and molecular weight.

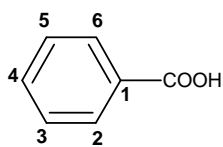
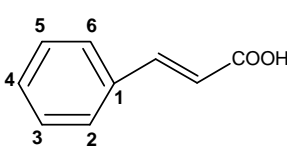
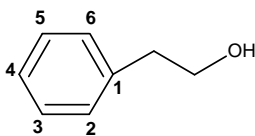
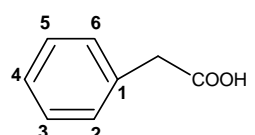
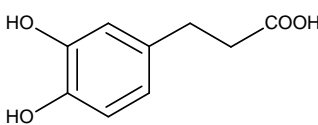
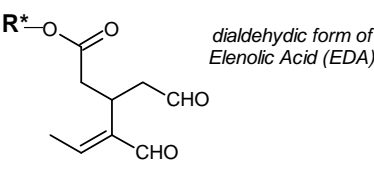
Compound	Substituent (MW)	Structure
Benzoic and derivatives acids		
3-Hydroxybenzoic acid	3-OH (138)	
<i>p</i> -Hydroxybenzoic acid	4-OH (138)	
3,4-Dihydroxybenzoic acid	3,4-OH (154)	
Gentisic acid	2,5-OH (154)	
Vanillic acid	3-OCH ₃ , 4-OH (168)	
Gallic acid	3,4,5-OH (170)	
Syringic acid	3,5-OCH ₃ , 4-OH (198)	
Cinnamic acids and derivatives		
<i>o</i> -Coumaric acid	2-OH (164)	
<i>p</i> -Coumaric acid	4-OH (164)	
Caffeic Acid	3,4-OH (180)	
Ferulic Acid	3-OCH ₃ , 4-OH (194)	
Sinapinic Acid	3,5-OCH ₃ , 4-OH (224)	
Phenyl ethyl alcohols		
Tyrosol [(<i>p</i> -hydroxyphenyl)ethanol] or <i>p</i> -HPEA	4-OH (138)	
Hydroxytyrosol [(3,4-dihydroxyphenyl)ethanol] or 3,4-DHPEA	3,4-OH (154)	
Other phenolic acids and derivatives		
<i>p</i> -Hydroxyphenylacetic acid	4-OH (152)	
3,4-Dihydroxyphenylacetic acid	3,4-OH (168)	
4-Hydroxy-3-methoxyphenylacetic acid	3-OCH ₃ , 4-OH (182)	
3-(3,4-Dihydroxyphenyl)propanoic acid	(182)	
Dialdehydic forms of secoiridoids		
Decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA)	R ₁ -OH (304)	 <p style="text-align: right;"><i>dialdehydic form of Elenolic Acid (EDA)</i></p>
Decarboxymethyl ligstroside aglycon (<i>p</i> -HPEA-EDA)	R ₁ -H (320)	

Table 1. Cont.

Compound	Substituent (MW)	Structure
Secoiridoid Aglycons		
Oleuropein aglycon or 3,4-DHPEA-EA	R ₁ -OH (378)	<p style="text-align: center;">Structure</p> <p style="text-align: center;">Elenolic Acid (EA)</p> <p style="text-align: center;">aldehydic form of Elenolic Acid (EA)</p>
Ligstroside aglycon or <i>p</i> -HPEA-EA	R ₁ -H (362)	
Aldehydic form of oleuropein aglycon	R ₁ -OH (378)	
Aldehydic form ligstroside aglycon	R ₁ -H (362)	
Flavonols		
(+)-Taxifolin	(304)	
Flavones		
Apigenin	R ₁ -OH, R ₂ -H (270)	
Luteolin	R ₁ -OH, R ₂ -OH (286)	
Lignans		
(+)-Pinoresinol	R-H (358)	
(+)-1-Acetoxypinoresinol	R-OCOCH ₃ (416)	
(+)-1-Hydroxypinoresinol	R-OH (374)	
Hydroxyisochromans		
1-Phenyl-6,7-dihydroxyisochroman	R ₁ ,R ₂ -H (242)	
1-(3'-Methoxy-4'-hydroxy)phenyl-6,7-dihydroxyisochroman	R ₁ -OH,R ₂ -OCH ₃ (288)	

Why are the phenolic compounds present in virgin olive oil so important? Why is their determination so interesting and difficult?

Last year, Boskou published an interesting review [51] wherein the sources of natural phenolic antioxidants were discussed, and the following idea was highlighted: “Widely distributed in the plant kingdom and abundant in our diet, plant phenols are today among the most talked about classes of phytochemicals”. To answer to the question of “why are phenolic compounds so interesting?”, the author of the review summarized several issues which have been studied in depth during the last decade:

- The levels and chemical structure of antioxidant phenols in different plant foods, aromatic plants and various plant materials.
- The probable role of plant phenols in the prevention of various diseases associated with oxidative stress such as cardiovascular and neurodegenerative diseases and cancer.
- The ability of plant phenols to modulate the activity of enzymes, a biological action not yet understood.
- The ability of certain classes of plant phenols such as flavonoids (also called polyphenols) to bind to proteins. Flavonol–protein binding, such as binding to cellular receptors and transporters, involves mechanisms which are not related to their direct activity as antioxidants.
- The stabilization of edible oils, protection from formation of off-flavors and stabilization of flavours.
- The preparation of food supplements.

Focusing on phenolic compounds of virgin olive oil and bearing in mind the reasons for being so important, attention must be paid to the fact that this class of compounds has not been completely characterized due to the complexity of their chemical nature and the complexity of the matrix in which they are found. Moreover, one of the current problems for developing rapid and reproducible analysis of phenolic compounds is the absence of suitable pure standards, in particular secoiridoid molecules and lignans.

Health aspects linked to phenols in VOO

VOO is an integral ingredient of the Mediterranean diet and accumulating evidence suggests that it may have health benefits which include reduction of risk factors of coronary heart disease, prevention of several types of cancers, and modification of immune and inflammatory responses. VOO can be considered as example of a functional food, with a variety of components that may contribute to its overall therapeutic characteristics [52]. Its nutritional and healthy values and pleasant flavour have contributed to an increase in consumption of VOO which has fostered cultivation of olives outside the traditional olive oil producing region of the Mediterranean basin into newer areas such as Australia, Argentina and South Africa. The nutritional value of VOO arises from high levels of oleic acid, and from minor components such as phytosterols, carotenoids, tocopherols and hydrophilic phenols [53].

VOO contains at least 30 phenolic compounds. The major phenolic compounds are oleuropein derivatives, based on hydroxytyrosol which are strong antioxidants and radical scavengers. Recently there has been a surge in the number of publications that has investigated their biological properties. Bisignano *et al.* [54] found that hydroxytyrosol and oleuropein have antimicrobial activity against

several bacterial strains that are causal agents of intestinal or respiratory tract infections in humans. In a more recent *in-vivo* study, Glatzle and co-workers [55] demonstrated that enteral immunonutrition with VOO more effectively reduced septic pulmonary dysfunction compared to a fish oil-enriched lipid formula at the same concentration.

It has recently been found that hydroxytyrosol is renally excreted: while some of hydroxytyrosol is unchanged, some is also metabolized to the following metabolites: glucuronide conjugate, sulphate conjugate, homovanillic acid, homovanillic alcohol, 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetaldehyde [56-57]. The radical scavenging potencies of these metabolites of hydroxytyrosol have also been investigated using the radical assay DPPH. The glucuronide conjugate was more potent than hydroxytyrosol while the sulphate conjugate was nearly devoid of radical scavenging activity. When phenol-rich VOO characterized by increasing concentrations of catecholic compounds were administered to human volunteers, Visioli and co-authors [58] observed a dose-dependent urinary excretion of hydroxytyrosol and its metabolite homovanillic alcohol. In a later study, the same authors [59] noticed that the urinary levels of unconjugated tyrosol and hydroxytyrosol correlated with their intake, except at the highest dose, which increased the quantity of glucuronide conjugate. Tuck *et al.* investigated the *in vivo* fate of hydroxytyrosol and tyrosol after intravenous and oral dosing of these tritium labelled compound to rats [60]. No significant differences in the amount of phenolic compounds eliminated in urine between the intravenous dosing method and the oral oil-based dosing method for either tyrosol or hydroxytyrosol were found.

Phytochemical compounds such as oleuropein and oleuropein aglycon have been intensively studied for some promising results with respect to their effects on human health and their potential medicinal properties. It has been found that diets containing olive oil phenols may increase *in vivo* resistance of LDLs to oxidation; the effectiveness of oleuropein has been explained in part through its ability to act as an antioxidant and in part through a hypocholesterolaemic effect [61]. In an investigation by Coni *et al.* [62] it was found that when fed a diet rich in oleuropein to rabbits, the ability of LDL to resist to oxidation increased, thanks to its antioxidant capacity; moreover, they found a significant reduction of the plasmatic levels of total, free and ester-derivatives of cholesterol. Oleuropein aglycon, the bitter component of olives and olive oil, is among the first example of how selected nutrients from an VOO-rich “Mediterranean diet” can directly regulate HER2-driven breast cancer disease [63]. As oleuropein aglycon exhibits synergistic anti-tumor effects when concurrently given to breast cancer cells chronically exposed to trastuzumab (Tzb; Herceptin™) for several months, this further underscores the potential clinical relevance of these findings.

On the basis of their shared throat irritant properties (pungency), Beauchamp *et al.* [64-65] examined whether *p*-HPEA-EDA, now referred to as “oleocanthal”, might mimic the pharmacological effects of ibuprofen, a potent modulator of inflammation and analgesia. It was found that, like ibuprofen, both enantiomers of *p*-HPEA-EDA caused dose-dependent inhibition of COX-1 and COX-2 activities (cyclo-oxygenase enzymes that catalyze key steps in the biochemical inflammation pathways derived from arachidonic acid) but had no effect on lipoxygenase *in vitro*. These authors hypothesized that long-term consumption of oleocanthal may help to protect against some diseases by virtue of its ibuprofen-like COX-inhibiting activity (by reducing the risk of developing some cancers and by lowering platelet aggregation in the blood). However, as noted by Fogliano and Sacchi [66], no data is available about the concentration of the various aglycons, including oleocanthal in plasma and urine after VOO consumption; absorption and bioavailability studies indicate however that tyrosol and

hydroxytyrosol are likely to be bio-available. It is worth mentioning that acid hydrolysis of oleocanthal would produce the elenolic acid, a dialdehyde compound even more similar to ibuprofen than oleocanthal itself.

In the context of the Mediterranean diet and coronary heart diseases, it has also been shown that VOO rich in phenols increases the resistance of LDL to oxidation, both *in vitro* and *ex vivo* [67-69]. The study carried out by Bogani *et al.* [70] confirmed the anti-thrombotic and anti-inflammatory effects of VOO phenolic components, in a postprandial setting; in fact, the results showed significant reductions in serum concentration of inflammatory markers (TXB₂ and LTB₄) at 2 and 6 h after consumption of VOO, but not after consumption of either olive oil or corn oil. They also evaluated the effects of these different oils on *in vivo* indexes of oxidative stress (plasma antioxidant capacity and urinary hydrogen peroxide levels) and showed the antioxidant activity of VOO phenolics after ingestion (increased plasma antioxidant capacity after 2 h of VOO consumption).

Foods containing high amounts of lignans such as flaxseed have been found to be protective against breast cancer, and in particular, to exert an anti-estrogenic effect; this latter observation might be explained by considering the structural similarities between the lignans and the synthetic antiestrogen tamoxifen [71].

Two hydroxy-isochromans, 1-(3'-methoxy-4'-hydroxyphenyl)-6,7-dihydroxyisochroman and 1-phenyl-6,7-dihydroxyisochroman, are formed by reaction between hydroxytyrosol and vanillin and benzaldehyde, respectively, (under very mild conditions). They have only recently been discovered in VOO [36] and are active in inhibited platelet aggregation and thromboxane release evoked by agonists (sodium arachidonate and collagen) that induce reactive oxygen species-mediated platelet activation [72].

A large number of studies, mainly experimental models, have been performed on certain minor components of olive oil. However, as commented in an excellent review by Covas *et al.* [73], the precepts of evidence-based medicine require high-level scientific evidence to be provided before nutritional recommendations for the general public can be formulated. Scientific evidence required is provided by randomized, controlled, double-blind clinical trials (level I evidence), and to some extent by large cohort studies (level II evidence). Basic research, despite its usefulness in permitting adoption on a mechanistic approach, does not provide evidence for nutritional recommendations. Of course, the level of evidence of a particular study depends not only on its design, but also on its quality (external and internal validity, homogeneity of the sample, and statistical power). Finally, evidence is built by the agreement of the results of several similar studies. In the same review, the authors highlighted that in experimental studies, olive oil phenols have been shown to:

- 1) have antioxidant effects, greater than those of vitamin E, on lipid and DNA oxidation [74-77];
- 2) prevent endothelial dysfunction by decreasing the expression of cell adhesion molecules [78], increasing nitric oxide (NO) production and inducible NO synthesis [79] and quenching vascular endothelium intracellular free radicals [80];
- 3) inhibit platelet-induced aggregation [81];
- 4) enhance the mRNA transcription of the antioxidant enzyme glutathione peroxidase (GSH-Px). It should be mentioned however that to regard this last point controversial results have been obtained depending on the tissue in which the gene expression was evaluated [75, 82].

Other potential activities of VOO phenolic compounds include chemopreventive activity [77]. The anticarcinogenic activity of phenols may be due not only to their antioxidant properties, but also to

their ability to reduce the bioavailability of food carcinogens and to inhibit their metabolic activation [83-84]. There are several mechanistic considerations of the role of phenolic compounds as anticarcinogens, as reviewed by Yang *et al.* [85].

Phenols as related to oxidative stability of VOO

Oxidation is an inevitable process that starts after the VOO has been extracted and leads to deterioration that becomes more pronounced during oil storage. Initially lipids are radically oxidised to hydroperoxides, which are odourless and tasteless [86] and do not account for sensory changes. However, decomposition occurs through homolytic cleavage of the hydroperoxide group with production of various volatile compounds, known as secondary oxidation products, which are responsible for typical unpleasant sensory characteristics. Oxygen, light, temperature, metals, pigments, unsaturated fatty acid composition, as well as the quantity and kind of natural antioxidants, are all factors that can influence the free radical mechanism of the autoxidation process in a different manner [87-88].

Natural antioxidants exhibit complex properties between air-oil and oil-water interfaces that significantly affect their relative activities in different lipidic systems. The presence of hydrophilic phenolic compounds in VOO and their high antioxidant activity can be explained by the so-called “polar paradox” [89] which dictates that “polar antioxidants are more effective in non polar lipids, whereas non-polar antioxidants are more active in polar lipid emulsions”. According to Frankel [90] in a bulk oil system the hydrophilic antioxidants, such as polar phenols, are oriented in the air-oil interface (a low quantity of air is always trapped in the oil) and become more protective against oxidation than the lipophilic antioxidants, like tocopherols, which remain in solution in the oil.

In a study carried out by Paiva-Martins and co-workers [91] it was found that when food is processed with VOO in the presence of water, olive phenolic extracts with higher quantities of 3,4-DHPEA-EA and 3,4-DHPEA-EDA would be better than VOO extracts with higher quantities of hydroxytyrosol, despite the higher antioxidant activity of hydroxytyrosol in bulk oil.

Moreover, the orientation of phenolic compounds in the oil-water interface and the active surface of water droplets influence the protection against the oxidation of oil. Recently, some researchers [92-95] have determined that VOO contains a low quantity of water (ranging from 450 mg kg⁻¹ to 3,000 mg kg⁻¹ depending on the extraction technology), that increases when samples were not filtered. Part of the total water content presents in VOO is free and available for chemical and enzymatic reactions and also keeps hydrophilic phenols in solution. This can explain the hydrolytic process that occurs both to phenols (by esterases) and triacylglycerols (by lipase) during prolonged VOO storage. Thus, more rapid oxidation of the unfiltered oil could be expected. Instead, according to Gomez-Caravaca *et al.* [95] and Tsimidou *et al.* [96], stability of unfiltered samples, when measured in terms of resistance to accelerated oxidation (value by OSI or Rancimat instruments) was in all cases significantly higher than that of the corresponding filtered oils. This coincided with a higher total phenolic content in unfiltered VOO. Undoubtedly, a loss of a significant fraction of phenols during filtration is related to the reduction of oxidative stability.

Chain-breaking antioxidants, such as phenolic compounds, react with lipid radicals to form non-reactive radicals, interrupting the propagation chain. In fact, these compounds are able to donate an electron or a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation

and stabilize the resulting phenoxyl radical by delocalizing the unpaired electron [97-100]. Phenolic compounds exert their antioxidant abilities in VOO by scavenging peroxy and alkoxy radicals, and by chelation of transition metals ions present in trace quantities [101]. Paiva-Martins and Gordon [102-103] have studied the antioxidant effects of pure phenolic compounds (hydroxytyrosol, hydroxytyrosol acetate, oleuropein, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA) by both the diphenylpicrylhydrazyl (DPPH) assay and the ferric reducing antioxidant potential (FRAP) assay in bulk oil and in emulsions (both with and without ferric ions). The compounds showing the best antioxidant activity in oil in water emulsions in the presence of iron were 3,4-DHPEA-EA and 3,4-DHPEA-EDA, which in contrast to hydroxytyrosol and oleuropein did not show pro-oxidant activity. However, when the radical scavenging activity was measured for these compounds, 3,4-DHPEA-EA showed a much higher activity than 3,4-DHPEA-EDA, suggesting that chelation of iron was of major significance in determining the antioxidant activity of these compounds in the presence of iron and water. According to this latter behaviour, Bendini *et al.* [104] showed evidence that 3,4-DHPEA-EDA has the ability to chelate copper in bulk oil.

The stability of VOO is improved by synergistic interactions between various antioxidants present (both phenolic and non-phenolic) and the lipid composition. Such cooperative activity seems to explain the antioxidant synergism observed when α -tocopherol and ascorbic acid or ubiquinol are used in combination. Recent investigations have also demonstrated an antioxidant synergism between α -tocopherol and some phenolics (green tea catechins and quercetin) [105-106]. Bendini *et al.* [104] hypothesized that phenols having an ortho-dihydroxyl structure, and in particular an isomer of 3,4-DHPEA-EDA, were able to reduce the oxidized forms of tocopherols (tocopheryl radicals and quinones). This was substantiated by Pazos *et al.* [107] who demonstrated that phenols, as well as several benzoic acids and epicatechin gallates, were potentially active in the regeneration of α -tocopherol via reduction of α -tocopheroxyl radical. Moreover this capacity was found to be directly proportional to the ability of phenolic compounds to transfer a single H atom.

There have been numerous studies on the relative antioxidant potency of the individual olive oil phenols, although it may vary depending on the methods used for evaluation. Many authors have frequently studied the ability of antioxidant molecules or extracts to scavenge some free radicals, and in this regard, several stable, coloured free radicals (DPPH and ABTS) are widely used due to their intense absorbance in the visible region. In this case, the hydrogen-donating activity can be determined.

However, as a general guide to their potency, oleuropein and hydroxytyrosol have been shown to be more effective than vitamin E [108] and butylated hydroxytoluene (BHT) or other synthetic antioxidants approved for use in foods [109-111].

From comparison with the principal phenolic constituents of VOO, it has been claimed that hydroxytyrosol is the most active antioxidant compound [112]. Both hydroxytyrosol and oleuropein have been shown to be scavengers of superoxide anions, and inhibitors of the hypochlorous acid-derived radicals, but hydroxytyrosol was more effective than oleuropein in this regard [113]. Both compounds also scavenged hydroxyl radicals, but in this case oleuropein showed greater activity [114]. Gordon *et al.* [115] investigated the antioxidant activity of hydroxytyrosol acetate by scavenging of DPPH radicals in comparison with that of the phenolic extract from VOO and the pure components hydroxytyrosol, oleuropein, 3,4-DHPEA-EA, and α -tocopherol in bulk oil and oil-in-water emulsions. In this study the authors showed that hydroxytyrosol acetate had a weaker DPPH radical scavenging

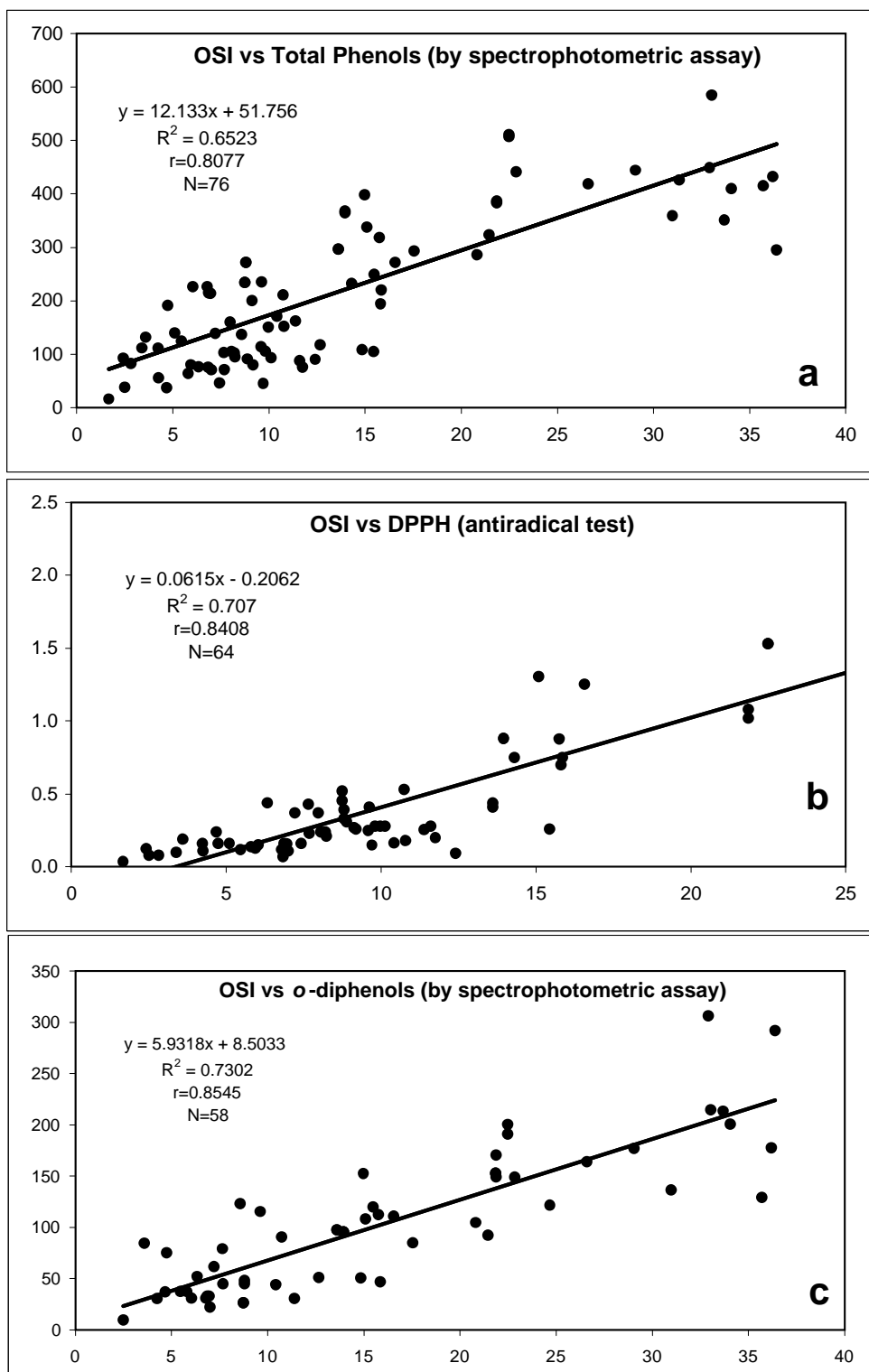
activity than hydroxytyrosol and 3,4-DHPEA-EA was slightly less effective than hydroxytyrosol acetate in oil but was the most effective hydroxytyrosol derivative in an emulsion oil in water.

It is well known that the high oxidative stability of VOO is primarily due to *o*-diphenols such as hydroxytyrosol and its oleosidic forms [108]. Aparicio *et al.* [116], using statistical analysis of data relative to 79 VOO of olives *cv.* Hojiblanca and Picual, measured correlations between oxidative stability (valued by Rancimat) and several compositional variables. The phenols ($R^2=0.87$), *o*-diphenols ($R^2=0.77$), and the oleic/linoleic ratio ($R^2=0.71$) had the highest values, followed by chlorophylls ($R^2=0.68$), total tocopherols ($R^2=0.65$) and carotenoids ($R^2=0.59$). Principal components analysis confirmed that phenols, oleic/linoleic ratio, and tocopherols had the maximum correlation with oxidative stability. From these results, the phenolic content would contribute around 51% of the stability of VOO, and particularly 30% for phenols and 21% for *o*-diphenols whereas, the oleic/linoleic ratio would account for only 27%. Since a hypothetical synergy effect was detected between these chemical variables, it is more prudent to conclude that 78% of the stability is due to the combined effect of both variables. The authors surmised that the contribution of total tocopherols was around 9%, whereas the remaining percentage of 13% could be attributed to chlorophylls and carotenoids.

In experiments carried out by Carrasco-Pancorbo and co-authors [117], the antioxidant activity of several single phenolic compounds of VOO (hydroxytyrosol, tyrosol, elenolic acid, 3,4-DHPEA-EDA, (+)-pinoresinol, (+)-1-acetoxypinoresinol, oleuropein aglycon and ligstroside aglycon) was evaluated by different chemical approaches: radical assay (DPPH), accelerated oxidation in a lipid model system (OSI, oxidative stability index), and an electrochemical method (flow injection analysis FIA-amperometry and cyclic voltammetry). These authors verified that, as is generally assumed, the presence of a single hydroxyl group on benzenic ring conferred only limited antioxidant activity. On the other hand, the presence of a catechol moiety enhances the ability of the phenolic compounds to act as antioxidants. The results obtained in all three tests showed that hydroxytyrosol, 3,4-DHPEA-EDA and oleuropein aglycon were the strongest in terms of antioxidant power. Elenolic acid, which does not have a phenolic ring, was one of the compounds that presented the weakest antioxidant activity, as also reported by Briante *et al.* [118]; this compound together with (+)-pinoresinol, tyrosol, ligstroside aglycon and (+)-1-acetoxypinoresinol showed pro-oxidant effect when tested by OSI. Similar results were found by Nenadis *et al.* [119]: tyrosol, hydroxytyrosol and their secoiridoid derivatives were examined calculating the bond dissociation enthalpy (BDE) of phenolic hydroxyl groups and the ionization potential (IP) as descriptors to predict the H-atom-donating and electron-donating abilities of antioxidants, respectively. Catechol derivatives had the lowest BDE values (77.7-80.1 kcal mol⁻¹), whereas the lignans, pinoresinol and 1-acetoxypinoresinol, and other monophenols had much higher BDE values (85.1-88.0 kcal mol⁻¹), which suggested a lower potential for radical scavenging.

In a recent work, Lorenz *et al.* [120] investigated the antioxidant and radical scavenging properties of several phenolic isochromans. All hydroxy-isochromans tested exceeded the scavenging effect of trolox (an hydrophilic analogue of α -tocopherol). They found excellent ROS/RNS (reactive species of oxygen/nitrogen) scavenging features of the hydroxy-isochromans and also concluded that their simple synthesis added to their interest as candidates for pharmaceutical interventions that protect against the deleterious action of ROS/RNS.

Figure 1 Correlations among OSI values (in hours), phenolic amounts and antioxidant activity (DPPH test) by spectrophotometric assays. a, OSI vs Total Phenols (mg gallic acid kg^{-1} VOO); b, OSI vs DPPH (mmol trolox kg^{-1} VOO); c, OSI vs *o*-diphenols (mg gallic acid kg^{-1} VOO). Analyses were carried out over three years; in each figure the number of samples is reported (N). Three replicates were prepared and analyzed for each sample.



Franconi *et al.* [121] tested the antioxidant activity of two VOO (Seggianese and Taggiasca characterized by different quali-quantitative phenolic profiles) on human LDL by measuring malondialdehyde and conjugate diene generation induced by copper ions. In both tests antioxidant potency correlated with total phenols; moreover, reduction of malondialdehyde and generation of conjugate diene was dependent on the amount of total phenols. High levels of secoiridoids enhance the antioxidant activity, suggesting that VOO rich in these compounds could have health-protecting properties consistent with a low extent of LDL oxidation.

Recently, the interest in oxidized forms of VOO phenols has significantly increased, especially in relation to determination of freshness/ageing status [122-123]. Moreover, characterization of these oxidized phenolics could represent an analytical instrument to investigate the thermal processes of the oils during refinement [124]; this could also provide the means to verify fraudulent practices such as "gentle deodorization" (under soft refining conditions) or blending of VOO with other oils. In 2005 Rios *et al.* [122] compared the performance of HPLC-APCI-MS and GC-IT-MS analytical techniques to evaluate the oxidation products of elenolic acid, oleuropein and ligstroside aglycons. Five oxidation phenols were identified with gas-chromatography. Armaforte *et al.* [125] showed that SPE procedure (usually used to extract the phenolic fraction from VOO) may be a not appropriate analytical step when VOO contains significant polar oxidation products (from phenols or lipids); in fact, these latter compounds could interfere with the retention mechanism of phenols during their extraction. These authors also proposed an index to establish the degree of freshness of VOO. This value, or TPAR (ratio between total peak area of reduced and oxidized forms of phenols) is close to 1 for fresh samples whereas it decreases rapidly in VOO with an increasing content of oxidized phenols.

Carrasco-Pancorbo and co-authors [124] by studying the phenolic profiles of the oils after a drastic heating treatment (at 180°C) found several "unknown" compounds, (by using HPLC-UV, HPLC-MS and CE-UV) that were probably linked to phenol oxidation. In particular, seven peaks significantly increased when the thermal treatment was longer (from 1 to 3 h) and their presence was also confirmed in refined olive oils. The concentration of hydroxytyrosol, elenolic acid, 3,4-DHPEA-EDA and 3,4-DHPEA-EA decreased more quickly with the thermal treatment than other phenolic compounds present in olive oil, confirming their high antioxidant power; moreover 3,4-DHPEA-AC and *p*-HPEA-EA were more resistant to heat treatment, whereas the amount of (+)-pinoresinol and (+)-1-acetoxypinoresinol were almost unchanged.

Sensory properties elicited by phenols in VOO

Virgin olive oil is a natural fruit juice obtained directly from olives without any further refining process. Its flavour is characteristic and is markedly different from those of other edible fats and oils. The combined effect of the taste, odor (directly via the nose or indirectly through the retronasal path via the mouth) and chemical responses (pungency, astringency, metallic, cooling or burning) gives rise to the sensation generally perceived as "flavor" [126]. VOO, when extracted from fresh and healthy olive fruits (*Olea europaea* L.) and properly processed and stored, is characterized by an unique combination of aroma and taste that is highly appreciated [127-128]. The sensory aspect, due to the use of VOO as a seasoning on cooked and especially raw foods, has great repercussions on its acceptability. Thus, since sensory quality plays an important role in directing the preference of consumers, many attempts have been made to clarify the relationships between the sensory attributes

in a VOO as perceived by assessors and its volatile and phenol profiles, which are responsible for aroma and taste, respectively [128].

Few individuals, except for trained assessors of VOO, know that bitterness and pungency perceived by taste are positive attributes for a VOO. These two sensory characteristics are strictly connected by the quali-quantitative phenolic profile of the product. An example of the positive correlations between amount of phenols and bitter and pungent intensities is shown in Figures 2a and 2b.

Some phenols mainly elicit the tasting perception of bitterness; however, other phenolic molecules can stimulate the free endings of the trigeminal nerve located in the palate and also in the gustative buds giving rise to the chemesthetic perceptions of pungency, astringency and metallic attributes. Thus, the intensity of bitterness and pungency is mainly related to the olives cultivar and the ripening stage and, as reported by many authors, are especially abundant in oils obtained from unripe fruits. For instance, Caponio *et al.* [129] showed that in *Coratina* and *Oliarola Salentina* VOO, oleuropein and its aglycon form both decrease as ripening of the olives progressed. From this data, the bitter to pungent taste would appear to be mainly ascribable to oleuropein aglycon since greater amounts of this phenolic compound are present in the *Coratina* oils with respect to *O. Salentina* oils, which are known to have a sweet taste. In order to attenuate such these taste sensations, the authors suggested the need to postpone harvesting of *Coratina* olives.

Figure 2 Sensory profile and phenolic content of two different VOO (HPh, high phenols oil and LPh, low phenols oil). **a**, sensory profiles of samples by Quantitative Descriptive Analysis (QDA); the intensity of each descriptor is evaluated on a 0-5 points scale; different perception routes: (1) orthonasal, (2) retronasal. **b**, single and total phenolic content of samples; A, hydroxytyrosol; B, tyrosol; C, vanillic acid; D, unknown phenolic compound with a retention time of 30.69 min; E, unknown phenolic compound with a retention time of 36.27 min; F, 3,4-DHPEA-EDA; G, (+)-pinoresinol; H, (+)-1-acetoxypinoresinol + *p*-HPEA-EDA; I, 3,4-DHPEA-EA; L, *p*-HPEA-EA.

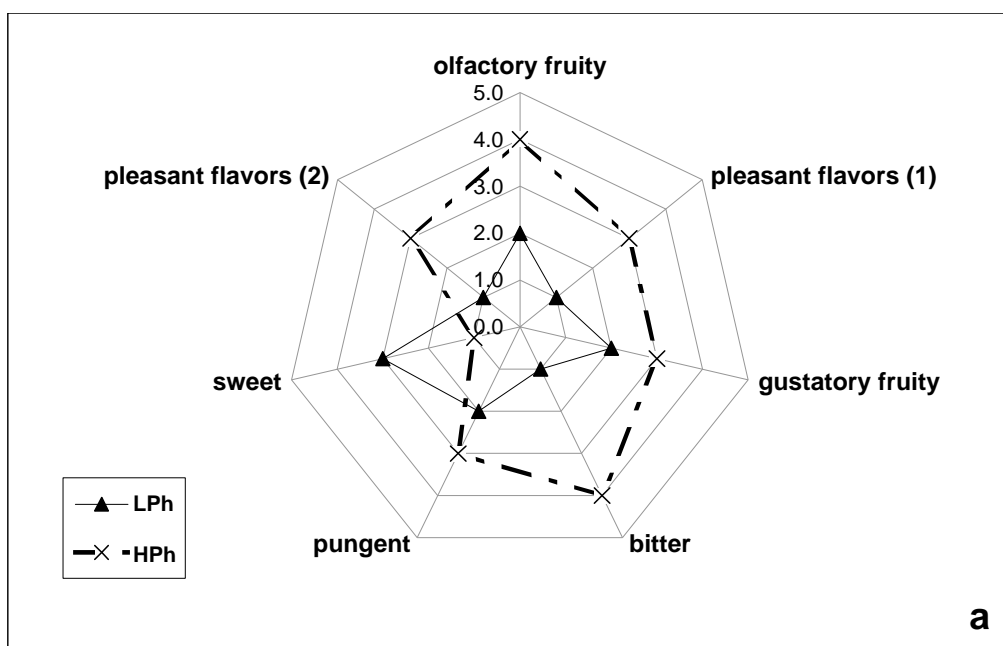
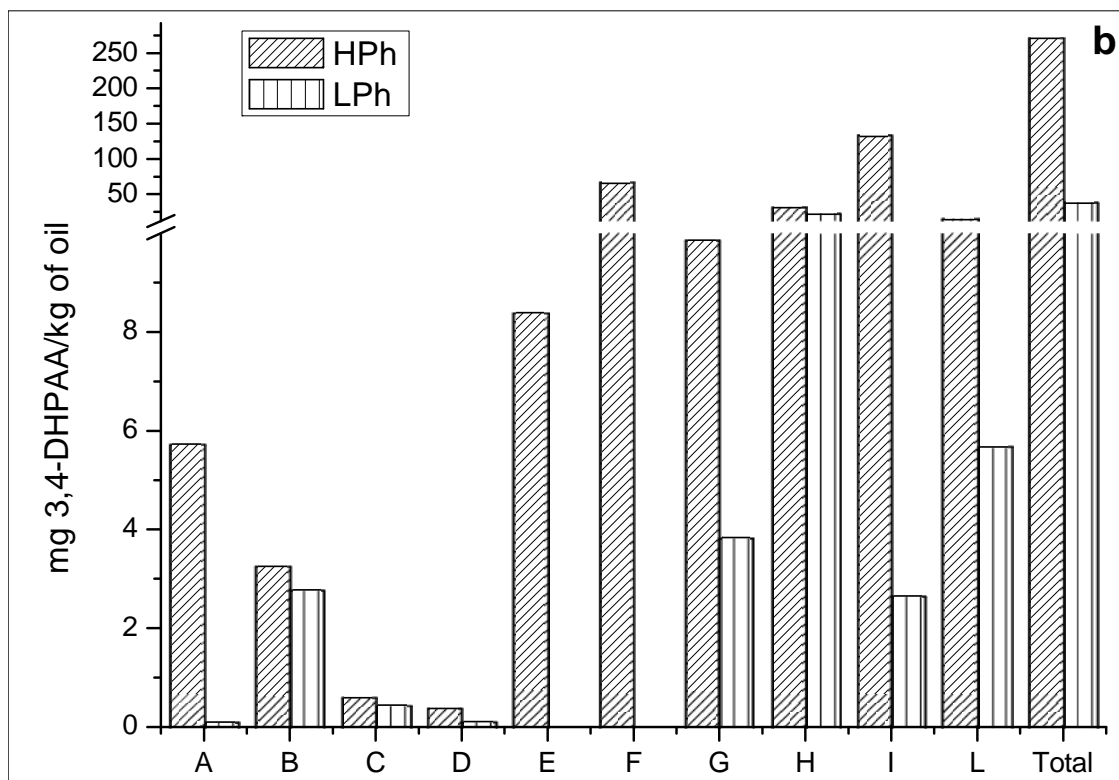


Figure 2. Cont.



A Quantitative Descriptive sensory Analysis (QDA) carried out by Rotondi *et al.* [130] confirmed a decreasing trend of the positive olive oil descriptors, such as bitterness and pungency, when *Nostrana di Brisighella* olives ripened. The highest statistically significant intensity was at the beginning of fruit skin pigmentation. The decrease in bitterness and pungency was also related to a reduction in total phenols and *o*-diphenols levels. In particular, a positive correlation between the secoiridoids content and bitterness and pungency was observed.

With the aim of reducing the bitterness intensity in VOOs, disfavored by many consumers when present at high intensity, some authors [131] developed postharvest technology based on hot-water treatments of olive fruits (cultivars Manzanilla, Picual, and Verdial) in the temperature range of 60–68°C or [132] with air-heating (40°C during 24, 48, and 72 h). These treatments promote a reduction in bitterness that is directly related to the time and temperature of treatment, probably due to a partial inhibition of glycosidases and esterases; in fact, these enzymes are involved in the release of secoiridoid derivatives from oleuropein during the crushing malaxation process. However, this heat treatment also affected other quality traits such as oxidative stability and color and could produce a change in the aroma profile of the VOO as well.

The standard method of analyzing the bitter taste of olive oil is by sensory analysis using a panel of tasters [133]. However, an analytical panel is often not likely to be available, since a permanent staff of trained tasters and a highly specialized panel chief is necessary. Many consumers from extra-European countries are not accustomed to the typical high intensity of bitterness or pungency of fresh VOO and, consequently, must be blended with less bitter VOO. For this reason, methods for the evaluation of the bitterness level based on physical-chemical determinations would be very useful for the industry. Several authors have found a strong relationship between these sensory attributes and the content of phenolic compounds in the olive oils. In 1992 Gutiérrez *et al.* [134] proposed an analytical method for

measurement of bitterness, based on solid phase extraction (SPE) of phenols and their spectrophotometric detection at 225 nm; this parameter termed IB or index of bitterness, was highly correlated to the sensory intensity of bitterness and is still the most widely used for its determination. Some years later, Mateos and co-workers [135] showed that several non-bitter phenolic compounds could also absorb at 225 nm; consequently, they asserted that this index was not appropriate for comparing bitterness of VOO obtained from blend of olive varieties characterized by very different phenolic profiles (e.g. Picual and Arbequina). Moreover, Mateos suggested that evaluation of the bitterness level of a VOO could be described by the experimental equation obtained from the regressions between intensity of bitterness and the concentrations of oleuropein aglycon using a Panel test and chromatographic analysis, respectively.

Some researchers suggest that secoiridoid derivatives of hydroxytyrosol are the main contributors to olive oil bitterness. Recently, a procedure called taste dilution analysis (TDA) was reported by Frank and co-authors to underlie the sensory threshold of bitter for oleuropein derivatives [136]. Bitterness was assessed by preparing serial dilutions of samples in water and then tasting in order of increasing concentration until the concentration at which the diluted sample can be differentiated from water as judged in a triangle test is found. When an isomer (or isomers) of oleuropein aglycon was prepared by β -glucosidase hydrolysis of oleuropein isolated from olives and evaluated by assessors, it was found to be bitter with a threshold of 50 μ mol. Using the same evaluation technique, no bitterness was observed for hydroxytyrosol or elenolic acid.

Andrewes *et al.* [137] assessed the relationship between polyphenols and olive oil pungency. *p*-HPEA-EDA was the key source of the burning sensation found in many olive oils. In contrast, 3,4-DHPEA-EDA, tasted at an equivalent concentration, produced very little burning sensation. This is a clear example of the different sensory properties of a secoiridoid derivative of hydroxytyrosol and tyrosol. In 2005, Beauchamp and co-authors [64] measured the pungent intensity of *p*-HPEA-EDA isolated from different VOO confirming this molecule is the principal agent in VOO responsible for throat irritation. These researchers also tested the throat-irritant properties of its synthetic form (named “oleocanthal”, with oleo- for olive,-canth- for sting, and -al for aldehyde) dissolved in non-irritating corn oil. They found an effect comparable to that of the purified compound from VOO and a dose-dependent activity.

In 2003, Gutierrez-Rosales and co-authors [138] isolated the major peaks found in the phenolic profile of VOO using preparative HPLC; after dissolving in water these molecules purified were then tasted to evaluate the intensity of bitterness. It was concluded that the peaks corresponding to the 3,4-DHPEA-EDA, 3,4-DHPEA-EA and *p*-HPEA-EDA were those mainly responsible for the bitter taste of VOO. As previously reported, Mateos *et al.* [135] verified the better correlation between the aldehydic form of oleuropein aglycon and bitterness.

Recently some researchers [139] have studied the temporal perception of bitterness and pungency in monovarietal VOOs; analyses were performed by a trained sensory panel utilizing a time–intensity (TI) evaluation technique; bitterness curves had a faster rate of rising and declining than pungency curves: the curves for bitterness reached a maximum after approximately 16–20 s, whereas the maximum of the perception of pungency is registered between 26 and 29 s and is independent of the maximum intensity of the perception.

As already discussed, several authors have associated some phenols with bitterness, thus obtaining models and relationships between individual phenols separated by HPLC and bitterness intensity [135,

138,140]. In these reports, bitterness was measured by a panel test or calculated from K_{225} values. Moreover tyrosinase- and peroxidase-based biosensors are being developed for the bitterness assessment [141], and are showing interesting possibilities. However, HPLC is often not available in many olive oil mill laboratories because of economic reasons, as well as specialized technical staff and biosensors. As an alternative, Beltrán and co-authors [142] proposed that measurement of phenol content can be used. This is a simple analytical method that involves liquid-liquid extraction and colorimetric measurement using Folin-Ciocalteu reagent [143]. In their experimental work, the authors analyzed the relationship between phenol content and K_{225} for oils from four of the most important olive cultivars worldwide (Frantoio, Hojiblanca, Picual, Arbequina); 360 samples were used to develop the model. As a practical application, bitterness intensities were evaluated by sensory analysis of 25 VOO samples, and were then estimated by applying the prediction model. In order to provide an easy tool for bitterness estimation, VOO bitterness was classified by its phenol content into four categories (results expressed as mg of caffeic acid per kg of oil and intensity of bitterness between 0 and 5 values): phenol contents equal or lower than 220 mg kg⁻¹ corresponded to non-bitter oils or oils with almost imperceptible bitterness (intensities 0–1.5); slight bitterness corresponded to 220–340 mg kg⁻¹ (intensities 1.6–2.5); bitter oils have a phenol contents ranging from 340 to 410 mg kg⁻¹ (intensities 2.5–2.99); and a phenol contents higher than 410 mg kg⁻¹ corresponds to quite bitter or very bitter oils (intensities higher than 3). In general, the authors determined that the oils were classified correctly into the same bitterness categories by both methods at 92%, achieving 100% of correct classification for the lowest and highest bitterness categories.

New analytical approaches to characterization of the phenolic profile and applied studies during the last decade

In order to utilize VOO as a source of phenolic compounds, to develop complete compositional databases and to obtain more accurate data about the intake of antioxidants further chemical characterization is needed. Identification and quantitation, based traditionally on HPLC (with different detectors, such as UV, fluorescence, coulometric electrode array detection, amperometric detector) [144-150], GC-FID [151-154] and, more recently CE-UV, can be aided today by MS and NMR, which is a focus of the present review.

Liquid chromatography/mass spectrometry (LC-MS) has been widely accepted as the main tool in identification, structural characterization and quantitative analysis of phenolic compounds in olive oil. Using a mass spectrometer for detection offers some undoubted advantages, such as independence of a chromo- or fluorophore, lower LOD than UV in most cases [155], the possibility to obtain structural information and easy separation of coeluting peaks using the information about mass as a second dimension.

The sensitivity of response in MS is clearly dependent on the interface technology employed. In LC-MS analysis of phenolic compounds, atmospheric pressure ionization interfaces, i.e. APCI and electrospray ionization (ESI), are used almost exclusively today, and both positive and negative ionization are applied. In general, phenolic compounds are detected with a greater sensitivity in the negative ion mode, but the results from positive and negative ion modes are complementary, and the positive ion mode shows structurally significant fragments [156].

On the other hand, optimal ionization depends not only on the interface parameters, but also on the mobile phase of the liquid chromatography. As a first rule, the use of non-volatile salts in the mobile phase (common in other chromatographic methods) should be avoided, as they would interfere with the ionization source. The mobile-phase composition and its pH need also careful optimization as they may influence the ionization efficiency of the analytes.

The selection of the analyzer, apart from its accessibility, is determined by the required sensitivity and selectivity and the general objectives. LC-atmospheric pressure ionization (API)-MS typically only yields a single strong ion, which reduces its ability to make analyte accurate identifications. In the most cases, single-stage MS is used in combination with UV detection to facilitate the identification of phenolic compounds in olive oil samples with the help of standards and/or reference data. Ion Trap or QqQ provide the possibility of doing MS/MS or MSⁿ, which can be used for structure elucidation or for additional selectivity to gain sensitivity by reducing the chemical noise [157]. MS/MS and MSⁿ involve two (or more) stages of mass analysis, separated by a fragmentation step. TOF MS, which is one of the most advanced MS analyzers, provides excellent mass accuracy [158] over a wide dynamic range if a modern detector technology is chosen. The latter, moreover, allows measurements of the correct isotopic pattern [159], providing important additional information for the determination of elemental composition [160].

Table 2 provides an overview of methodologies based on LC-tandem mass spectrometry used for the analysis of phenolic compounds in olive oil. The table does not include several publications in which the analysis of olive fruit, leaves, pulp and pomace, olive tree wood, as well as olive oil waste waters were carried out by using HPLC-MS [14, 49, 156, 161-168]. Other important issues are the presence of phenolic metabolites of VOO in the human low density lipoprotein fraction [169-170].

High-resolution spectroscopic techniques, and particularly NMR spectroscopy, are finding interesting applications in the analysis of complex mixtures of various food extracts that contain phenols.

During the past decade proton nuclear magnetic resonance spectroscopy (NMR) has been successfully used in olive oil analysis [171-172]. Currently available high-resolution spectroscopic techniques, coupled with the facilities of computerized mathematical or other treatment of data have found interesting applications in the field of agricultural and food science without the necessity for a separative technique coupled with NMR, as commented by Gerathanassis [173]. Additionally the usefulness of ¹H NMR spectroscopy has been increasingly recognized for its non-invasiveness, rapidity, and sensitivity for a wide range of compounds in a single measurement. However, difficulties may arise in relation to the information obtained from spectra of multicomponent mixtures such as olive oil. Strong signal overlap, dynamic range problems, diversity of intensities due to various concentrations of the food constituents, and the inherent lack of scalar coupling information between different moieties lead to ambiguous or incomplete assignments, thus hindering detection even with the use of multidimensional NMR [174]. One possible approach to these problems involves the combination of the advantages of NMR spectroscopy with those of chromatography. Coupled techniques such as LC-NMR or LC-NMR/MS may provide information on overall composition and enable the identification of individual phenols in complex matrices. Moreover, on-line solid phase extraction (SPE) in LC-NMR for peak storage after the liquid chromatography separation prior to NMR analysis or similar techniques have been recently applied.

Table 2. Summary of separation of phenolic compounds in the polar fraction of VOO using HPLC-MS methods.

Time of analysis	Mobile phase	Stationary phase	Type of elution	Extraction System	Detection System	Observations	References
120 min	A: H ₂ O B: MeOH	Spherisorb ODS 25 cm x 4.6 mm i.d.; 10µm	Gradient	Combination between LLE [42] and SPE	UV in HPLC. MS off-line	Separation of the polar fraction of VOO in two parts. Antioxidant activity assessment	[175]
93 min	A: H ₂ O + CH ₃ COOH 0.5% B: MeCN	Spherisorb ODS 2, 25 cm x 4.6 mm i.d.	Gradient	LLE with methanol/water (80:20 v/v)	UV; MS (ESI) in positive ion mode	Flavonoids such as luteolin and apigenin were detected as phenolic components of VOO	[41]
25 min	H ₂ O:CH ₃ CN (82:18 v/v) + CH ₃ COOH 0.02%	Nucleosil ODS, 25 cm x 2.1 mm or 25 cm x 1.1 mm i.d. 5 µm	Isocratic	LLE with buffer; SPE with phenyl cartridges (acidification)	UV, fluorescence, MS, MS/MS HPLC-APCI (negative ion mode)	Phenolic acids	[30]
60 min approx.	A: H ₂ O + HCOOH 0.045% B: MeOH + HCOOH 0.045%	Nucleosil ODS, 25 cm x 2.1 mm i.d. 5 µm	Gradient	-Phenolic acids as Cartoni [30] -HYTY and TY: 3 g oil across cartridge phenylic	MS; MS/MS	Olives and VOOs. MS/MS using Multiple Reaction Monitoring (MRM) (high specificity and sensitivity in MS spectra)	[176]
HPLC method and conditions of Cortesi et al. [177]		C18 column (RP) Alltech 25 cm x 4.6 mm i.d.	Gradient	LLE: Montedoro et al. [1] using butylated hydroxytoluene (BHT)	MS; MS/MS	Analysis of oleuropein aglycon by APCI-MS. Phenolic compound profile	[178]
HPLC method of Romani et al. [179]		Lichrosorb RP18, 25 cm x 4.6 mm i.d. 5 µm	Gradient	LLE with EtOH/water (70:30 v/v), the water was acidified with formic acid (pH 2.5)	DAD; MSD	HPLC analysis of phenolic acids, secoiridoids and flavonoids	[180]
60 min	A: H ₂ O + CH ₃ COOH 2mM B: MeOH + CH ₃ COOH 2mM	Nucleosil ODS, 25 cm x 2.1 mm i.d. 5 µm	Gradient	LLE with methanol/water (80:20 v/v), acidification and passed through a C18 cartridge	MS and MS/MS (API/MS in negative ion mode)	Identification of a new class of phenolic compounds in olive oils: hydroxy-isochromans	[35]

Table 2. Cont.

Time of analysis	Mobile phase	Stationary phase	Type of elution	Extraction System	Detection System	Observations	References
HPLC method and conditions of Brenes et al. [44]					UV; electrochemical, fluorescence, MS.	Use of a lignan (1-acetoxypinoresinol) to authenticate Picual VOOs. Use of GC too.	[35]
50 min or 70 min	A: H ₂ O + H ₃ PO ₄ 0.5% B: MeOH/ MeCN (50:50 v/v)	Lichrospher 100 RP18, 25 cm x 4.0 mm i.d. 5 µm	Gradient	SPE (diol-bound phase)	UV, HPLC-MS in ESI(positive ion mode)	Dialdehydic and aldehydic forms of oleuropein aglycon and ligstroside aglycon	[181]
65 min	A: H ₂ O + CH ₃ COOH 2% B: MeOH/ MeCN (50:50 v/v)	C18 Luna column, 25 cm x 3.0 mm i.d. 5 µm	Gradient	Comparative study of 5 extraction methods (LLE and SPE)	UV, DAD; MS	HPLC and CE methods. (HYTY, TY, oleuropein, ligstroside aglycon and decarboxymethyl oleuropein aglycon)	[182]
65 min	A: H ₂ O + CH ₃ COOH 2% B: EtOH	Phenomenex Luna (phenyl- hexyl)phase; 25 cm x 4.6 mm i.d. 5 µm	Isocratic	LLE with methanol/water (80:20 v/v) Montedoro et al. [1]	UV; MS (ESI in negative ion mode)	Isolation of individual polyphenols to study sensory properties	[137]
60 min approx.	A: H ₂ O + HCOOH 0.09% B: MeOH + HCOOH 0.09%	Nucleosil ODS, 25 cm x 2.1 mm i.d. 5 µm	Gradient	Separation of phenolic compounds in two fractions after C18 cartridge. Group A: 12 g oil Group B: 3 g oil	UV; fluorescence; MS; MS/MS	Improve of extraction system of [176]. Determination of isomer of dihydroxy- and dimethoxybenzoic acids. Comparison among LOD in HPLC- UV, HPLC-FL and HPLC-MS/MS	[183]
75 min	A: H ₂ O + CH ₃ COOH 0.5% B: MeCN	C18 Luna column, 25 cm x 3.0 mm i.d. 5 µm	Gradient	LLE with methanol/water (60:40 v/v)	DAD; MS (ESI in negative ion mode)	Effect of olive ripening degree on the oxidative stability and organoleptic properties of olive oil	[184]
HPLC method of Rotondi et al. [184]			Gradient	LLE with methanol/water from olive oil. SLE from olive fruits.	DAD; MS (ESI in positive and negative ion mode)	HPLC and CE analysis. 3 simple phenols, a secoiridoid derivative and 2 lignans	[185]

Table 2. Cont

Time of analysis	Mobile phase	Stationary phase	Type of elution	Extraction System	Detection System	Observations	References
60 min	A: H ₂ O + 0.2% acetic acid B: MeCN	Lichrospher 100, 12.5 cm x 4.0 mm i.d. 5 μm	Gradient	LLE with methanol (500 mg of oil)	Refractive index detector; MS	TY, Vanillic acid, Lut and Apig. Squalene (with Refractive Index detector). Quantitation in 7 samples.	[45]
45 min	A: H ₂ O + 0.2% acetic acid B: MeCN	Inertsil ODS-3, 15 cm x 4.6 mm i.d. 5 μm	Gradient	LLE with methanol/water (80:20, v:v) (45 g of oil)	UV; ESI-MS	Antioxidant activity of olive pulp and olive of Arbeq. cv	[46]
40 min	A: H ₂ O + 0.2% acetic acid B: MeCN	C18 Luna column, 15 cm x 2.0 mm i.d. 5 μm	Gradient	Diol cartridge (3 g of oil)	UV (DAD); MS; MS/MS (QqQ)	Quantification of 23 compounds in 3 olive oils. Possible models of derived secoiridoids (nine basic models of Lig and Ol aglycons found in bibliography)	[186]
70 min	A: H ₂ O + 0.5% acetic acid B: MeCN	C18 Luna column, 25 cm x 4.6 mm i.d. 5 μm	Gradient	LLE with methanol/water (60:40, v:v) (60 g of oil)	UV (DAD); MS	Isolation of several phenolic compounds and study of their antioxidants properties (DPPH, OSI and electrochemical method)	[117]
50 min	A: H ₂ O + HCOOH (pH 3.2) B: MeCN	C18 Luna column, 25 cm x 3.0 mm i.d. 5 μm	Gradient	LLE with ethanol/water (7:3, v:v) (25 ml of oil)	UV (DAD); ESI-MS	Evaluation of lignans free and linked HYTY and TY in VOO. TLC to determine the presence of lignans.	[187]
30 min	A: H ₂ O + 0.1% acetic acid B: MeCN	RP C18 2.1 x 100 mm, 3.5 mm particle size; XTerra MS	Gradient	SPE-Diol (60 g of oil) diluted 1:10	ESI-TOF (TOF)	Determination of all the well-known phenolic compounds of oil and more than 25 "new" compounds	[188]

One of the pioneers in this field was Montedoro [3] who identified four new phenolic compounds in olive oil in 1993. This paper reported the NMR, UV and IR characterization of the compounds under study, and finally, concluded that the newly identified compounds were an isomer of oleuropein aglycon, the dialdehydic form of elenolic acid linked to hydroxytyrosol, and the dialdehydic form of elenolic acid linked to tyrosol. The results obtained by Limioli [189] and Bariboldi [190] were useful in contributing to a more in-depth understanding of the secoiridoid fraction of VOO. Following these results, several authors have used NMR to analyze phenolic compounds in olive oils, which summarize the reports which include methodologies combining HPLC and NMR (Table 3).

Table 3. Summary of separation of phenolic compounds in the polar fraction of VOO using HPLC-NMR (as coupled techniques or by NMR as off-line technique after HPLC).

Time of analysis	Mobile phase	Stationary phase	Type of elution	Extraction System	Detection System	Observations	References
45 min	A: H ₂ O + CH ₃ COOH 2% (pH 3.1) B: MeOH	Erbasil C ₁₈ , 15 cm x 4.6 mm i.d.	Gradient	LLE with methanol/water	UV; NMR and IR	Spectroscopic characterization of secoiridoid derivatives	[3]
60 min	A: H ₂ O + CH ₃ COOH 0.2% B: MeOH	Spherisorb ODS 2, 25 cm x 4.6 mm i.d.	Gradient	Same as Montedoro et al. [1]	Photodiode array; MS; NMR	Simple phenols, flavonoids, secoiridoids	[44]
HPLC method of Montedoro et al. [1]		Column RP18 Latex; 25 cm x 4.0 mm i.d. 5 μm	Gradient	LLE with methanol (500 g of olive oil)	UV; MS (ESI) in negative and positive ion mode; NMR	Identification of lignans as major components in polar fraction of olive oil. Preparative thin-layer chromatography (PLC).	[34]
HPLC method of Montedoro et al. [1-3]		Column RP18 Latex; 25 cm x 4.0 mm i.d. 5 μm	Gradient	LLE with absolute methanol and methanol/water (80:20 v/v)	UV; MS (ESI) in negative and positive ion mode; NMR	Use of TLC, GC, GC-MS Study of antioxidant/anticancer capacity	[191]
50 min	A: H ₂ O + CH ₃ COOH 3% B: MeCN:MeOH (50:50 v/v)	Lichrosphere 100 RP18, 25 cm x 4.0 mm i.d. 5 μm	Gradient	Comparative studies of LLE and SPE using diol-phase cartridges; unwanted substances washed out with hexane and hexane/ethyl acetate (90:10, v/v)	UV; DAD NMR (for ligstroside aglycon)	Phenols, flavones and lignans. Colorimetric determination of <i>o</i> -diphenols. GC-MS	[192]

Table 3. Cont,

60 min	A: H ₂ O + CH ₃ COOH 0.5% B: MeOH/ MeCN (50:50 v/v)	Lichrospher 100 RP ₁₈ , 25 cm x 4.0 mm i.d. 5 μm	Gradient	Comparative study of LLE and SPE (diol and C ₁₈ -phase)	Photodiode array detector; MS, NMR.	Simple phenols, secoiridoids and lignans	[193]
30 min	A: H ₂ O + 0.1% trifluoroacetic (TFA- <i>d</i>) B: MeCN+ 0.1% (TFA- <i>d</i>)	Phenomenex RP-C18, 25 cm x 4.6 mm i.d. 5 μm	Gradient	LLE with methanol/water (80:20 v/v) (50 g of oil)	LC-SPE- NMR system	Complete characterization of 27 phenolic compounds in olive oil. 7 compounds not detected in the past	[174]

Recently, Christophoridou *et al.* [174] reported the first application of the hyphenated LC-SPE-NMR technique using postcolumn solid-phase extraction to direct identification of new phenolic compounds in the polar fraction of VOO. The addition of a post-column SPE system to replace of the loop system of the LC-NMR, resulted in higher sensitivity (significant increase of the signal to noise [S/N] ratio); in fact, S/N improvements by up to a factor of 4 could be demonstrated with this new technology [16]. The spectra recorded were one dimensional (1D) ¹H-NMR and two dimensional (2D) NMR. The presence of phenols was confirmed from the respective LC-SPE-NMR spectra, which were assigned on the basis of existing ¹H-NMR databases and with total correlation spectroscopy (TOCSY). The most interesting findings of this study were the verification of the presence of the lignan syringaresinol, the presence of two stereochemical isomers of the aldehydic form of oleuropein and the detection of homovanillyl alcohol.

As commented above the researches that studied olive mill waste, brines olive drupes, tissues of olive cultivars, alperujo, olives, olive leaves were not included in Table 3 [49, 161, 194-199]. Servili *et al.* [200] in a HPLC investigation of the phenols present in olive fruit, VOO, vegetation waters and pomace, and subsequently by 1D- and 2D-NMR achieved the complete spectroscopic characterization of demethyloleuropein and verbasoside extracted from olive fruit.

There are also several interesting reports describing the analysis of olive oil by ionspray ionization tandem mass spectrometry (IS-MS/MS) and ESI-MS/MS with NMR, without the use of a previous separative technique [201-203]. For the purposes of this review it is important to include a recent publication by Christophoridou *et al.* [204], where the authors demonstrate the potential of ³¹P-NMR spectroscopy to detect and quantify a large number of phenols in VOO extracts. This novel analytical method is based on derivatization of the hydroxyl and carboxyl groups of phenolic compounds with 2-chloro-4,4,5,5 tetramethyldioxaphospholane and the identification of the phosphitylated compounds on the basis of the ³¹P chemical shifts.

Even if the characterization and quantification of phenolic compounds have been successfully carried out by GC and HPLC, the use of faster analytical techniques and screening tools, allowing a rapid screening of phenolic compounds from VOOs, is strongly recommended. Although compared with GC or HPLC, CE is a relatively new technique in food analysis. A large variety of foods have

already analyzed by this technique, as CE can represent a good compromise between analysis time and satisfactory characterization for some classes of phenolic compounds in VOO.

CE is characterized by high separation efficiency, small sample and electrolyte consumption, and the separation requires only several minutes. This last characteristic is the main advantage versus chromatographic methods, which makes CE useful for routine analysis as well as for controlling and monitoring processes in a number of industrial fields [205-213]. Moreover, CE is relatively well suited to analysis of samples with complex matrices, like VOO.

CE technique can be coupled with different detectors (UV, FIL, electrochemical detectors, MS...). To date, for the analysis of phenolic compounds in VOO, there are several papers reporting the use of CE with ultraviolet detection; it is possible to study results obtained by using CE-MS in only two papers (Table 4).

Along these lines, the use of CE as an analytical separation technique coupled with mass spectrometry as a detection method can provide important advantages in the analysis of phenolic compounds of olive oil because of the combination of the high separation capabilities of CE and the power of MS for identification and confirmation method.

Using mass spectrometric detection, differences in optical detection must be considered. First, the separation electrolyte has to be volatile, reducing the choice of buffering system primarily to ammonia, acetate, or formate. While there are reports documenting nonvolatile buffers from UV-CE, only low buffer concentrations can be used and thus lower sensitivity must be accepted. Generally, nonaqueous solvents are well-suited for hyphenation with MS and add another parameter to modify selectivity.

As commented before for HPLC coupled with MS, CE can also be coupled with different MS analyzers (i.e., with quadrupole, ion trap, time-of-flight, etc.) and use several ionization methods (APCI, ESI, MALDI). ESI is one of the most versatile ionization methods and is the natural method of choice for the detection of ions separated by capillary zone electrophoresis. Regarding the analyzers, ion trap (IT) and TOF systems are the two analyzers more common in the lab of food analysis [214], although single-quadrupole MS is still often used as an easy and affordable detector.

Of particular interest is the coupling of CZE to ESI-TOF-MS. This coupling combines the abovementioned benefits of CZE separation with the high selectivity due to mass accuracy of 5 ppm, which opens the possibility of determining elemental compositions. The analysis of the true isotopic pattern by ESI-TOF-MS has recently been shown to provide an additional analytical dimension for identification [160].

During the last decade, concerning phenolic compounds present in VOO, it is possible to find reports in which applicative work is carried out, as well as other where a new analytical method is developed. Herein, the publications including CE-UV and CE-MS are summarized (see Tables 4 and 5).

Table 4. Summary of optimized conditions of capillary electrophoresis methods where VOO samples are analyzed. V, voltage; T, temperature, i. d., internal diameter of capillary; L_{ef} , effective length of capillary; [Buffer]; buffer concentration.

References	Instrumental Variables						Experimental Variables			
	λd [nm]	V [kV]	T [°C]	i. d. [μm]	Lef [cm]	t_{inj} [s]	Type of Buffer	[Buffer] [mM]	pH	Organic modifiers and other variables
[182]	200	27	30	50	40	3 s (0.5 p.s.i)	Sodium Tetraborate	45	9.6	-
[215]	CZE method of Bendini et al.[182]									
[185]	CZE method of Bendini et al.[182]									
[31]	210	25	25	75	50	8 s (0.5 p.s.i)	Sodium Tetraborate	25	9.6	-
[29]	200	18	25	50	36	2 s (1.5 p.s.i)	Sodium Tetraborate	40	9.2	-
[32]	210	-25	25	75	50	8 s (0.5 p.s.i)	Sodium Tetraborate	50	9.6	20% 2-propanol
[216]	214/250	25	25	75	100	8 s (0.5 p.s.i)	Sodium Tetraborate	30	9.3	
[217]	214/MS (ESI-IT)	25	25	50	100	10 s (0.5 p.s.i)	NH ₄ OAc	60	9.5	5% 2-propanol Sheath liquid (60:40 v/v 2-propanol/ water and 0.1% v/v of TEA at a flow rate of 0.28 mL/h)
[218]	200/240/280/330	28	22	50	40	8 s (0.5 p.s.i)	Sodium Tetraborate	45	9.3	
[219]	CZE method of Carrasco-Pancorbo [218]									
[188]	MS (ESI-TOF)	30	25	50	85	10 s (50 mBar)	Ammonium hydrogen carbonate	25	9.0	Sheath liquid (2-propanol/ water 50:50 v/v at a flow rate of 4 $\mu\text{L}/\text{min}$)

Table 5. Summary of extraction systems used and compounds detected in VOO samples with the application of each method. HYTY, hydroxytyrosol; TY, tyrosol; DHPE, 2,3-dihydroxyphenylethanol; VA, vanillic acid; DOA, decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA); Ac Pin, (+)-1-acetoxypinoresinol; Lig Agl, ligstroside aglycone; Ol Agl, oleuropein algycone; EA, elenolic acid

References	Initial quantity of oil→Final quantity of solvent (MeOH/H ₂ O (50:50 v/v)) in the extraction process (kind of extraction)	Detected compounds in olive oil	Other relevant aspects
[182]	2 g → 1 mL (LLE [220])	HYTY, TY, unidentified secoiridoids compounds	1 st paper where CE is used for the analysis of phenolic compounds from oils
[215]	2 g → 1 mL (LLE [220])	HYTY, TY, DHPE, unidentified oleuropein aglycone derivatives	
[185]	2 g → 0.5 mL (LLE [220], as modified in [184])	HYTY, TY, VA, DOA, Ac Pin	
[31]	60 g → 0.5 mL (LLE [31])	13 phenolic acids + taxifolin (flavanonol)	Potent extraction system which permits detection of small quantities of phenolic acids
[29]	10 g → non specified (Combination of LLE-SPE [29])	5 phenolic acids	
[32]	60 g → 0.5 mL (LLE [31])	13 phenolic acids + taxifolin (flavanonol)	Co-electroosmotic CE
[216]	60 g → 2 mL (SPE-Diol [216])	TY, Pin, Ac Pin, DOA, Lig Agl, HYTY, Ol Agl, EA	Use of standards obtained by semipreparative-HPLC
[217]	60 g → 2 mL (SPE-Diol [216])	11 phenols (simple phenols, lignans, complex phenols and EA)	1 st paper in which CZE-ESI-IT MS is used for the analysis of phenolic compounds from oils
[218]	60 g → 2 mL (SPE-Diol [216])	26 compounds belonging to all the different families of phenolic compounds present in olive oil	26 compounds in less than 10 min. 1 st paper in which flavonoids are detected by CE, and 1 st “multicomponent” method for the determination of olive oil phenols
[219]	60 g → 2 mL (SPE-Diol [216])	Applicative work using a previously method [218]	Interesting from a quantitative and applicative point of view
[188]	60 g → 2 mL (SPE-Diol [216]) and diluted 1:10	All the “well-known” phenolic compounds and 28 other analytes	1 st paper in which CZE-ESI-TOF MS is used for the analysis of phenolic compounds from oils. TOF permits the “identification” of new compounds in the oil’s profile

Concluding remarks and future outlook

The amount of phenolic compounds is a very important parameter when evaluating the quality of VOOs. Phenols are closely related with both the resistance of the oil to oxidation and the typical bitter and pungent tastes. Furthermore, some studies have shown that the amount of phenols, particularly those with a catecholic structure, together with a favorable monounsaturated to polyunsaturated fatty acid ratio, is related to several healthy attributes. These different aspects make VOO a very valuable and appreciated dietary lipidic condiment, and add importance to the determination of its phenolic compounds, both qualitative and quantitatively. The most commonly methods used for phenolic determination in VOO are based on GC and HPLC, and more recently on CE, coupled with different detector systems (UV, FLD, amperometric or coulometric). If the literature regarding phenolic compounds of VOO is analyzed in detail, it is evident that this class of compounds has not been completely studied, because of the complexity of their chemical nature and the complexity of the matrix in which they are found. During the last ten years, MS and NMR have become indispensable to study the quali-quantitative profiles of phenols and their oxidative forms, and detectors with the power to identify compounds and provide the analyst with information about the molecular structure are essential.

Apart from the interest on knowing in composition of the polar fraction of VOO, the determination of these compounds also helps to understand their health benefits that include reduction of risk factors of coronary heart disease, prevention of several varieties of cancer and modification of immune and inflammatory responses. It is also of interest to distinguish what phenolic molecules are responsible for bitterness, pungency, astringency and metallic sensations and to evaluate the antioxidant activities of the polar fraction.

Although excellent progress has already been made, it is expected that the use of different methodologies of potent techniques coupled with rapid, reliable and sophisticated detectors will become more common in the near future; there are still many “unknown” compounds present in the polar fraction of olive oil and it is very important to carry out collaborative studies to join the efforts of the scientific community.

Abbreviations: 3,4-dihydroxyphenyl-ethanol or hydroxytyrosol (3,4-DHPEA); 3,4-dihydroxyphenyl-ethanol acetate or hydroxytyrosol acetate (3,4-DHPEA-AC); 3,4-dihydroxyphenyl-ethanol linked to elenolic acid (3,4-DHPEA-EA); 3,4-dihydroxyphenyl-ethanol linked to dialdehydic form of elenolic acid (3,4-DHPEA-EDA); 1-acetoxypinoresinol (Ac Pin); butylated hydroxytoluene (BHT); Capillary Electrophoresis (CE); cyclooxygenase (COX); diphenylpicrylhydrazyl (DPPH); elenolic acid (EA); electrospray ionization mass spectrometry (ESI-MS); Flame Ionization Detector (FID); gas chromatography (GC); High Performance Liquid Chromatography (HPLC); HPLC-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS); ion-trap mass spectrometry (IT-MS); low-density lipoprotein (LDL); Liquid-Liquid- extraction (LLE); limit of detection (LOD); mass spectrometry (MS); tandem mass spectrometry (MS/MS); multiple-stage mass spectrometry (MSⁿ); nuclear magnetic resonance (NMR); Oxidative Stability Instrument (OSI); *p*-hydroxyphenyl-ethanol or tyrosol (*p*-HPEA); *p*-hydroxyphenyl-ethanol linked to elenolic acid (*p*-HPEA-EA); *p*-hydroxyphenyl-ethanol linked to dialdehydic form of elenolic acid (*p*-HPEA-EDA); pinoresinol (Pin); triple

quadrupole (QqQ); Solid-Phase Extraction (SPE); virgin olive oil (VOO); secoiridoid (SID); time of flight mass spectrometry (TOF-MS); Total Peak Area Ratio (TPAR); ultraviolet (UV).

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