Review Article

Hydrophilic antioxidants of virgin olive oil. Part 1: Hydrophilic phenols: A key factor for virgin olive oil quality

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Virgin olive oil (VOO) consumption is increasing all over the world due to its excellent organoleptic and nutraceutical properties. These beneficial traits stand from a prominent and well-balanced chemical composition, which is a blend of major (98% of total oil weight) and minor compounds including antioxidants. The main antioxidants are phenolic compounds, which can be divided into lipophilic and hydrophilic phenols. While lipophilic phenols such as tocopherols can be found in other vegetable oils, most hydrophilic phenols in olive oil are exclusive of the *Olea europaea* species endowing it with a chemotaxonomic interest. This review is focused on VOO antioxidant profile and, particularly, on hydrophilic phenols that are divided into different sub-families such as phenolic acids and alcohols, hydroxy-isochromans, flavonoids, secoiridoids, lignans and pigments. Analytical methods for qualitative and/or quantitative determination of these compounds are assessed. The implementation of efficient sample preparation protocols, separation techniques such as liquid chromatography, GC and capillary electrophoresis, as well as detection techniques such as ultraviolet absorption, fluorescence or MS are critical to succeed in the quality of the results. The effects of hydrophilic phenols on increasing VOO stability, its nutraceutical interest and organoleptic properties are also considered.

Keywords: Analytical methods / Antioxidant activity / Hydrophilic phenols / Sensory characteristics / Virgin olive oil

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Abbreviations: 3,4-DHPEA, 3,4-dihydroxyphenylethanol; **3,4-DHPEA-EA,** isomer of oleuropein aglycone; **3,4-DHPEA-EDA,** dialdehydic form of elenolic acid linked to 3,4-DHPEA; **API,** atmospheric pressure ionization; **CE,** capillary electrophoresis; **DMF,** *N,N*-dimethylformamide; **DNA,** deoxyribonucleic acid; **EA,** elenolic acid; **F–C,** Folin–Ciocalteu method; ***p*-HPEA,** *p*-hydroxyphenylethanol; ***p*-HPEA-EA,** ligstroside aglycone; ***p*-HPEA-EDA**, dialdehydic form of elenolic acid linked to *p*-HPEA; **ICAM-1,** intercellular adhesion molecule-1; **IT,** ion trap; **LC,** liquid chromatography; **LLE,** liquid–liquid extraction; **NO,** nitric oxide; **OOL,** oleic–oleic–linoleic; **OOO,** oleic–oleic–oleic; **POL,** palmitic–oleic–linoleic; **POO,** palmitic–oleic–oleic; **QqQ,** triple quadrupole; **SFA,** saturated fatty acids; **SOO,** stearic–oleic–oleic; **SPE,** solid-phase extraction; **TOF,** time-of-flight; **TPI,** total polyphenol index method; **UV,** ultraviolet; **UV–Vis,** ultraviolet-visible; **VCAM-1,** vascular cell adhesion molecule-1; **VOO,** virgin olive oil

1 Olive oil in the Mediterranean diet

Virgin olive oil (VOO) is the natural juice of olive fruit; it can be consumed as it is – freshly pressed from the fruit. It conserves the taste, aroma, vitamins and properties of olive fruit.

In the current Mediterranean diet, VOO constitutes the primary source of fat intake replacing all types of commonly used animal fats that are detrimental to human health [1]. Numerous epidemiological studies have confirmed that people who closely follow 'the Mediterranean diet' live longer than other Europeans and Americans due to the lower incidence of chronic and degenerative diseases [2, 3]. Although lifestyle factors (such as more physical activity) may play a part in promising longer life in the Mediterranean area [4], several studies pointed out that VOO is the main factor responsible for the health and nutritional benefits of the Mediterranean diet [5].

The chemical composition of VOO is characterized by the presence of two main groups: the major components (≈98%), and the minor components (≈2%) [6]. Major components include mainly TAGs and small concentrations of DAGs, MAGs and some free fatty acids. The key aspect of this fatty fraction is the high concentration of MUFA, especially oleic acid. In fact, high percentage of MUFA to the detriment of PUFA reduces the risk of atherosclerosis [7], and protects from different kinds of cancer [5].

On the other hand, minor components include a number of heterogeneous compounds (more than 230). Referring to its origin, Boskou [6] divided these components into two groups: those non-chemically related to fatty acids such as hydrocarbons, alcohols, sterols, volatile compounds and antioxidants, and fatty acid derivatives such as phospholipids, waxes and sterol esters, as the most important. Despite the small concentration of minor components (only 2% of VOO weight), they play a key role in the quality and behaviour of the different VOOs and in their characterization [8].

Antioxidants in VOO are represented by tocopherols, pigments (carotenoids, chlorophylls) and phenolic compounds. These compounds provide a defence mechanism that delays aging and prevents carcinogenesis, atherosclerosis, obesity, liver diseases and inflammations, among other health disorders [9, 5].

Different reviews have been reported about antioxidants in VOO describing their benefits and analytical methods for their characterization, they are cited in the following sections. The objective of this review was to present wide and up to dated information on phenolic composition of VOO: the traditional and recent methods for their determination and quantification, and recent knowledge about phenols effect on oil oxidative stability, on its organoleptic characteristics and on human health. This information will be of interest for both researchers working on the impact of olive oil composition on human health, and agronomists, breeders and producers searching for improving olive oil quality.

2 Virgin olive oil antioxidants

Antioxidants are the compounds that more differentiate VOO from other vegetal oils. The most abundant antioxidants in VOO are lipophilic and hydrophilic phenols. Antioxidants affect the healthy and sensory characteristics of VOO [9], and improve its resistance to oxidation [10]. Besides, these compounds are important to plant physiology, contributing to resistance to microorganisms and insects [11]. These compounds have previously been very well described [3, 9]; thus, the following part will present a summary of the most relevant considerations about VOO composition in antioxidants.

Lipophilic phenols in VOO are *tocopherols*, which are heteroacids of high molecular weight. Among them, α-tocopherol is the most abundant (90%), β- and γ-tocopherols are also present [12]. The percentages of tocopherols are strongly variable according to pedoclimatic factors, agronomic conditions, fruit ripening and cultivars. The profile and composition in tocopherols are a criterion of VOO purity. In addition to their antioxidant effect on human health, tocopherols exert a synergic effect, together with other phenolic compounds, on the oxidative stability of VOO [13].

Two classes of natural pigments have been identified in VOO: *chlorophylls* and *carotenoids*. The former encompass chlorophylls type a and b and their derivatives (pheophytins a and b and pheophorbides). During fruit ripening, the concentration of chlorophylls decreases drastically [14].

Carotenoids include lutein as main component, β-carotene, and other xanthophylls such as violaxantin and neoxantin [15]. Their concentration decreases progressively during ripening, but less drastically than chlorophylls do. Research on carotenoids demonstrated their utility in varieties and origin differentiation. Other studies suggest adopting the ratio of minor carotenoids to lutein to determine the ripeness stage of olive fruit. Finally, it is worth noting that the concentration of pigments is also highly dependent on olive processing systems and storage conditions [14].

Hydrophilic phenols, such as phenolic acids, phenolic alcohols, hydroxy-isochromans, flavonoids, secoiridoids and lignans (Fig. 1), are specially important in VOO quality due to their antioxidant activity and their effect on organoleptic characteristics and VOO shelf-life. Among these secondary metabolites, secoiridoids and lignans are the most concentrated. Average values of the common phenolic alcohols, phenolic acids and secoiridoids of VOO are illustrated in Table 1.

Phenolic acids include caffeic, vanillic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, *p*-hydroxybenzoic and gallic acids; also ferulic and cinnamic acids have been quantified, but in lower quantities (less than 1 mg analyte/kg VOO). Phenolic acids are secondary aromatic plant metabolites spread in a wide range of plants and they have been associated with colour and sensory qualities of foods. They are also used as potential markers of geographical origin and olive cultivars [16, 17].

The main *phenolic alcohols* in VOO are 3,4-dihydroxyphenyl ethanol, also known as 3,4-DHPEA or hydroxytyrosol, and *p*-hydroxyphenyl ethanol *p*-HPEA or tyrosol. These alcohols are at low concentration in fresh oils, which increases during storage as they result from the hydrolysis of VOO secoiridoids containing 3,4-DHPEA and *p*-HPEA in their molecular structures [18].

Two *hydroxy-isochromans* have been identified and quantified in VOO: 1-phenyl-6,7-dihydroxyisochroman and 1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxyisochroman.
They are formed during oil extraction by reaction between hydroxytyrosol and benzaldehyde or vanillin [10].

Flavonoids include flavones, flavonols, flavanones, flavanols, anthocyans and derived glucosides (such as luteolin-7-glucoside and rutin) [3]. Luteolin and apigenin are present in VOO [19].

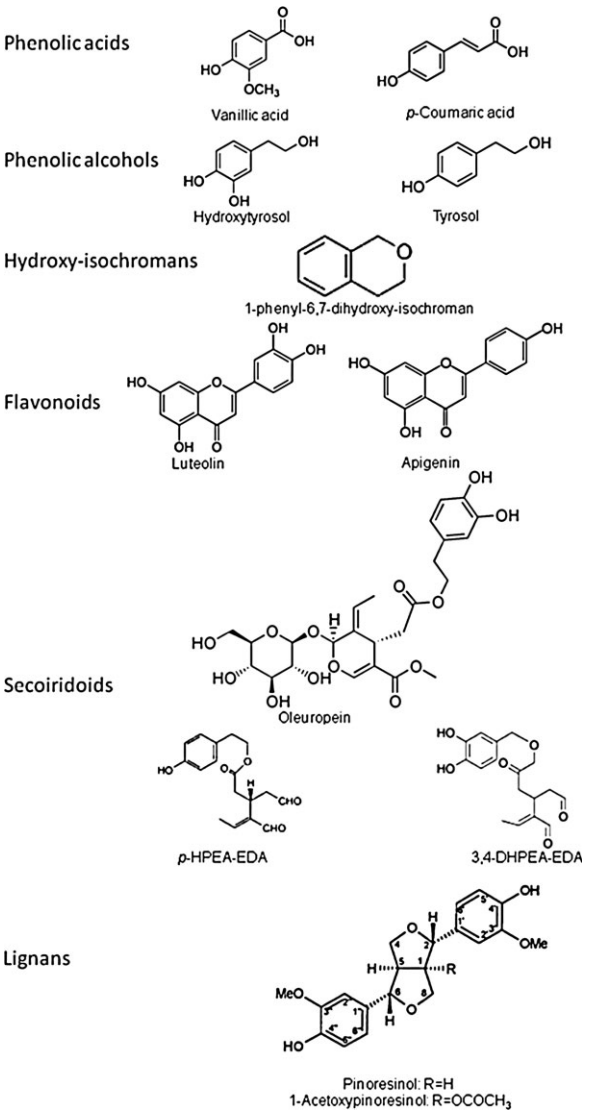

Figure 1. Example of structures of the most important hydrophilic phenolic groups present in VOO.

The most abundant *secoiridoids* in intact olive fruit are oleuropein, demethyloleuropein, ligstroside and nüzhenide. Verbascoside, a derivative of the hydroxycinnamic acid, has also been detected in VOO as a characteristic compound [20]. During mechanical oil extraction, several hydrolysis reactions of oleuropein, demethyloleuropein and ligstroside take place due to the activity of endogenous β-glucosidases [21] and originate aglycone derivatives known as VOO secoiridoids.

From a chemical standpoint, secoiridoids are characterized by the presence in their molecules of elenolic acid (EA) or some of its derivatives. The most abundant secoiridoids in VOO are the dialdehydic form of decarboxymethyl EA linked to hydroxytyrosol or tyrosol termed 3,4-DHPEA-EDA and *p*-HPEA-EDA, an isomer of oleuropein aglycone (3,4-DHPEA-EA) and the ligstroside aglycone (*p*-HPEA-EA) [22–26].

Table 1. Mean values (mg/kg) of the common phenolic alcohols, phenolic acids and secoiridoids of VOO calculated using 116 oil samples (Unpublished results reported by Servili and Montedoro [9], reproduced with permission of Wiley Interscience)

	Median	Lower quintile	Upper quintile
3,4-DHPEA	1.9	1	3.9
p-HPEA	2.6	1.2	6.4
Vanillic acid	0.2	0	0.3
Caffeic acid	0.4	0.2	0.7
3,4-DHPEA-EDA	185.7	63.2	839.7
p-HPEA-EDA	22.4	15.4	33.3
3,4-DHPEA-EA	163.6	85	310

The concentration of secoiridoids is largely affected by agronomic factors (cultivar, ripening stage, geographic origin of olive fruit and olive trees irrigation) and by oil extraction conditions during crushing, malaxation and VOO separation [27–29].

Lignans were identified for the first time in VOO by Brenes et al. [30], this group encompasses (+)-pinoresinol and (+)-1-acetoxypinoresinol. These compounds are present in olive fruit and are released to VOO during the mechanical extraction process. The concentration of lignans is affected by cultivars and agronomic conditions [27].

2.1 Antioxidants determination

Analytical methods for determination of phenols in VOO depend on the degree of information demanded. This information level can encompass from the determination of the overall content of these compounds as a qualitative index to the profiling analysis of individual phenolic compounds in VOO. In any case, two basic steps are usually required for analysis of VOO phenols: extraction from the oil sample and separation/detection. The state-of-the-art of the different techniques involved in each step are described and discussed below.

2.1.1 Sample preparation

2.1.1.1 VOO samples availability and storage

Samples of VOO are frequently acquired from olive oil mills located in industries or from markets trying to select a representative population. When control on the production processing is desired, for instance to obtain VOO from preset cultivars, olives should be processed in a pilot extraction plant. One of the most used systems in this case is the

Abencor unit [31] although low scale mills can also be used. The Abencor unit consists of three essential sub-units: the hammer mill, the thermobeater, for controlled-temperature processing, and the pulp centrifuge. After fruits crushing in the mill, olive paste is mixed in the beater, which is immersed in a thermostated bath. Generally, malaxation temperature is set within the range 27–29°C, which is mandatory to avoid degradation of VOO thermolabile components. Subsequently, the oil is fastly separated from the paste by centrifugation. Finally, VOO is separated from vegetation aqueous phase by decanting for 24 h at RT and then transferred into dark glass bottles. VOO samples can be stored in the dark at 4°C until analysis to minimize the influence of enzymatic action without affecting sample integrity.

2.1.1.2 Sample pre-treatments prior to isolation of phenolic compounds

Preliminary operations before extraction of phenolic compounds usually start by measurement of oil volume or by weighting. The latter option is preferred as more accurate taking into account VOO viscosity. This operation is mandatory when the purpose of the analysis is quantitation. Due to VOO viscosity, it is frequent to dilute samples with an organic solvent, usually hexane [23, 32–34], which is homogenized by stirring or vortexing. In this way, handling of VOO samples by analysts is favoured.

Due to the presence of conjugated phenolic compounds, a hydrolysis step has been proposed in some cases to minimize interferences in subsequent steps of the analytical method such as chromatographic separation, particularly when the appropriate standards are not commercially available. In this context, acid or alkaline hydrolysis can be used [22, 35], which has traditionally been used for determination of phenolic acids and aglycones, flavonoids and derived glycosides and phenolic esters. The alkaline hydrolysis has recently been examined due to the stability of plant phenols under these conditions and to the successful involvement of alkali treatment in the commercial processing of many plant-derived foods.

2.1.1.3 Extraction of phenolic compounds from VOO

The final goal of a step for isolation of phenolic compounds is the preparation of a sample extract uniformly and quantitatively enriched in these compounds and free from interfering matrix components [36].

Two main techniques have traditionally been used for extraction: liquid–liquid extraction (LLE) [22, 37–40] and solid-phase extraction (SPE) [41, 42–45]. These techniques do not differ only in solvents and/or physical state of the acceptor phase, but also in the amount of sample taken for analysis, volume of solvents, and other aspects, as commented below [32].

Liquid–liquid extraction. Liquid–liquid extraction is based on transference of the phenolic fraction from VOO to a more hydrophilic phase such as pure methanol or methanol–water mixtures with different alcohol concentrations from 50 to 100% [22, 38–40, 46, 47].

Ultrasound and microwaves can be used as auxiliary energies to accelerate and improve LLE. The main advantages of ultrasound- or microwave-assisted LLE are shortened extraction time, reduced reagent and sample volume and improved extraction efficiency [48].

Other solvents and additives have also been proposed. Thus, the use of *N,N*-dimethylformamide (DMF) has provided interesting results in terms of efficiency and sample manipulation [49]. One other example is the method proposed by Cortesi et al. [50] who used 80:20 v/v tetrahydrofuran–water followed by centrifugation, or the addition of tensioactives such as 2% v/w Tween-20, used to liberate the phenolic compounds from the lipoprotein membranes [22, 51].

After extraction, clean-up and/or pre-concentration steps can be implemented to achieve the appropriate selectivity and sensitivity, respectively. One alternative to remove potential interferences is to store extracts overnight at subambient temperature followed by filtration or centrifugation [52]. VOO extracts can also be cleaned with pure solvents such as hexane, petroleum ether and chloroform as well as with solvent mixtures such as 9:1 hexane–petroleum ether, 9:1:5 hexane–petroleum ether–chloroform or 1:4 acetonitrile–hexane [53]. Extracts cleaning can also be carried out using sorbent columns such as Sephadex [22, 23] and Policlar AT/Celite 560, which enable proper fractionation of extracts (1:2) [54]. Pre-concentration is usually performed by evaporation of the extractant under vacuum or a nitrogen stream [52] at ambient or moderate temperature to avoid degradation. The dried residue is then reconstituted with the appropriate solvent, which will be selected depending on the purposes of the analysis, frozen and stored until next step.

Solid-phase extraction. The main advantage of SPE, as compared with LLE, is its dual action associated to isolation of the target compounds: clean-up and pre-concentration. The proved versatility of this technique is based on the range of sorbents with different characteristics that can be used (e.g. C18, diol, amino, C8) as well as eluents (e.g. methanol, ethanol and methanol–water mixtures) [20, 32, 42–45, 55–57]. SPE was for the first time applied for isolation of phenolic compounds from VOO by Mannino et al. [58] using C18 sorbent packed in cartridges and methanol as eluent. Further experiments using C8 [42] and C18 sorbents [59] revealed that reversed-phase SPE is less suited for isolation of phenolic compounds than normal phase SPE due to incomplete extraction of the target compounds [60] and partial oil separation in the former case [42]. A complete study was carried out by Hrncirik and Fritsche [32] by comparison between LLE using 60:40 methanol–water v/v as

extractant and SPE with two different sorbents such as diol-bond and C18. The results obtained in this study showed that LLE led to higher extraction efficiencies of total phenolics (93%) than SPE-diol (68%) and SPE-C18 (38%). Another comparison, between LLE using 80:20 methanol–water v/v as extractant and SPE with C18 cartridges and methanol as eluent was performed by Montedoro et al. [22]. In this case, SPE was more efficient than LLE to separate simple phenols; however, LLE provided higher efficiencies for isolation of secoiridoid derivatives. From these examples, it can be deduced that the sorbent variety, the packing process and the diversity of protocols can justify the differences between the results obtained by different groups.

2.1.2 Overall and/or individual determination of phenolic compounds

Identification as well as overall and/or individual quantitation of phenolic compounds in VOO samples are of quality interest due to their effect on olive oil quality and beneficial effects. The main limitation in the quantitation of phenolic compounds as a quality parameter is that concentrations of olive phenols reported in the literature greatly differ depending on agronomic factors such as cultivar, fruit ripening, pedoclimatic conditions, irrigation and technological factors as crushing, malaxation and olive oil separation. This variation can sometimes encompass even orders of magnitude [17]. On the other hand, these discrepancies may also be caused by the wide variety of analytical methods used (particularly methods for overall determination, drastically influenced by the standard selected) and/or by the expression of the results in different formats [42].

2.1.2.1 Total phenols determination

Overall determination of phenols after separation of the lipidic fraction involves selection of a single standard for relative quantitation. One of the standards more frequently used for this purpose is caffeic acid. This involves that predominance of phenols with molecular weight lower than that of the standard yields an overall phenols concentration higher than when the predominance corresponds to phenols with molecular weight higher than that of the standard.

Folin–Ciocalteu method. Folin–Ciocalteu (F–C) is one of the most widely used methods for the quantification of total phenols of VOO. It is based on the reaction of F–C reagent [51, 54] with functional hydroxyl groups present in phenolic compounds [33]. The typical protocol of this method could be consulted in Cerretani et al. [61] application. Briefly, it consists of running the calibration curve with a pure phenolic compound, extraction of the phenols from the sample and measurement of the absorbance after formation of the coloured compounds between the reagent and phenols in the extract.

The most important limitation associated to the F–C assay is its low selectivity as the coloration reaction can occur with any oxidizable phenolic hydroxyl group. In addition, this method does not give quantitative information about given phenols because it does not distinguish between individual compounds differing in molar mass and structure. It has also been reported that the temperature influences the colour of the reaction product; this colour may change in few minutes making the application of this method on large sequences of samples difficult. Moreover, precipitate formation during the reaction time may generate errors in the colorimetric measurements.

Total polyphenol index method. This method has been rarely used for overall quantitation of phenols, as it provides less selective information than F–C method. In the latter approach, absorption is monitored at 700–765 nm, where background contribution is particularly limited; while the total polyphenol index (TPI) is based on absorption at 280 nm, where a number of interferences can contribute to the absorbance.

It is worth noting that in both methods, F–C and TPI, calibration is carried out with a standard phenol such as oleuropein [62], caffeic acid [32, 63] or gallic acid [23, 24], and the amount of total phenols is expressed according to this representative standard.

NIR spectroscopy determination. Mailer [64] reported the possibility of using a FOSS NIRSystems® 6500 spectrophotometer with a liquid cell holder as a fast, simple and non-destructive method to evaluate the phenolic content in VOO. The resulted correlation between values obtained by F–C method using caffeic acid as standard and NIR spectra showed a cross-validation correlation coefficient of 0.82, which could be improved with a wider distribution of values. It can be concluded that this technique is feasible for routine analysis of total phenols, but it still needs further validation and improvement of accuracy to make it really competitive.

Fluorescence-based determination. Fluorescence spectrometry, being a rapid technique and saving costs and time of analysis, may provide a powerful tool for analysing non-destructively VOO phenolic compounds. In this context, Gracia et al. [65] assessed the possibility of using this technique for the determination of total phenols in VOO. The results showed an acceptable correlation ($r = 0.84$) between the values obtained by the reference method (F–C at 726 nm, with the results expressed as mg of caffeic acid) and fluorescence spectra at ranges of excitation between 200 and 500 nm and emission monitoring at 525 nm.

NMR spectroscopy. NMR spectroscopy has also been used as complementary technique for structural assignment of VOO phenols. However, NMR spectra of phenols are

frequently complex and identification of the isolated compounds is complicated in the absence of appropriate standards. Also, the limited sensitivity [66] and the need for quantities of samples higher than those required by chromatographic counterparts constitute the greatest limitations of NMR spectrometry. Recently, high resolution 1H NMR spectroscopy has been employed for the development of a versatile and rapid method for identification and quantitation of various classes of phenolic compounds such as simple phenols, flavonols, lignans and some secoiridoids. The strategy for identification of phenolic compounds is based on the NMR chemical shifts of a large number of model compounds assigned by using two-dimensional (2D) NMR spectroscopy [67]. Accordingly, a good agreement of HPLC–DAD/MS and 1D and 2D NMR spectroscopy has been obtained by Valli et al. [68], when comparing the sum of monoaldehydic and dialdehydic forms of phenolic compounds in VOO. It is worth noting that a previous extraction of phenolic fraction from VOO is mandatory to eliminate the interferences on the measurement caused by the saponifiable fraction of the oil. Anyway, the most promising application of NMR is targeted at qualitative analysis by generation of characteristic fingerprinting registers to improve VOO quality.

2.1.2.2 Individual separation and determination

The great diversity of phenolic compounds, the scant selectivity of the traditional methods and the present and growing importance of VOO phenols necessitate the replacement of overall quantitation methods by others providing individual information about each phenol. In this section, the contribution of liquid and gas chromatographic methods and, in a lesser extent, capillary electrophoresis (CE) methods, has enabled to elucidate the profile of phenolic compounds in VOO. In this sense, the combination of separation techniques with MS has increased the selectivity of analytical methods with identification purposes as compared to conventional methods based on spectrophotometric or fluorimetric detection.

GC separation and profile determination. GC is a separation technique endowed with a high resolution capability based on the interaction of the sample components with a stationary phase and along a temperature gradient. GC assumes that the analytes are volatile at the temperature of the analysis and remain stable during analysis. That is why GC is generally used for volatile compounds or those that can become volatile by derivatization of specific functional groups. Due to the low volatility and thermal stability of phenolic compounds, a suited derivatization step is mandatory in order to increase volatility and thermostability of the analytes.

Derivatization of phenolic compounds is mostly based on silylation reactions, which consist of substitution of active hydrogen of –OH, –SH, $–NH_2$ functional group by a silyl group. In this way, phenolic compounds are generally separated as more volatile trimethylsilyl esters; thus decreasing the temperature required for chromatographic separation.

Solinas [37] reported one of the first protocols for determination of VOO phenolic compounds by GC after silylation. The method was initiated with separation of the target analytes from the non-polar matrix by LLE with methanol. Then, clean-up of the methanolic extract followed by azeotropic distillation to remove the extractant, low-pressure column chromatography of the pre-concentrate to clean-up the extracts and, finally, GC analysis of the trimethylsilyl esters. This method was able to characterize the simplest compounds, but conjugated phenols present in VOO were not identified due to detection based on universal flame ionization, which does not allow identification unless standards are used. However, the advances experienced by MS supported this technique as an excellent tool with identification purposes when hyphenated to GC for GC-MS analysis. This capability is based on the detection of molecular mass for each compound with different accuracy levels depending on the mass analyser and the structural information revealed by fragmentation of precursor ions. Thus, a GC-MS for identification of phenolic compounds in VOOs was developed by Angerosa et al. [52], and the results showed that the assignment of the phenolic nature to minor polar compounds extracted by methanol from VOO was made possible by the presence of a main peak at m/z 192 or at m/z 280, that was related only to tyrosol and hydroxytyrosol according to their molecular mass. One year later, Angerosa et al. [25] proposed an improvement to this method by using chemical ionization (CI) that allowed them to obtain precursor ions, which were not detectable in the electron impact mode previously used. The information obtained by this method about molecular masses was very useful for the complete characterization of aglycones from glycosides occurring in VOO.

The main drawback of GC-MS methods for analysis of phenolic compounds is that silylation increases considerably (+73 mass units per target functional group) the molecular mass of analytes, which may be displaced beyond the range of the mass analyser (theoretically up to values of 1000 m/z units) [69]. In addition, derivatization often produces mixtures of partially derivatized phenolic compounds [25], thus decreasing sensitivity and involving errors.

More improvement to GC-MS was obtained by the introduction of the ion trap (IT) technique that provides the possibility of doing MS/MS or MS^n, which can be used for structure elucidation or for additional selectivity to gain sensitivity by reducing the chemical noise [70]. Thus, GC-tandem MS (GC-MS/MS) was used, providing excellent sensitivity and selectivity for target compound analysis [57].

However, a disadvantage of this approach may be the unwanted side-reactions and other events that may occur in an IT upon introduction of samples containing relatively

large amounts of compounds that co-elute with the analytes [71]. Nonetheless, these methods improve previous possibilities for quantitative evaluation of oxidation products of linked phenolic forms, and thus, offer potential applications to measure the oxidation degree of olive oils [72].

Liquid chromatography profile determination. Liquid chromatography (LC) is nowadays the most popular technique for VOO phenols analysis because of the combination of resolution (approaching at levels similar to those obtained by GC), efficiency, versatility and speed of analysis. This technique is especially suited for separation of non-volatile compounds. Among the different chromatographic modes, reverse-phase LC is the preferred option for VOO phenols separation [73] using as stationary phase mainly a non-polar octadecylsilane (C18) bonded phase. Chromatographic columns with length in the range 10–30 cm, with particle size from 5 to 2.1 μm, are frequently used, although lower particle sizes up to 1.8 μm can be used to improve resolution [17].

Gradient or isocratic operation modes can be used for LC separation. The isocratic mode has demonstrated to provide more adequate resolution due to the selectivity effect of one or more components of the mobile phase, but the use of a gradient mode is mandatory due to the complexity of the phenolic profile of most VOO samples. Thus, binary gradients from pure or acidified water to a less polar organic solvent such as acetonitrile or methanol are commonly employed [74]. Acidification of the aqueous phase by addition of acetic, formic or phosphoric acid is generally recommended to avoid dissociation of phenolic compounds, decrease asymmetry of chromatographic peaks and reduce peak-tailing effects [75].

The most conventional detection system used in LC analysis of VOO phenols is that based on UV–Vis absorption, preferably with diode array detectors since phenolic compounds show different absorption maxima and can be easily identified by monitoring at the corresponding wavelength [22, 38]. Detection is mainly performed at specific wavelengths such as 260, 280 and 325 nm [54].

Apart from spectrophotometric detection, Cartoni et al. [76] proposed the use of fluorimetric detection to analyse phenolic acids in VOO. This detector was compared with electrochemical and UV detectors [49] concluding that fluorescence detectors present clear advantages in terms of sensitivity and selectivity being specially promising for characterization of lignans. Its main limitation is that not all phenolic compounds of VOO are able to fluoresce [77].

Liquid chromatography coupled to MS has also been used for the analysis of VOO phenols [78, 79]. Accordingly, some modifications were introduced to this technique such as LC-MS with pneumatically assisted electrospray (ion spray) (LC-ESI-MS) that was able to elucidate several structures of phenols [80, 81]. Afterward, Bianco et al. [82] described a highly specific and sensitive method by HPLC-atmospheric pressure ionization (API)-MS/MS that was able to measure quantitatively and qualitatively a great number of phenolic compounds in VOO; however, this method typically yields only a single strong ion, which reduces its ability to make analyte accurate identifications. More recently, IT and triple quadrupole (QqQ) analysers were also experimented in LC. The IT analyser enables to elucidate phenols structure by selective isolation of ions in the trap and subsequent fragmentation MS^n. The latter is composed by two quadrupoles as mass filters and a hexapole as collision cell for fragmentation of precursor ions placed between the two quadrupoles. In a study performed in VOO and olive-tree materials such as alperujo (the semisolid residue resulting from the production of olive oil) leaves, small branches and stones, Japón-Luján et al. [83] illustrated an extra level of selectivity in identification and sensitivity in the determination of phenols. In fact, methods based on LC-MS with a QqQ analyser enables to lower detection limits up to pg/mL or fg/mL, which is crucial for detection of low-concentrated phenols. Finally, time-of-flight (TOF-MS), one of the most advanced MS analysers, is a powerful tool for identification of olive phenols by determination of elemental composition with excellent mass accuracy and confirmation of the correct isotopic pattern [84–86]. Although continuous efforts are made to improve identification of olive phenols, the current trend is focused on taking benefits from TOF mass accuracy and resolution for qualitative analysis. For this reason, LC-TOF-MS (and also LC-Q-TOF MS/MS) are ideal complementary techniques to NMR in the way to improve VOO quality by analysis of phenolic extracts or by direct analysis of VOO.

Capillary electrophoresis profile determination. Although GC and LC are very successful techniques for separation of VOO phenols; they need complex and accurate sample preparation, and are generally time-consuming. CE requires minimal sample preparation and represents a good compromise between analysis time and satisfactory characterization for the same classes of VOO phenols.

Capillary electrophoresis is characterized by fast analysis time, high separation efficiency and small sample and electrolytes consumption (low analysis cost). These characteristics and the suitability of CE to analysis of samples with complex matrices make CE useful for routine analysis of VOO phenolic compounds.

The most efficient operative mode to separate phenolic compounds is the borate-based CE, which makes use of a borate run buffer at alkaline pH. The best compromise in terms of resolution of the phenolic compounds and total analysis time was obtained with buffer at pH 9.5 as reported Carrasco-Pancorbo et al. [17]. To date, the most widely used detector in CE is based on UV absorption [87]. Although the coupling to MS analysers such as QqQ, IT, TOF, etc., has revalorized the potential of this technique [88].

2.2 Beneficial effects of hydrophilic phenols on VOO quality

Virgin olive oil is a premium and highly priced product thanks to the nutraceutical properties of its components. Among them, it is worth emphasizing the role of antioxidants, which are of paramount importance to keep a long oil shelf-life and provide excellent organoleptic characteristics (taste and colour) and beneficial effects on human health. Several reviews described previously the beneficial effects of antioxidants on VOO quality [3, 5, 9, 10, 27]. The following section will be limited to the basic aspects, focusing on the most recent results.

2.2.1 Effect of hydrophilic phenols on oxidative stability of VOO

Oxidative stability of VOO mainly depends on the characteristics of its fatty acid composition (mainly unsaturated fatty acids), and on the quantity of phenolic compounds having a catechol group, such as hydroxytyrosol and its secoiridoid derivatives, that are endowed with a high antioxidant activity [10, 89–91].

The presence of phenolic compounds in VOO is therefore extremely important, because they combat lipid oxidation in its initial stages through a variety of mechanisms based on radical scavenging, hydrogen atom transfer and metal-chelating. Even in small quantities, phenols in VOO are fundamental for protecting glycerides from oxidation; in fact, by virtue of their favourable oxidation potential, they exert an intense protective action by exposing themselves to oxidation instead of the lipid substrate.

Phenols, particularly secoiridoids, generally decrease during VOO storage due to the hydrolysis of secoiridoids derivatives in the hydroxytyrosol, tyrosol and EA and formation of oxidized phenols. These reactions lead to a decrease in bitterness and pungent intensity, positive attributes that are characteristic of a fresh VOO [91]. Moreover, high secoiridoids content may extend VOO shelf-life due to the positive relationship between the ratio of secoiridoid derivatives/EA derivatives and oxidative stability of a given oil [92].

Additionally, *o*-diphenolic compounds such as hydroxytyrosol (3,4-DHPEA), decarboxymethyl oleuropein aglycones (3,4-DHPEA-EDA) and oleuropein aglycone (3,4-DHPEA-EA) are, in this order, the most potent antioxidants [93]. Nevertheless, the contribution of monohydroxylated phenols as tyrosol and ligstroside aglycone is scarce [13, 93]. In addition, Mateos et al. [13] concluded that *o*-diphenols prevent oxidation of α-tocopherol. Regarding flavonoids group, Mateos et al. [13] reported an antioxidant activity for luteolin similar to that of hydroxytyrosol; however apigenin did not show any antioxidant activity. According to lignin contribution to VOO oxidative stability, the results are contradictory [47, 94].

Finally, several studies confirmed the stability of phenols on heating and appointed the strong effect of 3,4-DHPEA-EDA and 3,4-DHPEA-EA on VOO stability [95]. The amount of these compounds decreased during heating preserving the oil from oxidative reactions.

2.2.2 Effect of VOO hydrophilic phenols on human health

VOO phenols are endowed with healthy effects on human health. In fact, these compounds are well absorbed by the intestine and enter the blood circulation [96] to constitute one of the bases of nutritional and therapeutic effects of VOO.

It is well known that the oxidative stress produced by free radicals is responsible for the development of several diseases such as atherosclerosis, different kinds of cancer, inflammatory diseases and Parkinson's disease. The high correlation found between total phenols content determined colorimetrically and free radical scavenging activity supports the antioxidant effect of these compounds [97].

Phenolic compounds have also a protective effect against cardiovascular diseases [98] by protecting against low-density lipoprotein (LDL) oxidation that is a crucial step in the progress of atherogenic processes [96, 99]. In addition, consumption of VOO with high phenolic content significantly increases high-density lipoprotein (HDL) [100], considered as protective factor against cardiovascular diseases.

Antiatherosclerotic effects of VOO are mainly due to the presence of oleuropein and hydroxytyrosol [99]. However, it is noteworthy that the results of human studies on in vivo antioxidants effects on lipid oxidation, performed up to year 2005, are controversial [101–103], mainly because some studies concluded that the concentration of phenols present in VOO and plasma are too low to produce a quantifiable and biologically significant effect on LDL oxidiation capacity [96]. However, Covas et al. [100] confirmed the protective effect of phenolic compounds in humans by noting the existence of a linear correlation between the phenolic content of the consumed VOO and the increase in HDL cholesterol in parallel with a decrease in the lipid oxidative damage.

Phenolic compounds also exert a beneficial effect on the thrombotic pathways by inhibiting endothelial activation involved in monocyte recruitment. In this context, oleuropein increased nitric oxide (NO) production in macrophages. NO is an important bactericidal and cytostatic factor with vasorelaxing and anti-aggregating properties [104]. Moreover, Paiva-Matins et al. [105] demonstrated, in vitro studies, that low doses of 3,4-DHPEA-EDA, an oleuropein derivative, protect red blood cells from oxidative mediated hemolysis. In addition, both oleuropein and hydroxytyrosol inhibited vascular cell adhesion molecule-1 (VCAM-1) and reduced the stimulated expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1), those three (CVAM-1, ICAM-1 and E-selectin) are thought to play a pivotal role in monocyte recruitment [106]. Besides, it was reported that

flavonoids may contribute to prevention of cardiovascular diseases as luteolin was demonstrated to be a strong scavenger [33], and quercetin inhibits platelet aggregation both in vitro [107] and ex vivo [108, 109], although its activity in vivo has yet to be confirmed.

On the other hand, phenols have a potential role in cancer prevention [110, 111], particularly in breast cancer prevention [5]. In fact, human carcinogenesis is mainly due to deoxyribonucleic acid (DNA) damages caused by oxidative stress [112, 113]. Recent studies concluded that phenolic compounds have the ability to induce cell differentiation, inhibit cell cycle progression and exert antiproliferative effects [114, 115].

Other preventive and therapeutic effects have been reported to some phenolic compounds (oleuropein, verbascoside, hydroxytyrosol and tyrosol), such as antiviral and antibacterial activity, beneficial effects on insulin sensitivity and diabetes [116, 117], on Parkinson's diseases [103] and on autoimmune diseases such as rheumatoid arthritis [118]. Besides, dialdehydic form of deacetoxy-ligstroside aglycone (also called oleocanthal) has an antinflammatory action comparable to that of the structurally similar drug ibuprofen [55]. Also, apigenin-7-glucoside was demonstrated to be efficient to fight against Alzheimer's [119, 120] or liver diseases [121, 122]. Luteolin-7-glucoside avoids the abnormal proliferation of aortic vascular smooth muscle cells, a common cause of pathogenesis such as atherosclerosis and restenosis [123]. Also, the two aglycone forms, apigenin and luteolin, are strong inhibitors of murine and human T-cell responses [124]. Phenolic compounds are also involved in preventing obesity and metabolic syndrome [125, 126].

Finally, it is noteworthy that clinical confirmation of these beneficial effects is still needed.

2.2.3 Effect of hydrophilic phenols on VOO sensory qualities

In addition to their effect on VOO stability and human health, phenolic compounds, together with volatile compounds, are the main responsible for the sensory attributes of VOO (e.g. bitter, astringent, pungent, throat-catching), providing this oil with its delicate and unique flavour highly appreciated by consumers [5, 44, 127]. Phenolic compounds are responsible for the taste, in particular the positive bitter and pungent organoleptic attributes [128].

Montedoro et al. [129] reported that hydroxytyrosol, tyrosol, caffeic acid, coumaric acid and *p*-hydroxybenzoic acid exhibit the greatest effect on the sensory characteristics of olive oil. In fact, recent studies showed that the sensory intensity of the bitterness attribute in VOO is associated mainly to secoiridoid derivatives of hydroxytyrosol, particularly 3,4-DHPEA-EDA and 3,4-DHPEA-EA [130] and intensity of sensory perception of pungency is mainly related to the content of the deacetoxy form of *p*-HPEA-EDA [131].

2.2.4 Possibility of oils enrichment with natural antioxidants: Nutraceutical uses

Concerns about safety of use of synthetic antioxidants in food industries have increased interest in searching for natural antioxidants from plant sources. In addition, this interest is increased because foods preserved with natural antioxidants are at present more appreciated by consumers because they could be considered as functional foods, and thus, endowed with beneficial effects on health when consumed on a regular basis as part of a varied diet, at effective levels [132]. Accordingly, various antioxidants as tocopherols, ascorbic acid, lycopene, rosemary extracts and some flavonoids have been successfully extracted from plants and are now available in the markets to replace synthetic products. Moreover, phenolic compounds of olive fruit and VOO have been widely explored due to their powerful antioxidant effect and potentially beneficial effects on human health [133]. Japón-Lujan and Luque de Castro [134] experimented the enrichment of olive, sunflower and soya oils with phenolic extracts from olive leaves (with oleuropein, verbascoside, apigenin-7-glucoside and luteolin-7-glucoside as more concentrated phenols). They showed that olive oil was the most enriched, but the other two oils could also be enriched; improving in this way quality and healthiness of the target oils and suggesting the future possible use of this process at a industrial scale. Subsequent studies from the same team with other oils (olive-pomace, sunflower, high oleic-acid contents sunflower, coconut and linseed) showed a given enrichment order as a function of phenols polarity and molecular weight – higher distribution factors for more polar and lower molecular weight phenols. Concerning oil composition, oils with higher concentration of PUFA yielded higher phenols distribution factors; oils with higher concentrations of saturated fatty acids yielded the lower distribution factors [135].

Other experiments on mass transfer of natural phenolic compounds to refined olive oil also showed that antioxidant activity depends on the concentration of the phenolic compounds and their chemical structure [136]. These authors found that the most positive effect is obtained using the 3,4-dihydroxy and 3,4,5-trihydroxy structures linked to an aromatic ring that conferred to the other moiety a higher proton dislocation, facilitating in this way the scavenging activity.

Other studies on sunflower oils showed that extracts obtained from the olive tree and residues from olive-oil production (leaves, fruits and pomace) have a remarkable antioxidant activity retarding oxidative rancidity of these oils [133].

Finally, it is worth noting that some studies on the effect on taste and flavour of enriched refined olive oil with phenolic leaf extracts were made by Paiva-Martins et al. [137], and the official panel at the Agronomy Institute of Porto did not found significant differences between the flavour of oil before and after enrichment and a better score in the taste quality was obtained by the enriched oil.

3 Concluding remarks

Virgin olive oil is considered the pillar source of fat in the Mediterranean diet; it is recognized for its nutraceutical and organoleptic properties. These belong to its unique and well-balanced composition that includes antioxidants such as hydrophilic phenols. This group of compounds has valuable antioxidant activity endowing the oil with high stability during storage, besides their effects on human health and on VOO sensory characteristics.

Different techniques and methodologies have been used for determination of total phenols and quantitation of individual phenolic compounds. Despite the use of traditional and sophisticated technologies, this group of compounds has not been completely identified due to the complexity of their chemical nature and the heterogeneity of VOO.

For all these reasons, the challenge of the scientists should be moved towards the following developments: (i) more systematic studies focused on the improvement of phenols recovery; (ii) more collaborative studies between recognized groups with a view in the determination of all phenols present in VOO; (iii) a more reliable and more sensitive universal method for phenols determination to make possible the comparison between different studies.

On the other hand, it is of equal importance to distinguish between individual phenols responsible for major resistance to oxidation, and those responsible for healthy properties and for bitter and pungent sensations of VOO, aimed at characterization of this highly valued product.

Finally, it is worth noting the interest of testing the effect of these bioactive phenolic compounds in vivo in humans with the aim of understanding their biochemistry and transformation by human enzymes.

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