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Phenolic Fraction of Virgin Olive Oil: An Overview on Identified Compounds and Analytical Methods for their Determination

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ABSTRACT

Among edible oils, virgin olive oil (VOO) has nutritional, technological and sensory characteristics that make it a unique and basic ingredient of the Mediterranean diet. These properties of VOO are attributed not only to the fatty acid composition (high level of oleic acid), which is not significantly different from refined olive oil (ROO), but especially to the high content of phenolic compounds, which are reduced by refining (bleaching and deodorization phases). Phenolic compounds, acting as natural antioxidants, increase the resistance of the oil to storage and heating. Moreover, phenols are the main contributors to the typical tastes of VOO (bitter and pungent attributes), and may also contribute to the prevention of several human diseases. Polar phenolic compounds belong to different chemical classes: phenolic acids, phenyl ethyl alcohols, flavonoids, lignans and secoiridoids. Approximately 50 compounds present in the phenolic fraction have been separated and identified to date, although researchers have not reached a consensus for the correct identification/denomination for many of these compounds. Due to variety of analytical approaches carried out on the complex phenolic pattern of VOO by high performance techniques, some disagreements have been emphasized. The aim of this review paper is to summarize and critically analyze the available information about phenolic compounds in VOO.

Keywords: antioxidant, oxidation, chemical properties, chemical structure, identification, official methods

CONTENTS

INTRODUCTION

Compared to other vegetable oils normally subjected to a refining process, virgin olive oil (VOO) is well-known to have a higher quality in terms of health and sensory aspects, and its characteristic oxidative stability. These properties are related not only to the high monounsaturated/polyunsaturated fatty acid ratio of its lipid matrix, but especially to the presence of several minor compounds having a hydrophilic phenolic structure. The quali-quantitative profile in polar phenols of a VOO depends on a wide variety of variables that range from agronomic factors (olive cultivar, olive ripening, environment conditions, agricultural and harvesting methods) to technological variables (milling, malaxation and separation phases) and storage/distribution parameters (time, temperature, light, packaging). In a recent review (Servili *et al.* 2004) several agronomic and technological aspects that influence the presence of phenolics in VOO were well discussed. The role of the olive cultivar is particularly important because it affects the absolute concentration of the specific hydrophilic phenols of VOO (Cerretani *et al.* 2006). Moreover, a clear negative effect on the total phenolic concentration is related to fruit ripening, so in general the phenol content shows a decrease during olive ripening (Rotondi *et al*. 2004). Relationships between water availability during olive growing and phenolic concentration of VOO were investigated and, also if the results did not lead to univocal conclusions, generally a negative correlation between the level of water used during irrigation treatment and the concentration of phenols was observed (Tovar *et al.* 2001). As studied by Gómez-Caravaca *et al.* (2008), these antioxidant compounds are consumed if the

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quality of olives is bad as a consequence of olive fly attack (*Bactrocera oleae*). From a technological point of view, a good compromise should be found between time and temperature during malaxation phase to obtain a VOO with an optimal concentration in phenols. In fact, the secoiridoids peculiar of VOO originate by enzymatic hydrolysis of respective glycosides (mainly oleuropein) during crushing and malaxation, therefore these steps are the most important critical points for the presence of phenols in VOO during the mechanical extraction process. During malaxation the concentration of secoiridoid aglycons decreases in olive pastes and oils with increasing time (Angerosa *et al.* 2001; Gallina Toschi *et al.* 2004). Moreover, the presence or absence of olive stones during crushing (Cerretani *et al.* 2008), as well as the type of separation system (Cerretani *et al.* 2005b) can influence the concentration of phenols. In fact, the use of new technologies, such as oil mechanical extraction from destoned pastes, can improve the oil phenolic concentration. This effect is due to a partial removal of peroxide enzymes, particularly present in olive seed and consequently to a reduction of the enzymatic degradation of phenols in the oils (Cerretani *et al.* 2008). Moreover the different type of separation system are by pressing, percolation, or centrifugation. The latter can be carried out using a two-phase (no addition of lukewarm water) or a three-phase centrifugation system (requiring the addition of lukewarm water). The amount of water added to the olive paste before the centrifugation step influences the phenolic content in VOO. In particular, the addition of water determines firstly a decrease in simple phenols (the most polar) and successively in secoiridoids and lignans (Gallina Toschi *et al.* 2004).

Finally, correct packaging, in terms of the bottle's shape and size, packing material used to make the bottle and initial value of the oxygen partial pressure in the headspace, mainly contributes to preserve the antioxidant content (Del Nobile *et al.* 2003). In this respect, the use of inert gases in the head-space of the container, mainly nitrogen or argon, as a conditioner gas during storage to remove oxygen improves the stability of VOO thanks also to a lower decrease in phenolic molecules (Di Giovacchino *et al.* 2002). The shelf life of packaged VOO under various storage conditions may also be predicted by applying mathematical modelling and simulations (Kanavouras *et al.* 2006). Prevention of light exposure during storage of VOO is therefore very relevant to extend its shelf life because light radiation it is able to initiate auto-oxidation and to produce photo-oxidation, indirectly consuming the antioxidant compounds (Caponio *et al.* 2005; Luna *et al.* 2006). For this reason the opacity of the packaging material to light is fundamental for the preservation of th phenolic fraction (Méndez and Falqué 2007). As mentioned earlier, a consequence of the oxidative reaction is the degradation of the naturally occurring VOO minor components. The rate of degradation of α-tocopherol, squalene and phenolics in VOO during different storage conditions was reported by several authors (Lavelli *et al.* 2006; Gomez-Alonso *et al.* 2007).

In the last two decades, efforts have been made by researchers in order to correlate the chemical structures of these naturally occurring molecules in VOO with their antioxidant properties such as health benefits and sensory characteristics. As regards the antioxidant properties of phenolics in VOO, some concepts will be discussed in the following paragraph.

Interesting healthy activities have been reported in recent publications: for example it was confirmed by several authors (Visioli *et al.* 2002a, 2002b, 2004; Pérez-Jiménez 2005) that the phenolic fraction of VOO exerts preventive effects against reactive oxygen species (ROS) mediated degenerative diseases as cancer. Salvini *et al.* (2006) measured the reduction of DNA damage by consumption of VOO rich in phenols, particularly hydroxytyrosol; Deiana *et al.* (2007) evidenced the protective effect of phenolic fraction of VOO against lipid peroxidation *in vivo*, other researchers (Covas *et al.* 2006; Bogani *et al.* 2007), by evaluating inflammatory and oxidative stress markers, correlated VOO phenols with a lower incidence of cardiovascular diseases. Finally, Cicerale *et al.* (2009) reviewed different positive effects of VOO phenolics on certain physiological parameters, such as plasma lipoproteins, oxidative damage, inflammatory markers, platelet and cellular function by *in vitro* studies and also *in vivo* researches conduced on humans and animals.

Many recent publications (Andrewes *et al.* 2003; Gutiérrez-Rosales *et al.* 2003; Mateos *et al.* 2004; Beauchamp *et al.* 2005; Baccouri *et al.* 2008; Esti *et al.* 2009) have explored the correlations between sensory perceptions and chemical composition in phenols found in VOO. These interesting investigations have shown that some phenols, mainly those belonging to the secoiridoid class, are responsible for the tasting perception of bitterness, while others can stimulate the free endings of the trigeminal nerve located both in the gustative buds and the palate, inducing the chemesthetic perception of pungency.

It is important to underline that, based on their chemical characteristics, each phenolic molecule may have a different antioxidant capacity and health benefits, as well as distinct gustative attributes. Therefore, the quantitative abundance of individual phenolic compounds is fundamental for the many qualitative aspects of VOO.

Due to its stability against oxidative degradation, VOO is suitable for cooking and frying. Several investigations have been carried out to determine the types of changes that phenols undergo during heating, and both spectrophotometric (Albi *et al.* 1997; Pellegrini *et al.* 2001) and chromatographic analyses (Brenes *et al.* 2002a; Gomez-Alonso *et al.* 2003; Beltran-Maza *et al.* 1998; Nissiotis and Tasioula-Marhari 2002; Carrasco-Pancorbo *et al.* 2007a; Cerretani *et al.* 2009) have been utilised in this regard. Some researchers have subjected VOO, in presence or absence of food such as potatoes, to thermal treatments under temperatures (160-190°C) and heating times typical of domestic frying conditions (0.5-2 h) (Beltran-Maza *et al.* 1998; Pellegrini *et al.* 2001; Gomez-Alonso *et al.* 2003; Carrasco-Pancorbo *et al.* 2007a); others have performed thermal oxidation from 60 to 100°C in an oven during extended periods (Nissiotis and Tasioula-Marhari 2002). All the components identified in the phenolic fraction decreased in concentration with increasing duration of thermal treatment, although the rate of loss depended on the chemical structure and antioxidant activity. Generally, under heating, the *o*-diphenolic secoiridoids, which are hydroxytyrosol derivatives and have a high antioxidant capacity, showed more drastic decreases than those derived from tyrosol, whereas the most stable phenols were compounds belonging to the lignan class. In a recent publication (Carrasco-Pancorbo *et al.* 2007a), other authors detected several unknown peaks which were higher when the thermal treatment was longer; some of these unidentified compounds were also found in a commercial refined olive oil and in crude refined olive oil sampled after the bleaching phase of the refining process. In fact, during the refining process, and in particular as a consequence of the application of drastic conditions of heating (also if under vacuum) or the use of bleaching earths (time-temperature during bleaching and deodorization steps), most phenolic compounds are degraded and only a very small amount remain in the refined oil. Thus, the presence of many phenolics found in olive oil is mainly due to those from the virgin olive oil that is blended with the refined olive oil (EC Regulations have not established this percentage) (EC Regulation No 702/2007).

Another aspect that it is important to keep in mind is the fluctuation of VOO storage temperature that can occur during storage/marketing phases in either winter or summer: a temperature that is too high or too low (with respect to room temperature) may have significant effects on phenolic substances and indirectly affect both the shelf life and sensory characteristics of the oil. In fact, a low storage temperature may positively influence the oxidative stability of VOO by slowing the formation of oxidation compounds,

but at the same time may decrease the protective activity of the phenolics towards lipid oxidation. As previously shown (Bonoli-Carbognin *et al.* 2005; Cerretani *et al.* 2005a), this latter effect seems to be linked to destabilization of micro-droplets of water in the oil (during crystallization of more saturated triacylglycerols and waxes) in witch polar phenolic molecules are solubilised.

ANTIOXIDANT ACTIVITY OF PHENOLIC MOLECULES WITH PARTICULAR EMPHASIS ON THE ROLE OF VOO PHENOLICS

During VOO storage, even in small quantities, phenols are fundamental to protect glycerides from oxidation. In fact, due to their favourable oxidation potential, phenols are able to determine an effective antioxidant activity. Thanks to the studies carried out by many researchers in the last decades it has been established that VOO phenolics can inhibit glyceride oxidation as a consequence of several potentially synergistic mechanisms such as radical scavenging, hydrogen atom transfer and metal-chelating activity. As radical scavengers or chain breakers, they act by donating a hydrogen radical to alkoxyl and peroxyl radicals formed during the initiation step of lipid oxidation, slowing down the total rate of autoxidation. Hydrogen donation generally occurs through the hydroxyl group of a phenolic molecule, and the subsequently formed radical is stabilized by resonance delocalization of the phenolic ring structure. The presence of a second hydroxy group, especially at the *ortho* position, stabilizes the phenoxyl radical through an intramolecular hydrogen bond; thus phenols with a catechol moiety, known as *o*-diphenols, are particularly effective antioxidants. At the same time, *o*-diphenol structures have the capacity to bind metals such as iron and copper that are present in trace amounts in VOO. This metal chelating capacity is especially important in VOO since metals are powerful catalysts of lipid autoxidation. Several studies (Paiva-Martins and Gordon 2002, 2005; Bendini *et al.* 2006) have investigated the antioxidant effect of the *o*-diphenolic fraction of VOO through activity measurement of the entire *o*-diphenolic fraction and by testing the effects of individual catechols in lipid model systems (Litridou *et al.* 1997; Carrasco-Pancorbo *et al.* 2005).

It is important to stress that the high stability of VOO towards lipid oxidation is improved by the presence of various antioxidants as hydrophilic and lipophilic phenols, and for example, antioxidant synergism has been observed between *o*-diphenols and α-tocopherol. Most likely, phenols having an *ortho*-dihydroxyl structure can be potentially active in the regeneration of α-tocopherol via reduction of α-tocopheroxyl radical with a capacity that is proportional to the ability of phenolics to transfer a single H-atom (Baldioli *et al.* 1996; Servili *et al.* 1996; Bendini *et al.* 2006; Pazos *et al.* 2007).

Another aspect to keep in mind, which has important implications for the antioxidant activity of natural molecules in VOO, is related to the distribution of hydrophilic (polar phenols) and lipophilic (tocopherols) phenols in lipidic substrates. These compounds exhibit complex interfacial affinities between air-oil and oil-water interfaces that significantly affect their antioxidant power in VOO. Based on a mechanism known as the "polar paradox", intensively studied by Porter and Frankel (Porter *et al.* 1989; Frankel *et al.* 1994; Frankel, 1996), in a bulk oil system hydrophilic antioxidants, such as polar phenols, are oriented in the air-oil interface (a low quantity of air remains always inside of the VOO) and become more protective against oxidation than the lipophilic antioxidants, like tocopherols, which are solubilised in the oil. Therefore, in VOO polar phenols are more effective as antioxidants than tocopherols.

Recently, some researchers (Fregapane *et al.* 2006; Gómez-Caravaca *et al.* 2007; Lercker *et al.* 2007) have conducted studies on the presence of a low quantity of water in micro-emulsion in VOO (from 450 to 3000 mg kg^{-1} depending on the type of VOO extraction technology). Lercker and co-authors (Lercker *et al.* 2007) have suggested that polar phenols, in relation with their hydro-solubility, are arranged at the interface between water droplets and the lipid phase. On one hand, such an orientation of polar phenolic compounds in the oil-water interface, as in the air-oil interface, would explain how these hydrophilic molecules remain solubilised in a lipid system while providing high antioxidant protection against oil oxidation. Moreover, the close contact between water and polar phenols can elucidate a hydrolytic process (by chemical or enzymatic reactions) that occurs to complex molecules as secoiridoids during VOO storage and brings about the release of simpler structures as phenyl ethyl alcohols and elenolic acid (Cinquanta *et al.* 1997; Di Lecce *et al.* 2007). For instance, Cinquanta *et al.* (1997) studied the evolution of simple phenols during 18 months of VOO storage in the dark. They found a great increase in the tyrosol and hydroxytyrosol content due to hydrolysis of their complex derivatives in a first storage phase (from 0 to 6 months), and a rapid loss of hydroxytyrosol as compared with that of tyrosol at the end of the storage period (after 18 months), according to a higher antioxidant activity of the former (Cinquanta *et al.* 1997).

Most likely, it is due to the presence of this small amount of water that some enzymes naturally present in olive pulp during the oily paste malaxation are transferred in VOO; while polyphenoloxidase (PPO) is almost completely inactivated, other active enzymes such as lipoxygenase (LOX) and peroxidase (POD) maintain oxidoreductase activity during storage (Servili *et al.* 1998). For instance, LOX catalyses the formation of hydroperoxides and can also promote indirect oxidation of secoiridoids. In addition, β-glucosidase maintains its activity in VOO bringing about the production of aglycon derivatives of secoiridoids through hydrolysis of oleuropein and demethyloleuropein glycosides originally present in olive pulp (Servili *et al.* 1999a; Ranalli *et al.* 2003; Servili *et al.* 2003). As shown by several authors (Servili *et al.* 1999a; Brenes *et al.* 2001; Carrasco-Pancorbo *et al.* 2007a; Bendini *et al.* 2008; Boselli *et al.* 2009), the general effects attributable to phenolic compounds during VOO storage or as consequence of heating treatments are (**Fig. 1**): 1. lysis of complex phenols and increase of the content of low molecular weight phenolics like hydroxytyrosol and tyrosol (**Fig. 1A**); 2. increase of the dialdehydic forms of decarboxymethyl oleuropein aglycon and decarboxymethyl ligstroside aglycon (**Fig. 1B**); 3. hydrolysis of the acetic ester occurring for hydroxytyrosol (**Fig. 1C**); 4. cleavage of elenolic acid with loss of the carboxymethyl moiety and conversion of the monoaldehyde form to its dialdehyde form (**Fig. 1D**); 5. the appearance of unknown oxidation products of phenolics (especially the dialdehyde forms of decarboxymethyl oleuropein aglycon and decarboxymethyl ligstroside aglycon) (**Fig. 1E**).

Obviously, all the aforementioned modifications of phenolics, due to hydrolysis/oxidation reactions, also produce changes in the total antioxidant power of the phenolic fraction, and thus in oxidative stability of VOO.

CHEMICAL CLASSES OF POLAR PHENOLIC COMPOUNDS IN VOO

Approximately 50 compounds belonging to several chemical classes have been separated from the phenolic fraction of different VOO (Carrasco-Pancorbo *et al.* 2007b; Saitta *et al.* 2009). The polar phenolic compounds can be classified as:

- phenolic acids,
- phenyl ethyl alcohols,
- flavonoids,
- lignans
- secoiridoids.

The main compounds identified are reported in **Table 1**. For each compound, the table reports the chemical general structure, common compound name, empirical formula and molecular weight.

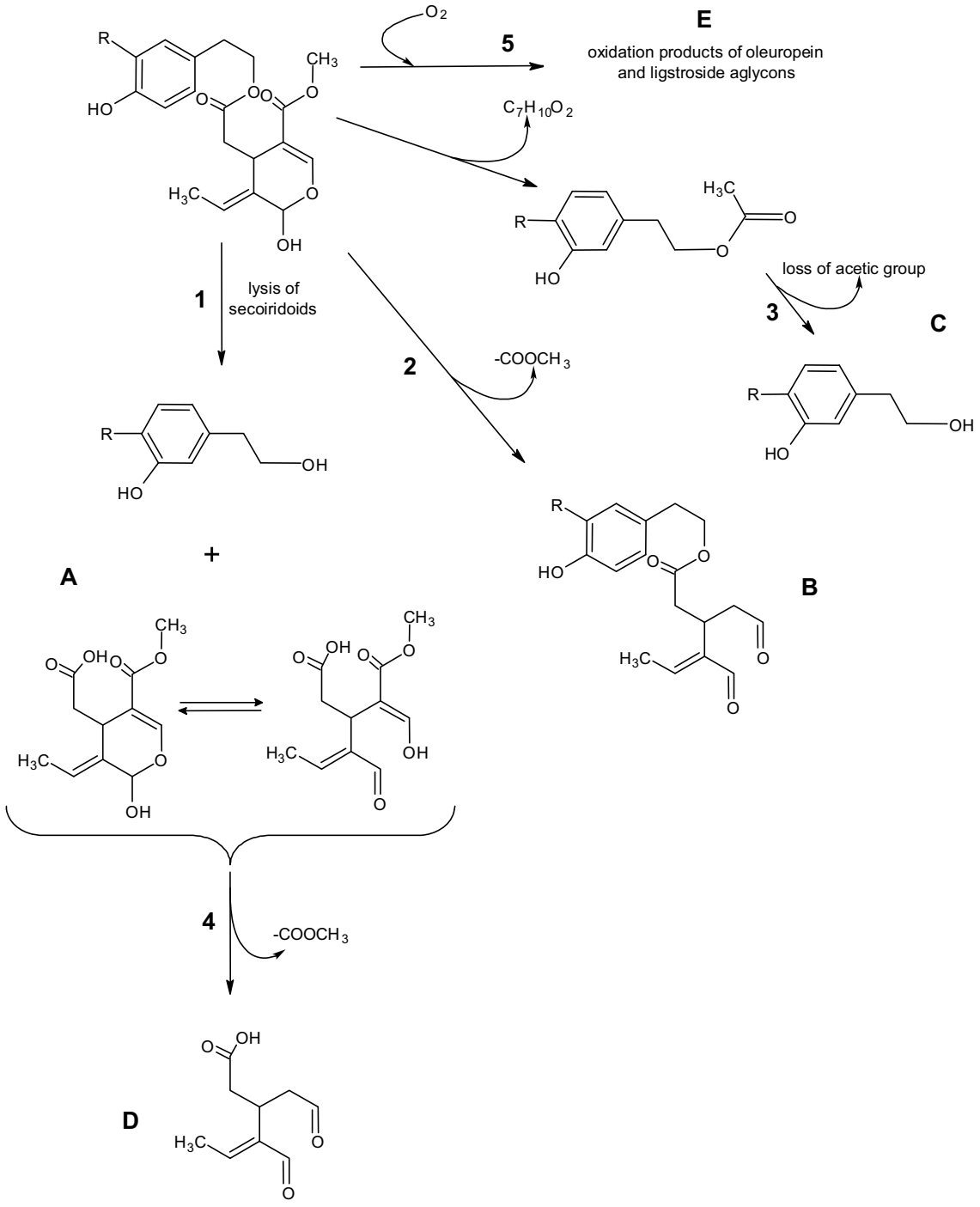


Fig. 1 Phenolic compounds transformations during virgin olive oils storage or heating treatments. (**A**) Lysis of complex phenols and increase of the content of low molecular weight phenolics like hydroxytyrosol and tyrosol; (**B**) Increase of the dialdehydic forms of decarboxymethyl oleuropein aglycon and decarboxymethyl ligstroside aglycon; (**C**) Hydrolysis of the acetic ester occurring for hydroxytyrosol; (**D**) Cleavage of elenolic acid with loss of the carboxymethyl moiety and conversion of the monoaldehyde form to its dialdehyde form; (**E**) The appearance of unknown oxidation products of phenolics (especially the dialdehyde forms of decarboxymethyl oleuropein aglycon and decarboxymethyl ligstroside aglycon).

Simple phenols in VOO: phenolic acids and phenyl ethyl alcohols

Simple phenols, such as phenolic acids and phenyl ethyl alcohols (**Table 1**), were the first group of phenols observed in VOO (Montedoro, 1972; Vasquez-Roncero, 1978). These compounds are characterized by a molecular weight generally lower than 200 g mol^{-1}. Phenolic acids can be divided in two major groups as derivatives of either benzoic or cinnamic acid (**Table 1**). These compounds are generally present in small amounts (<10 mg per kg of oil) and have been found by several authors (Cartoni *et al.* 2006; Carrasco-Pancorbo *et al.* 2006b; Baccouri *et al.* 2007; Carrasco-Pancorbo *et al.* 2007a, 2007b; Saitta *et al.* 2009). Previous and preliminary studies carried out using a variety of separation techniques (Nergiz and Unal 1991; Montedoro *et al.* 1992; Carrasco-Pancorbo *et al.* 2004) detected high amounts of these compounds, a discrepancy that was probably due to incorrect tentative identification based only on retention time and UV spectra.

With regards to phenyl ethyl alcohols, hydroxytyrosol and tyrosol are the main simple phenolic compounds present in VOO, and range from 0 to approximately 70 mg per kg of oil (Gallina Toschi *et al.* 2005; Cerretani *et al.* 2009; Saitta *et al.* 2009). Their amount in VOO is generally low in fresh oils, but increases during oil storage (Montedoro *et al.* 1992; Di Lecce *et al.* 2007; Boselli *et al.* 2009). Most recently, hydroxytyrosol acetate has been identified (Gordon

Table 1 Phenolic compounds in virgin olive oil: their chemical general structure (gray for hydrogen, cyan for carbon and red for oxygen atoms), common name, molecular formula and molecular weight.

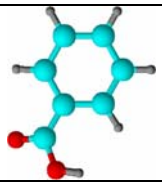
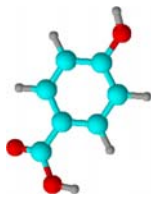
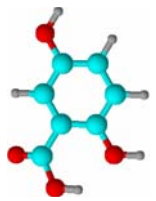
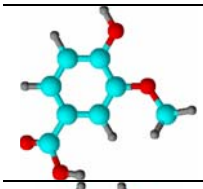
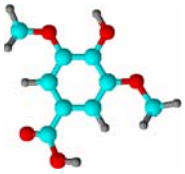
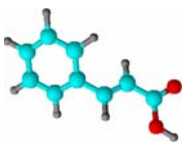
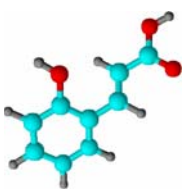
	Common compound name and empirical formula	Molecular weight (g/mol)
PHENOLIC ACIDS-BENZOIC ACID DERIVATIVES		
	Benzoic acid $C_7H_6O_2$	122.121340
	4-Hydroxybenzoic acid or *p*-Hydroxybenzoic acid $C_7H_6O_3$	138.120740
	Gentistic acid $C_7H_6O_4$	154.120140
	Protocatechuic acid $C_7H_6O_4$	154.120140
	Vanillic acid $C_8H_8O_4$	168.146720
	Gallic acid $C_7H_6O_5$	170.119540
	Syringic acid $C_9H_{10}O_5$	198.172700
PHENOLIC ACIDS-CINNAMIC ACID DERIVATIVES		
	Cinnamic acid $C_9H_8O_2$	148.158620
	*o*-Coumaric acid $C_9H_8O_3$	164.158020

Table 1 (Cont.)

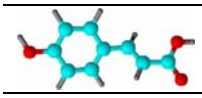
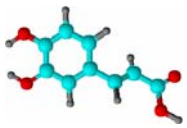
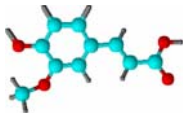
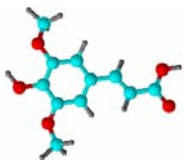
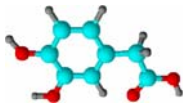
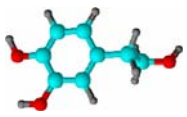
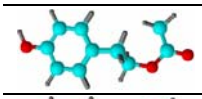
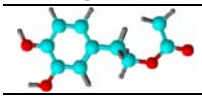
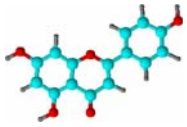
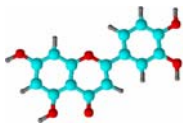
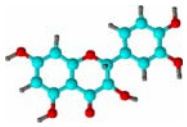
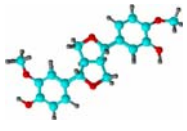
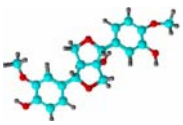
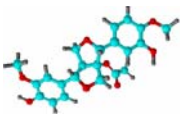
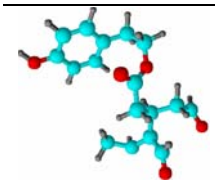
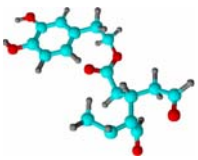
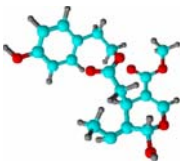
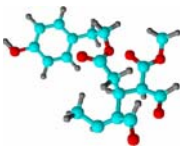
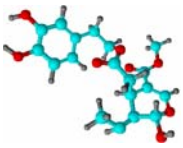
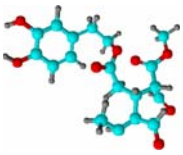
	Common compound name and empirical formula	Molecular weight (g/mol)
PHENOLIC ACIDS-CINNAMIC ACID DERIVATIVES		
	*p*-Coumaric acid $C_9H_8O_3$	164.158020
	Caffeic acid $C_9H_8O_4$	180.15742
	Ferulic acid $C_{10}H_{10}O_4$	194.184000
	Sinapinic acid or Sinapic acid $C_{11}H_{12}O_5$	224.209980
OTHER PHENOLIC ACIDS		
	Dopacetic acid or 3,4-Dihydroxyphenylacetic $C_8H_8O_4$	168.146720
PHENOLIC ALCOHOLS		
	Tyrosol or *p*-hydroxyphenyl-ethyl alcohol $C_8H_{10}O_2$	138.163800
	Hydroxytyrosol or 3,4-dihydroxyphenyl ethyl alcohol $C_8H_{10}O_3$	154.163200
	Tyrosol acetate $C_{10}H_{12}O_3$	180.200480
	Hydroxytyrosol acetate $C_{10}H_{12}O_4$	196.199880
FLAVONOIDS-FLAVONES		
	Apigenin $C_{15}H_{10}O_5$	270.236900
	Luteolin $C_{15}H_{10}O_6$	286.236300
FLAVANONOL		
	(+)- Taxifolin $C_{15}H_{12}O_7$	304.25158
LIGNANS		
	(+)-Pinoresinol $C_{20}H_{22}O_6$	358.385080

Table 1 (Cont.)

	Common compound name and empirical formula	Molecular weight (g/mol)
LIGNANS		
	(+)-1-Hydroxypinoresinol $C_{20}H_{22}O_7$	374.384480
	(+)-1-Acetoxypinoresinol $C_{22}H_{24}O_8$	416.421160
SECOIRIDOIDS		
	Oleocanthal or Dialdehydic form of decarboxymethyl elenolic acid linked to *p*-HPEA or *p*-HPEA-EDA $C_{17}H_{20}O_5$	304.337700
	Dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA or 3,4-DHPEA-EDA $C_{17}H_{20}O_6$	320.337100
	Ligstroside aglycon or *p*-HPEA-EA $C_{19}H_{22}O_7$	362.373780
	Dialdehydic form of ligstroside aglycon $C_{19}H_{22}O_7$	362.373780
	Oleuropein aglycon or 3,4-DHPEA-EA $C_{19}H_{22}O_8$	378.373180
	Dialdehydic form of oleuropein aglycon $C_{19}H_{22}O_8$	378.373180

et al. 2001; Carrasco-Pancorbo *et al.* 2007b) in VOO, and a higher antioxidant activity has been ascribed to hydroxyltyrosol as compared to phenolic compounds present in VOO (Carrasco-Pancorbo *et al.* 2005; Bešter *et al.* 2008).

Flavonoids in VOO

Flavonoids occur widely in plants and are chemically diverse groups of secondary metabolites that can be divided into subgroups including anthocyanidins, flavonols, flavones, flavanols, flavanones, chalcones, dihydrochalcones and dihydroflavonols (Treutter 2006). Many flavonoids absorb radiation most strongly in the ultraviolet (UV) region and form special UV patterns on flower petals (Havsteen 2002). The identification of the flavones apigenin and luteolin (**Table 1**) in VOO was reported for the first time by Rovellini and co-workers (1997). The UV spectra of these flavones show two major absorption maxima at about 250 and 350 nm. The amount of these compounds in VOO is very low and generally ranges between 0 and 10 mg/kg of oil (Bendini *et al.* 2007b). In 2004, Carrasco-Pancorbo and co-authors reported the presence of taxifolin (**Table 1**) in Spanish VOO in amounts less than 1 mg per kg of oil (Carrasco-Pancorbo *et al.* 2004).

Secoiridoids in VOO

Secoiridoids are the main class of phenols present in olive fruit and oil; in particular oleuropein and ligstroside are the two main secoiridoid glucosides present in olive fruit. During VOO production, their aglycon derivatives are formed by two pathways (Rovellini and Cortesi 2002):

- by β-glucosidase activity giving rise to aglycons with an aldehyde structure (Gariboldi *et al.* 1986);
- by hydrolytic mechanism, in which the final molecules, due to the loss of the glucose, present an hydroxyl group linked to the oxygenated ring of the elenolic acid. Moreover, these structures are subjected to opening of elenolic ring that includes the formation of elenolic species, which successively isomerize to a dialdehyde open structure as proposed by Montedoro and co-workers (1993).

Taking into account that secoiridoids represent the major compounds in the phenolic fraction of fresh VOO it is important to underline that both the agronomical (cultivar, cultivation area) and technological aspects of VOO production affect their concentration (Cerretani *et al.* 2006). Therefore, the definition of their average concentration is somewhat difficult, and may range from a few mg up to 800-900 mg/kg of oil (Servili *et al.* 2002). In agreement with several authors (Servili *et al.* 2002; Baccouri *et al.* 2007; Bendini *et al.* 2007b; Gómez-Alonso *et al.* 2007), the forms that are most abundant in fresh VOO are the dialdehydic form of oleuropein aglycon followed by the aldehydic form of oleuropein aglycon and the decarboxymethylated forms of oleuropein aglycon.

Derivatives from phenolic oxidation in VOO

During storage, phenolic compounds contained in VOO undergo oxidative degradation, and the products of oxidation can be found. The first study in this area was published by Rovellini and Cortesi (2002), who proposed several oxidized forms derived from phenolic compounds of VOO. In the initial study, the authors found oxidized forms of each phenolic compound linked to elenolic acid. In particular, the authors observed the presence of four oxidized derivatives of elenolic acid; oxidized dialdehydic forms of decarboxymethyl ligstroside and oleuropein aglycon; oxidized aldehydic forms of ligstroside and oleuropein aglycon and oxidized ligstroside and oleuropein aglycon. In each of previous cases, the oxidized forms had a molecular weight that was 16 g mol^{-1} greater with respect to the original parent compound (Rovellini and Cortesi 2002). Successively, Ríos *et al.* (2005), starting from oils oxidized at 100° C and under air flow, collected individual oxidation products from an oxidized sample by preparative HPLC, converted these compounds to trimethylsilyl (TMS) ethers and finally determined the structures of these oxidized forms by GC–MS. In this work, the oxidation products of secoiridoids were identified based on ions with *m/z* 255 for dialdehydic forms and with *m/z* 313 for aldehydic forms of decarboxymethyl ligstroside and oleuropein aglycons, coming from a loss of carboxyl group (Ríos *et al.* 2005). Armaforte *et al.* (2007) proposed an index to establish the degree of freshness of VOO. This value, termed TPAR (ratio between total peak area of reduced and oxidized forms of phenols), is based on the evaluation of oxidized forms of phenolic compounds and is close to 1 for fresh samples, whereas it decreases rapidly in VOO with an increasing content of oxidized phe-

nols. Most recently, other researchers (Boselli *et al.* 2009) observed that the oxidation products of the dialdehydic forms of ligstroside and oleuropein aglycon appeared after 9 months of storage at room temperature.

FIRST APPROACHES TO ANALYZE PHENOLS IN VOO: SAMPLE PREPARATION AND EXTRACTION PROCEDURES

The problems related to the determination of phenols in VOO are linked to the complexity of the family of molecules commonly defined as "phenols" which, as previously mentioned, comprises many subgroups characterized by different chemical and physical proprieties (e.g. molecular size, polarity, stability, extractability by solvents, UV absorption). The lack of homogeneity of phenols in VOO together with the quali-quantitative variability due to the cultivar, pedoclimatic and agronomic conditions, fruit ripening and conservation has rendered it difficult to find universally accepted procedures for isolation, quantification and determination of single components.

In reality a standard procedure, even for the quantification of total phenols in VOO, is not available, although a method has been recommended by the International Olive Council (Resolution no. RES-4/94-V/06); many researchers are looking for the analytical parameters and conditions that will provide comparable results in terms of recovery and quantification of the most representative classes.

One possible preparative step to remove interferences and to dose "simple phenols" (phenolic acids and alcohols) is acid or alkaline hydrolysis of the VOO sample before extraction of the polar fraction (Cortesi and Fedeli 1983; Montedoro *et al.* 1992). This treatment has also been proposed at the end of an extraction procedure to check its exhaustiveness (Brenes *et al.* 2000a), as an alternative to spiking the VOO with commercial phenols (Montedoro *et al.* 1992; Favati *et al.* 1994). Specifically, it consists in the treatment of the previously extracted VOO with 2 N HCl, followed by HPLC analysis and quantitative determination of the "residual" hydroxytyrosol and tyrosol (predominant simple phenols). More recently, other researchers (Mulinacci *et al.* 2006) proposed both an acid (0.5 M H_2SO_4, 2 h, 80°C) and an alkaline hydrolysis (5 M KOH in MeOH) to indirectly quantify secoiridoids (by measuring tyrosol and hydroxyltyrosol) and to improve the recognition of lignans, pinoresinol and acetoxypinoresinol in the total phenolic fraction. In fact, after alkaline hydrolysis, pinoresinol and acetoxypinoresinol are almost completely degraded and are no longer present on the HPLC profile.

Simple and complex phenols are generally extracted and concentrated from VOO by liquid-liquid extraction (LLE) or solid phase extraction (SPE). Other procedures, capable of using less organic solvents, such as supercritical fluid extraction (SFE), have been used to concentrate phenols from olive leaves (Le Floch *et al.* 1998; Tena *et al.* 1998) or other supports, but carbon dioxide, even if modified with 10% methanol, recovers only 45% of the amounts extracted by liquid methanol (Le Floch *et al.* 1998). At any rate, SFE does not seem suitable for extraction of phenols from VOO, even using high percentages of a polar modifier, due to the presence of 98% of simple lipids (mainly triacylglycerols) miscible with carbon dioxide.

LLE was the first preparative technique employed, and the pure VOO or the oil added with a lipophilic solvent (generally *n*-hexane) is extracted with methanol or methanol/water (Montedoro *et al.* 1971; Vasquez-Roncero 1978; Solinas and Cichelli 1981; Cortesi and Fedeli 1983; Solinas 1987; Montedoro *et al.* 1992; Angerosa *et al.* 1995; Owen *et al.* 2000; Tasioula-Margar and Okogeri 2001; Bendini *et al.* 2003; Hrncirik and Fritsche 2004). The phenolic fraction is recovered after washing the dried extract by solvent partition and used for the subsequent separation. Emulsions formed by methanol/water solution and VOO were reported to be problematic with the procedure (Angerosa *et al.* 1995) suggesting the use of pure methanol or other extracting solvents such as ethanol, tetrahydrofuran/water or *N*,*N*-dimethylformamide might be preferred (Brenes *et al.* 2000a).

Scheme 1 shows the flowchart of one of the first and most cited LLE methods employing methanol/water (80:20, v/v) and two other more recent procedures, provided as examples, requiring low amounts of oil, solvents, energy and time. Methanol/water (from 80:20 to 60:40 v/v) remains a first choice mixture in the renewed low-scale extraction procedure (LLE 3) (Pirisi *et al.* 1997; Rotondi *et al.* 2004).

The SPE technique, widely used for isolation of phenols in the last decade, should be considered highly selective, but more expensive and not always solvent saving. Many procedures have been proposed during recent years as many different SPE-phases have been made available. In particular, SPE short columns (generally 500 mg, 3 mL) composed of alkyl silicates (reverse phases, from C8 to C18) have

Scheme 1 Flowchart of three LLE procedures to extract phenols from VOO.

LLE 1 (Montedoro *et. al.* 1992)	LLE 2 (Brenes *et al.* 2000a)	LLE 3 (Pirisi *et al.* 1997; Rotondi *et al.* 2004)
VOO (14 g)	VOO (0.6 g)	VOO (2 g)
↓	↓	↓
methanol/water 80:20 (v/v), (14 mL, 4 times)	dimethylformamide (0.6 mL, 3 times)	*n*-hexane (1 mL) + methanol/water 60/40 v/v (2 mL, vigorus mixing and centrifugation 3000 g for 3 min, 3 times)
↓	↓	↓
vortex agitation and centrifugation (6000 g, 5 min, 4 times)	vortex agitation and centrifugation (6000 g, 5 min, 3 times)	washing of the combined hydroalcoholic fractions with *n*-hexane, evaporation in vacuum at 35°C
↓	↓	↓
Concentration of the extract (vacuum, 30°C)	addition of 0.2 mL I.S. (syringic acid) to the pooled DMF extract	solubilization of the residue in methanol/water 50/50 (v/v) (0.5 mL)
↓	↓	↓
solubilization of the residue in acetonitrile (15 mL)	washing with hexane (2 mL, twice)	filtration through a 0.2 μm nylon filter
↓	↓	↓
washing with hexane (15 mL, 3 times)	vortex agitation and centrifugation (6000 g, 5 min, twice)	HPLC analysis (DAD/MSD detection)
↓	↓	
evaporation of acetonitrile (vacuum, 30°C)	bubbling N_2 into the DMF extract (10 min)	
↓	↓	
solubilization of phenolic extract in methanol (1 mL)	HPLC analysis (electrochemical detection)	
↓		
HPLC analysis (UV detection)		

been tested (Papadopoulos and Tsimidou 1992; Mannino *et al.* 1993; Favati *et al.* 1994; Favati *et al.* 1995; Pirisi *et al.* 1997; Servili *et al.* 1999b; Pirisi *et al.* 2000; Liberatore *et al.* 2001). C8-SPE, C18-SPE, Diol-SPE and LLE extraction have been compared, and the latter two methods showed higher recoveries of total phenols, *o*-diphenols, tyrosol, hydroxytyrosol and secoiridoids than others (Bendini *et al.* 2003). Hrncirik and Fritsche (2004) demonstrated that Diol-SPE led to significantly higher recovery of total phenolics (68%) than SPE-C18, which was lower in both cases than LLE (93%) carried out with methanol/water 60/40 (v/v). Liberatore *et al.* (2001) compared the performance of C18-SPE and C18-SPE with a total suppression of residual silanol group (C18 EC). In the case of C18 EC, the suppression of residual polar Si-OH groups makes the mechanism of release of the analyte worse. Cerretani *et al.* (2005a) and Liberatore *et al.* (2001) both confirmed the higher effectiveness of C18-SPE in extracting complex phenols such as oleuropein aglycon and derivatives; Cerretani *et al.* (2005a) obtained high yield and a similar phenolic profile by HPLC-DAD analyses of VOO phenolic extract by LLE and C18-SPE. However, it has been recently reported (Armaforte *et al.* 2007) that the stationary phase of Diol-SPE interacts with oxidized phenols, retaining them, while at the same time causing partial retention of syringic acid (IS). Therefore, the accumulation of polar oxidation products (from phenols or lipids) in Diol-SPE may interfere with the retention of phenols. For this reason, Diol-SPE seems to be an effective alternative to LLE for fresh samples, but may be inappropriate for samples in which phenols, fatty acids or triacylglycerols are oxidized.

The following Diol-SPE procedure is reported in the colorimetric determination of *o*-diphenols, one of the two provisional applicable methods recommended by the International Olive Council (Resolution no. RES-4/94-V/06) that will be described. The preparative step is reported in the following manner (Cert *et al.* 2006):

"A sample of olive oil (6±0.001 g) is dissolved in 6 mL of hexane. A diol-bonded phase 6-mL cartridge is placed in a vacuum elution apparatus, and conditioned by the consecutive passing of 9 mL of methanol and 9 mL of hexane. Then the vacuum is released to prevent drying of the column. The oil solution is applied to the column and passed through the cartridge. The sample container is washed with two 4-mL portions of hexane that are run out of the cartridge. The sample container is washed again with 3 mL of the mixture hexane/ethyl acetate (90:10, v/v) that are run out of the cartridge and discarded. Then, a 25-mL conical flask is placed and the column is eluted with 15 mL of methanol. The solvent is evaporated in a rotary evaporator at room temperature under vacuum until dryness. The residue is dissolved in 5 mL of methanol/water (1:1)."

The course of the high resolution techniques for the separation and identification of phenols in VOO

The first approaches to identify and quantify VOO phenols were performed by gas chromatography (GC) with FID, and later with mass detection (Janer Del Valle and Vazquez Roncero 1980; Angerosa *et al.* 1995, 1996; Liberatore *et al.* 2001). HPLC, however, has quickly become the most widespread technique due to its greater versatility in terms of detectors (UV, fluorescence, amperometric and mass spectrometer) and to the possibility to directly analyze extracts without a derivatization step (Solinas and Cichelli 1982; Montedoro *et al.* 1992; Tsimidou *et al.* 1992a; Mannino *et al.* 1993; Ryan *et al.* 1999; Gutiérrez-Rosales *et al.* 2003; Bendini *et al.* 2006; Selvaggini *et al.* 2006).

To date, 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 VOO (Rovellini *et al.* 1997; Mateos *et al.* 2001; Rovellini and Cortesi 2002; Rotondi *et al.* 2004; Cardoso *et al.* 2005; Carrasco-Pancorbo *et al.* 2007a), even if some interesting studies using gas chromatography/mass spectrometry (GC-MS) have recently been published (Ríos *et al.* 2005; Boselli *et al.* 2007). For the analysis of phenolics by LC-MS, different atmospheric pressure ionization interfaces have been used both in positive and in negative mode. Despite chemical ionization, atmospheric pressure chemical ionization (APCI) in negative mode generally provides good results, while electrospray ionization (ESI) in positive mode appears to be more diagnostic, thanks to its ability to reveal structurally significant fragments (for example sodium and potassium adducts). Thus, the results from positive and negative ion modes can be considered complementary.

In this respect, the mobile-phase composition and its pH need careful optimization as they may influence the ionization efficiency of analytes. Generally, HPLC methods are performed by reverse phase employing C18 stationary phases and mobile phases running gradients. The most useful solvents are acidified water (with a small percentage of formic acid, acetic acid or phosphoric acid), methanol and acetonitrile, avoiding the addition of non-volatile salts that may interfere with the ionization source. This has been summarized in a recent review (Bendini *et al.* 2007a). Thanks to the most recent advances in technology, some researchers may study the molecular structures of VOO phenolics in-depth, gaining selectivity and sensitivity by using instrumentation (Ion Trap or QqQ) that can work in MS/MS or MSn. One of the most advanced MS analyzers is the TOF MS, which provides excellent mass accuracy and measurements of the correct isotopic pattern (Innocenti *et al.* 2006; Di Donna *et al.* 2007). The main disadvantage of GC and HPLC separation of VOO phenols is the long analysis time, which is usually more than 40 min. Thus, the establishment of a faster analytical technique that is equally sensitive and powerful in terms of separation capacity that would allow rapid screening of VOO phenols is highly desirable. In this regard, capillary electrophoresis (CE) can be considered a good compromise between analysis time and satisfactory separation. In fact, the separation of VOO phenols by CE requires only few minutes and is characterized by high separation efficiency; moreover, the technique uses very small amounts of both sample and solvent as it needs only small quantities of run electrolyte (aqueous buffer). For the identification step, it should be mentioned that detectors generally coupled with HPLC, such as UV, FLD and electrochemical detectors (Bendini *et al*. 2003; Bonoli *et al*. 2003; Carrasco-Pancorbo *et al*. 2004; Gómez-Caravaca *et al*. 2005; Carrasco-Pancorbo *et al*. 2006a) and very recently different MS analyzers (quadrupole, ion trap, time-of-flight) using different ionization mode (APCI, ESI, MALDI) (Carrasco-Pancorbo *et al*. 2006a; Carrasco-Pancorbo *et al*. 2007b), can all be coupled with CE. For a more exhaustive discussion about CE and its application to phenol analysis of VOO, readers may refer to a recent review (Bendini *et al.* 2007a).

GC and GC-MS for the analysis of phenols in VOO

The determination of phenols or, more generally, of the fraction known as MPC (minor polar components) from VOO can be carried out with GC or GC-MS (Angerosa *et al.* 1995). To obtain volatile molecules such as those required by GC analysis, the polar fraction obtained by LLE and concentrated should be silylated (Sweeley *et al.* 1963). This procedure may be useful to analyze hydroxy-derivatives, non-volatile or even thermolabile substances. Furthermore, it can help in the discrimination of phenols. However, silylation determines an increase in the molecular mass of analytes, possibly beyond the range of the mass analyzer (commonly *m/z* 650) and can produce partially derivatized compounds from a single molecule (Angerosa *et al.* 1996).

The columns generally used are the "low polar" SE-30 capillary columns or equivalent (Solinas *et al.* 1981, 1982; Solinas 1987; Angerosa *et al.* 1995) with a methylpolysiloxane phase and a low selectivity, or the SPB-5 (30 m, 0.32

μm ID, 0.10 film thickness) or equivalent (e.g. DB5-MS) (Angerosa *et al.* 1996; Liberatore *et al.* 2001; Saitta *et al.* 2002; Ríos *et al.* 2005). Normally, a 5% phenyl-methyl-polysiloxane phase is used, in which the presence of the phenyl groups provides induced dipole interaction, leading to high retention times for aromatic molecules such as phenols. Both the phases cited have excellent thermal stability (from 270-320°C). Even if some compounds such as fatty acids and monoglycerides can interfere with the assay, capillary GC-MS is able to separate and identify 27 phenols (Saitta *et al.* 2009). As such, it is a valid tool for the detection and the quantification of phenol oxidation products (Ríos *et al.* 2005; Boselli *et al.* 2009).

INTERNATIONAL OLIVE COUNCIL RECOMMENDATION FOR THE IDENTIFICATION AND QUANTIFICATION OF THE PHENOLICS IN OLIVE OIL

As already pointed out in previous sections, several investigators have studied the importance of the total or individual phenol contents with regards to VOO stability. In this respect, the most powerful molecules identified are the *o*-diphenols such as hydroxytyrosol and its oleosidic forms. However, monophenol tyrosol and its derivatives also showed slight antioxidant activity.

The Folin–Ciocalteu test, set up for wine analysis in 1965 by Singleton and Rossi, is a well-standardized method that is useful for routine determination of total phenols in several aqueous matrices, but can be also considered suitable for estimation of the reductive capacity of phenolic molecules (for example those previously extracted by VOO). The reagent is composed of a mixture of phosphotungstate and phosphomolybdate in a basic medium that is able to oxidize phenols through formation of molybdenum oxide, which has an intense absorbance around 750 nm. Total phenols determined by the Folin-Ciocalteu test are most frequently expressed in gallic acid equivalents, even if for VOO the expression with respect to other standards such as tyrosol, 3,4-dihydroxyphenylacetic acid or caffeic acid is also used. In 1992, Montedoro and co-authors (1992) proposed that VOO be divided into three groups with low, medium and high total phenol content that are characterized, respectively, by values less than 200, from 200 to 500 and more than 500 mg of gallic acid/kg of oil. The major limitation concerning the reaction with Folin-Ciocalteu reagent consists in the contemporary determination of all kinds of phenolic molecules (monophenols and *o*-diphenols) present in VOO extract, so the reagent is not highly selective. A wide variety of substances (particularly sugars, proteins, aromatic amines, sulphur dioxide, ascorbic acid and other enediols and reductones, organic acids, Fe^{2+}, sodium metabisulphite and sulphite) can also interfere with the reagent to give apparently elevated phenolic concentrations. Moreover, even if simple to perform, the analytical method is time-consuming (it is necessary to wait 8 h before spectrophotometric analysis). Notwithstanding, the determination of total phenols with the Folin-Ciocalteu method is widely applied in VOO analysis.

Despite the importance of phenols in VOO quality, to date, the existing legislation does not provide either official methods for the determination of total phenols or for the most active individual molecules, or the legal limits concerning their content. Taking into account the 40 Italian VOO with a protected designation of origin (PDO) and one with a protected geographical indication (PGI), it is interesting to note that the minimum value of total phenols is indicated among the analytical characteristics in their specifications for more than half of the products. These values range from 60 to 200 mg kg^{-1}, but there are no indications or reference values, and the method used for their determination is not specified. Only in "*Terra di Siena*" PDO are both the analytical reference and the expression of the results correctly reported.

Based on this serious legal gap and the urgent need to facilitate the determination of the phenolics present in VOO, and to define the most reliable method characterized by acceptable repeatability and reproducibility, in 2006 the members of the International Olive Council, with regards to the recommendation made by the Technical Committee at its 1st meeting, held on the occasion of the 94th session of the Council of Members, approved the provisional application of the following methods (Resolution no. RES-4/94-V/06):

1. Colorimetric determination of *o*-diphenols in VOO (according to the method proposed by Cert *et al.* 2006).
2. Determination of the biophenols in VOO by HPLC. Natural and oxidised derivatives of oleuropein and ligstroside, lignans, flavonoids and phenolic acids (according to the method proposed by the SSOG Technical Commission, based on a previous published method of Rovellini and Cortesi 2002).

Colorimetric determination of *o*-diphenols in virgin olive oils

This colorimetric assay provides rapid quantitative determination of phenolics that are more active as antioxidants, as for example the monoaldehydic and dialdehydic forms of oleuropein aglycon and hydroxytyrosol, which are characteristic components of VOO. For the *o*-diphenol assay, a preliminary step of separation and purification of the phenolic fraction by SPE (solid phase extraction) is necessary, followed by a reaction with sodium molybdate dihydrate as a reactive and finally spectrophotometric measurement at 370 nm using a common laboratory spectrophotometer (the result is expressed as millimol of catechol per kg of oil). Briefly, for the SPE step, the oil solubilised in *n*-hexane is inserted on the column, and due to *n*-hexane, passes through the diol-bonded phase cartridge (previously conditioned by methanol and *n*-hexane). To eliminate the lipid components, a mixture of *n*-hexane/ethyl acetate (90:10, v/v) is used to wash the cartridge that is then eluted with methanol to collect the phenolic fraction. After the solvent evaporation, the residue is dissolved in methanol/water (1:1, v/v). Colorimetric determination of *o*-diphenols has been applied to the phenolic fraction of VOO by the research group coordinated by Cert (Mateos *et al.* 2001). In a recent review, Bendini *et al.* (2007a) reported that the *o*-diphenols content in VOO ranges from 10 to 350 mg of gallic acid per kg of oil. With respect to Folin-Ciocalteu method, the *o*-diphenol assay is more time-saving (absorbance can be read after only 15 min after the end of the reaction), and it is selective for molecules having a catecholic structure.

Determination of biophenols in virgin olive oils by HPLC. Natural and oxidized oleuropein and ligstroside derivatives, lignans, flavonoids and phenolic acids

This assay is based on a previous published method (Rovellini and Cortesi 2002) in which the authors studied the identity of these phenolic molecules by HPLC-ESI-MS. Through interpretation of mass spectra, Rovellini and Cortesi (2002), based on a previous study (Montedoro *et al.* 1993), elucidated different structures that naturally occur in a VOO, and in particular found the presence of several secoiridoid derivatives of oleuropein and ligstroside aglycons. These compounds can have aldehyde and dialdehyde closed structures, dialdehyde open structures, in addition to oxidized forms, as previously mentioned (in paragraph "Secoiridoids in VOO"). To apply the method suggested by the International Olive Council, it is necessary to have a HPLC pump equipped with a basic UV detector. In fact, this procedure consists in direct extraction of the phenolic fraction from VOO using a methanol solution, and subsequent quantification by HPLC using a UV detector at 280 nm. The chromatographic separation is performed in reverse-phase mode using a C18 stationary phase and a ternary gradient (0.2% of phosphoric acid in water phase A, methanol phase B and acetonitrile phase C) as the mobile phase. Based on the

information about the maximum absorbance (max UV abs) values and relative retention times (RRT, calculated with respect to the retention time of syringic acid) is possible to identify 27 phenolic molecules. All the identified compounds can be expressed in mg of tyrosol/kg of oil, adding syringic acid as an internal standard.

Obviously, the use of a mass detector coupled with the HPLC may be useful for correct identification of different phenolic compounds that often partly overlap. On the other hand, the acquisition of UV spectra at three different wavelengths can be valuable; in fact, flavones such as luteolin and apigenin have absorption maxima at around 350 nm, whereas elenolic acid and its derivatives absorb at 240 nm and the other compounds absorb at 280 nm. In this respect, an HPLC with a multiple wavelength UV detector is sufficient.

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