Review Article

Hydrophilic antioxidants of virgin olive oil. Part 2: Biosynthesis and biotransformation of phenolic compounds in virgin olive oil as affected by agronomic and processing factors

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The biosynthesis of the phenolic fraction of olive fruits during ripening and the transformations occurring in this moiety during virgin olive oil (VOO) extraction are discussed in this paper. The influence of agronomical factors that can significantly affect the phenolic profile of VOO is also discussed. Particularly, it is worth emphasizing the role of genetic factors, cultivation and climatic conditions such as water availability, atmospheric temperature, altitude, health status of the fruits, alternate bearing in the olive, and some processing factors such as crushing, malaxation time and temperature or volume of water added during milling. Among these parameters, special attention has been paid to genetic factors due to the high variability observed among *Olea europaea* genotypes for all recorded traits. In this context, interesting experimental results have been obtained with cultivated and wild olive trees, and also with segregating populations resulting from olive breeding programs. To the authors' knowledge, reviews evaluating the influence of the main factors that contribute to the profile of hydrophilic phenols have not been previously published. The discussion concerning olive breeding programs is a major and novel aspect to be emphasized considering recent trends to obtain new olive cultivars that confer better organoleptic properties and better quality to VOO.

Keywords: Agronomic factors / Genetic variability / *Olea europaea* / Phenolic compounds / Processing factors

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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; **EA,** elenolic acid; ***p*-HPEA,** *p*-hydroxyphenylethanol; ***p*-HPEA-EA,** ligstroside aglycone; ***p*-HPEA-EDA,** dialdehydic form of elenolic acid linked to *p*-HPEA; **PAL,** phenylalanine ammonia lyase; **PPO,** polyphenoloxidase; **VOO,** virgin olive oil

1 Biosynthesis and biotransformation of phenolic compounds in olive fruit

The *Oleaceae* family contains simple phenolic compounds, carotenoids, tocopherols, chlorophylls and anthocyanins, compounds common to many fruits and vegetables, and a group of complex phenolic compounds specific of this family known as phenolic oleosides or secoiridoids. While meanwhile the biosynthesis pathways of the common compounds are well known, limited data are available for secoiridoids of the *Olea europaea* species. This lack of information can be due to the low rate of water uptake by plants in this genus and the subsequent difficulty for conducting biosynthesis studies [1].

Moreover, olive fruit is in dynamic state and the metabolites level at processes [2]. Moreover, studies on olive oil also add the difficulty due to the extraction process that modify the status of fruit metabolites, and yield chemical changes by exposure to oxygen, mild heat, etc. [3].

1.1 Biosynthesis and biotransformations of common compounds in olive fruit

Phenolic compounds are secondary metabolites derived from the Shikimate pathway and phenylpropanoid metabolism. The former is the metabolism responsible for the formation of the two aromatic amino acids phenylalanine and tyrosine. The non-oxidative glycolysis of glucose yields phosphoenolpyruvate and erythrose-4-phosphate; both constitute the initial reactants of shikimic acid, or Shikimate pathway [4]. The obtained phenylalanine represents the initial substrate of the general phenylalanine metabolism and refers in particular to the production of *p*-coumaric acid from phenylalanine (Fig. 1).

The key enzyme in phenolic biosynthesis is phenylalanine ammonia lyase (PAL), which initiates the biosynthesis of a wide range of phenylpropanoid secondary compounds, including lignin and flavonoid pigments. PAL catalyses the non-oxidative stereo specific elimination of ammonia from phenylalanine (or tyrosine) to yield *trans*-cinnamate [4]. This enzyme, which also plays a key role in the control of flux into total phenolics, is highly sensitive to environmental conditions, and especially to stresses such as temperature, wounding and ultraviolet (UV) light [5]. PAL activity varies greatly according to the degree of fruit ripening [5]; consequently, ripening index and environmental conditions constitute crucial factors to be taken into account in the selection of sampling time for qualitative and quantitative determinations of the target compounds (antioxidants).

On the other hand, 4-coumarate 3-hydroxylase, the enzyme responsible for formation of caffeate, has yet not been unequivocally identified [6], but the final products of the metabolism in which it is involved are caffeic acid and its conjugate, quinic acid, which are important antioxidants.

Carotenoids proceed from the prephytoene pyrophosphate, formed by the successive action of geranylgeranyl pyrophosphate and phytonene synthases on geranyl pyrophosphate. At this step, the enzymes lycopene synthase, phytoene desaturase and ξ-carotene add a double bond within the conjugated system to form lycopene; then, carotene cyclases can produce either β-carotene or lutein (via α-carotene) [7].

Concerning tocopherols biosynthesis from tyrosine, an aromatic amino acid, is oxidized to *p*-hydroxypyruvic acid, this converted into homogentisic acid, then condensed with phytyl diphosphate in a reaction catalysed by a prenyl transferase to yield 2-methyl-6-phytyl-plastoquinol. This is first methylated to form 2,3-dimethyl-5-phytyl-1,4-benzoquinol and then converted to γ-tocopherol by tocopherol cyclase. A further methylation reaction produces α-tocopherol, while modifications to the pathway produce β- and δ-tocopherols (http://www.lipidlibrary.co.uk/Lipids/tocol/index.htm.).

During morphologic development of fruit, an accumulation of chlorophylls, magnesium complexes of a substituted porphyrin occurs. The biosynthesis of chlorophylls involves more than 18 stages and has glutamic acid as initial reactant.

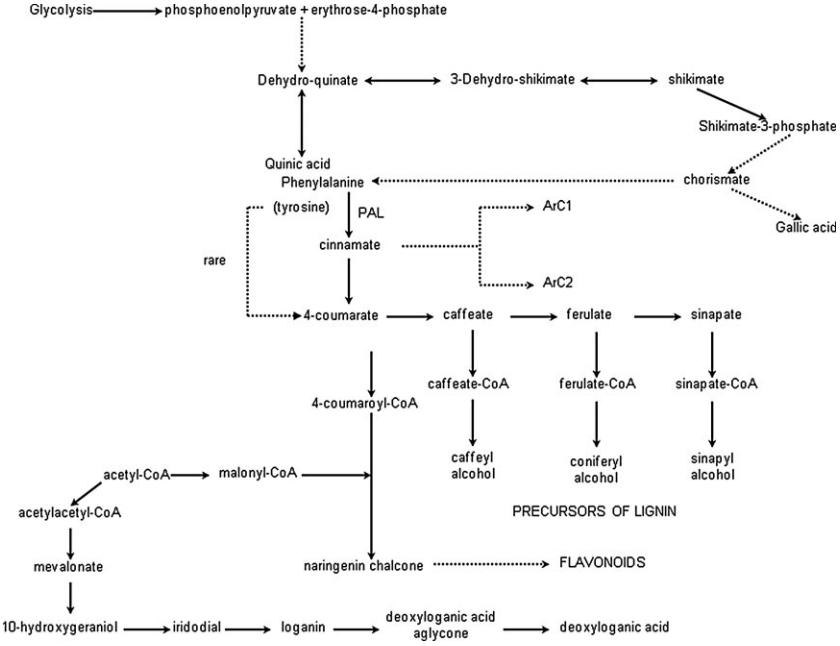

Figure 1. Phenolic biosynthesis pathway (Ryan et al., 2002 [5], reproduced with permission of Elsevier).

The final product of this metabolism is chlorophyll a, which results from the combined action of phytyl pyrophosphate and the enzyme chlorophyll synthetase [7].

During maturation, the appearance of violet or purple colour in olive fruit indicates the end of morphological development [8]. This colour change is due to the decrease in chlorophylls and oleuropein levels and formation of anthocyanins. The most common of these pigments found in olive fruit are cyanidin and delphinidin glycosides [9].

The synthesis and accumulation of anthocyanins require the presence of free sugars. The later are acquired by degradation of oleuropein during fruit ripening [10]. Besides, cytokinin increases with olive fruit ripening and enhances anthocyanins biosynthesis [11], which, according to Romani et al. [12], is varietal dependent.

The chloroplasts are the site for pigments biosynthesis, where the latter are fairly stable. However, olive oil production, including crushing and malaxation, breaks cell walls and exposes olive fruit matrix to enzymes, oxygen and mild heat, thus favouring many chemical and enzymatic processes [13]. As maturation progresses, chlorophylls disappear and carotenoids associated to them may simultaneously disappear simultaneously. Alternatively, the concentration of carotenoids may be maintained or, even increased, as a result of synthesis of new carotenoids [14].

1.2 Biosynthesis and biotransformations of phenolic oleosides

The exclusive presence in *O. europaea* of a number of coumarin-like compounds – secoiridoids – derived from iridoids by opening of the cyclopentane ring of the latter is of chemotaxonomic interest. Iridoids (e.g. loganin), produced via secondary metabolism of monoterpenes, are characterized by skeletons in which a six-membered heterocyclic ring is fused to a cyclopentane ring [3]. The opening of the cyclopentane ring leads to the formation of the secoxyloganin, which represent the parent compound of the secoiridoids.

In *Oleaceae* species, secoiridoid conjugates such as oleuropein involve a phenolic fraction as a result of esterification via a branching in the mevalonic acid pathway in which terpene synthesis (oleoside moiety) and phenylpropanoid metabolism (phenolic moiety) merge (Fig. 2).

Ryan et al. [5] described the biosynthetic pathway for the production of oleuropein and its derivative (dialdehydic form of elenolic acid linked to 3,4-dihydroxyphenylethanol (3,4-DHPEA-EDA)) via tyrosine (Fig. 3).

Oleuropein, demethyloleuropein, ligstroside and nüzhenide are the main oleosides in *O. europaea*. They are esters characterized by the presence in their molecule structure of elenolic acid (EA) in its glucosidic or aglyconic form [16]. For example, oleuropein is the ester of EA with 2-(3,4-dihydroxyphenyl)etanol (hydroxytyrosol), and ligstroside is the ester of EA with 2-(4-dihydroxyphenyl)etanol (tyrosol) (Fig. 4).

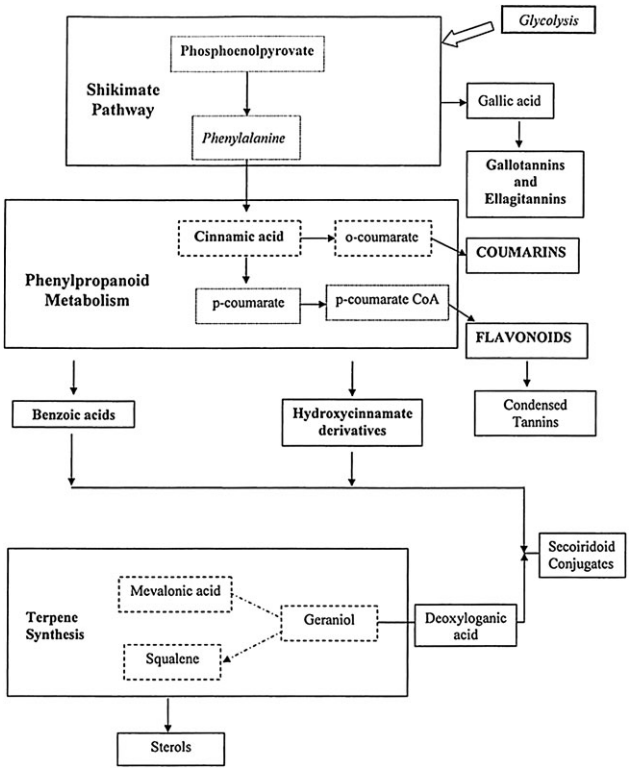

Figure 2. Schematic illustration showing the links between phenylpropanoid metabolism and mevalonic acid pathway (Obied et al., 2008 [15], reproduced with permission of the Royal Society of Chemistry).

Oleuropein is the major compound in fruit of many cultivars; its concentration reaches higher levels during the growth phase or green maturation of the fruit, and declines with physiological development of the fruit, may be due to the increased activity of hydrolytic enzymes [18, 19]. The decline in oleuropein concentration with ripening [10] may be associated to the accumulation of anthocyanins and the requirement for sugar. Indeed, Capasso et al. [20] found that the hydrolysis of oleuropein by β-hydroxylase results in the formation of glucose.

The decline continues rapidly during black maturation [21] and even can fall to zero when olive fruit is completely black, as reported by Bianco et al. [22] in *O. europaea*, cv. Leccino. These authors also noted the presence of small quantities of ligstroside in small green olive fruit, which disappear when they reach their normal size.

Degradation of oleuropein during maturation is accompanied by accumulation of demethyloleuropein and EA glucoside [18]. The former replaces oleuropein in about the same amount, and constitutes the major constituent of black olive fruit [23]. On the other hand, the presence of demethyloleuropein is varietal dependent, as reported by Amiot

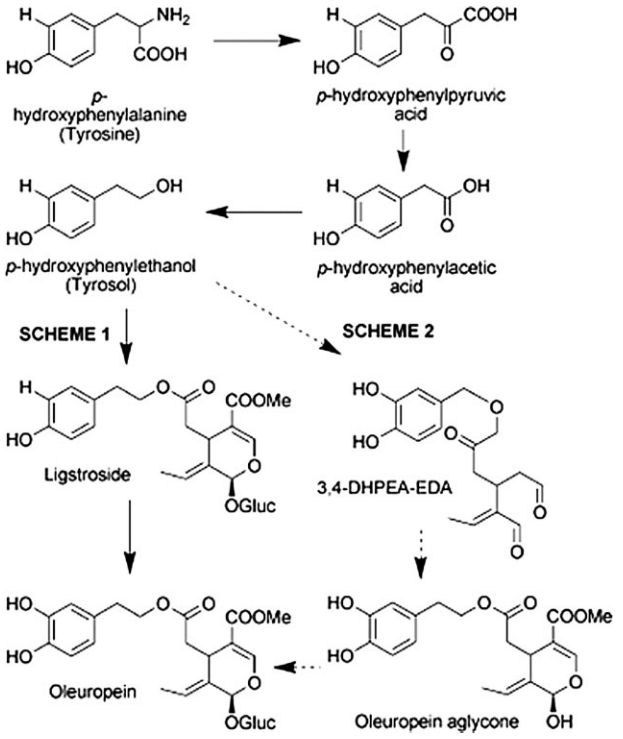

Figure 3. Biosynthetic pathway for the production of oleuropein and 3,4-DHPEA in *O. europaea* (Ryan et al., 2002 [5], reproduced with permission of Elsevier).

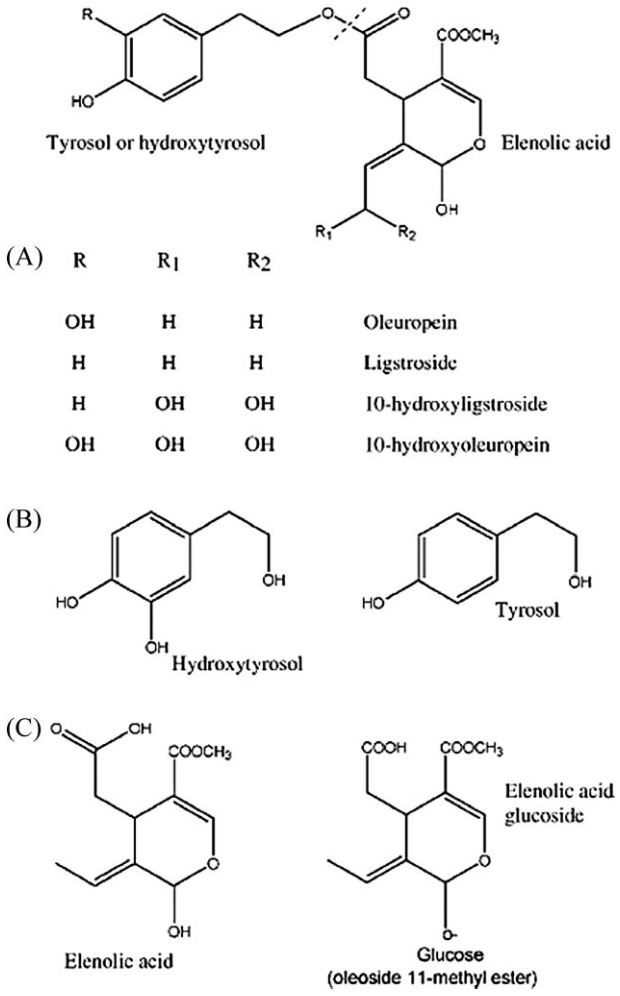

Figure 4. Chemical structures of: (A) oleuropein, ligstroside, 10 hydroxyligstroside and 10-hydroxyoleuropein – hydroxytyrosol and tyrosol derive from the hydrolysis of oleuropein; (B) hydroxytyrosol and tyrosol; (C) EA and EA glucosides (Tripoli et al., 2005 [17], reproduced with permission of Cambridge Journals).

et al. [18]. An inverse relationship between oleuropein and a non-secoiridoidal biophenol – verbascoside – during fruit ripening was suggested by Amiot et al. [10]. This relationship was not supported by Ryan et al. [24], who claimed that the metabolism of oleuropein in olive pulp varied with alternate bearing, and the concentrations of oleuropein and verbascoside increased with fruit ripening in high-fruiting season.

Nüzhenide, the major specific secoiridoid in olive seed, was found at higher concentrations at the beginning of the low-fruiting season than in the high-fruiting season. This phenomenon was also observed for oleuropein and verbascoside in olive seeds [15]. Therefore, it is very important to take into account the effect of the cultivar and alternate bearing on phenolic profiles.

3,4-DHPEA-EDA, isomer of oleuropein aglycone (3,4-DHPEA-EA) and dialdehydic form of elenolic acid linked to *p*-hydroxyphenylethanol (*p*-HPEA-EDA) are derivatives of secoiridoids glucosides present in olive fruit, produced by enzymatic and/or chemical degradation of the oleosidic secoiridoids, and released in the oil during the mechanical extraction process [25, 26].

The degradation of oleuropein occurs by two pathways; the first involves cleavage of oleuropein to EA or demethyloleuropein by specific endogenous esterases [27], which are both found in mature olive fruit. The second includes activation of β-glucosidases during crushing and malaxation of fruit, which may produce the aglycon from the glycoside [15]. It was presumed that demethyloleuropein acts as a precursor for the formation of 3,4-DHPEA-EDA during crushing. However, high concentrations of the latter were found in cultivars characterized by low demethyloleuropein concentration. These results indicate that the concentration of 3,4-DHPEA-EDA is not only dependant on the concentration of demethyloleuropein, but also on that of oleuropein decomposed by methylesterase.

During malaxation, the final composition of secoiridoid derivatives is mainly controlled by the activity of β-glucosidase. However, many authors [28–33] expressed the strong effect of peroxidase (POD) and polyphenoloxidase (PPO) on 3,4-DHPEA-EDA and 3,4-DHPEA-EA oxidation, while the effect on the oxidative degradation of *p*-HPEA-EDA was

lower. Thus, the enzymatic oxidative mechanism is the most important technological aspect that may control the concentration of secoiridoid derivatives in virgin olive oil (VOO).

2 Factors affecting the composition in antioxidants of VOO

The phenolic composition of VOO is the result of a very complex multivariate interaction between genotype and agronomic, environmental and technological factors [34, 35]. In fact, while genetic, agronomic and environmental factors determine the chemical and biochemical composition of olive fruit [36–39] the technological factors, mainly milling and malaxation, are the most critical steps during olive processing and oil extraction where the most important changes in VOO phenolic composition occur [40–42].

In this section, we summarise the effect of agronomic, environmental and technological factors on the phenolic composition of VOO. The genetic factor is studied in detail in the following section, as it is one of the main objectives of this paper.

2.1 Agronomic and environmental factors

2.1.1 Maturity index of olive fruit

The modification of phenolic profile throughout growth of the fruit and ripening process has given place to studies focused on describing the evolution of phenolic compounds in olive fruit during this period [9, 10, 43]. These studies concluded that the harvest time plays a key role in the phenolic composition of VOO.

In the first growing stages, oleuropein is the main phenolic compound in olive fruit; its concentration can reach up to 14% of net weight [10]. The concentration of this secoiridoid declines with physiological development of fruit in what is called 'green maturation phase' [10, 18]. This decline continues and become very significant during the black-maturation phase, when olive fruit turns to dark brown colour due to the presence of anthocyanins. This steady decrease in oleuropein content could be due to the activity of esterase enzymes (e.g. β-glucosidase) that cause degradation of the phenolic moieties, leading to their transformation into new phenolic and non-phenolic conjugates [43]. Degradation of oleuropein in olive fruit is accompanied by accumulation of demethyloleuropein and EA glucoside, of which only the former is a phenol [18]. Ragazzi and Veronese [23] claimed that oleuropein is converted into demethyloleuropein during fruit ripening. Accordingly, Gómez-Rico et al. [43] found that the content of demethyloleuropein doubled during fruit ripening of 'Arbequina' cultivar, thus constituting the major phenolic compound in this fruit; however, the same authors and others denied the hypothesis of Ragazzi and Veronese as they did not find this product in all cultivars [18, 43]. Moreover, in over-ripened olive fruit, the concentration of both oleuropein and demethyloleuropein underwent a high decrease [18]. Other phenolic compounds, such as ligstroside, are detected in small green olive fruit, but their concentration decreases gradually and falls to zero at the end of green maturation.

The drastic decrease in some phenolic compounds, mainly oleuropein, is accompanied by an increase in phenolic alcohols as hydroxytyrosol and tyrosol [44] and in the main hydroxycinnamic derivative in olive fruit, verbascoside [40]. An increase in flavonoids with fruit ripening (mainly in luteolin-7-glucoside, rutin and luteolin) was also detected by Artajo et al. [32]. Finally, the concentration of both chlorophylls and tocopherols decreases in parallel with fruit ripening, while that of carotenes disappears, remains unmodified or even increases, as a consequence of a new synthesis. The concentration of anthocyanins increases [45].

These changes in the phenolic profile of olive fruit is reflected in the composition of the corresponding VOO, as this is the juice of olive fruit obtained only by mechanical or physical methods under conditions, especially temperature, guaranteed to avoid alteration of the product.

In summary, the levels of hydroxytyrosol and tyrosol in the oil increase while the concentrations of both dialdehydic forms of EA linked to hydroxytyrosol or tyrosol diminish. This phenomenon is caused by β-glucosidase activity, which hydrolyses oleuropein to oleuropein aglycon and 3,4-DHPEA-EDA [21, 46]. Other simple phenols as vanillic acid, vanillin, *p*-coumaric acid and ferulic acid practically remain constant [18]. Among the group of glycosylated flavonoids, Artajo et al. [32] noted an increase in luteolin and apigenin in the oil with ripening. Finally, Brenes et al. [47] noted a decrease in pinoresinol concentration as ripening progresses, while acetoxypinoresinol increased in some cultivars ('Picudo' and 'Hojiblanca') and decreased in others ('Picual').

2.1.2 Cultivation zone

The cultivation zone of olive trees has a considerable effect on chemical composition of fruit and corresponding VOO; the effect is probably related to the pedo-climatic conditions; this effect is more evident at 'macroclimate' scale than at 'mesoclimate' scale [48].

The influence of temperature on this effect was studied, but with contradictory results; while Ripa et al. [49] concluded that the higher the degree-days cumulated from fruit set to harvest the lower the phenolic amount in the fruit, Tura et al. [48] reported that in some varieties as 'Casaliva', 'Leccino' and 'Frantoio', the amount of phenols increases with the increase in the degree-days cumulated from fruit set to harvest in some cool areas in Italy.

In addition, some studies in the Mediterranean basin on the effect of low temperatures on oil quality showed that when temperature falls down to zero during fruit ripening, damage

of the fruit takes place thus decreasing the quality of the oils. It was shown that oils produced from frost-damaged olive fruit had lower content of secoiridoids in comparison with normal drupes due to freeze-fracturing of cell walls in the drupes, leading to oxidation of phenolic compounds [50].

The effect of altitude on the composition of VOO was studied by Osman et al. [51] and Mousa et al. [37]; and they conclude that drupes grown at lower altitudes have higher phenols amounts than those produced at higher altitude. These authors suggest that this behaviour could be related to the more suitable temperature and sunlight intensity in lower altitudes leading to an increase of carbohydrate biosynthesis and acylbuilding blocks for phenols synthesis [52]. However, the effect of altitude was not unequivocal in other studies [53].

Also, the effect of soil on the phenolic profile of VOO is unclear, but some relationships between soil characteristics and VOO composition were outlined [54]. In this context, it was reported that oils from the cultivar 'Moraiolo' cultivated in stony soils produced oils with higher phenolic content than oils of the same cultivar cultivated in clay ones; however, this behaviour was related to the lower water availability in stony soils, rather than to soil texture [55, 56].

Finally, Torres and Maestri [57] demonstrated that, using the same extraction conditions, 'Arbequina' olive fruit grown in Córdoba (Argentina) produced oils with higher phenolic content than those grown in Spain. These results may be very useful to characterize the origin of monovarietal VOO by their phenolic composition.

2.1.3 Water availability

Water availability has a considerable effect on phenolic composition of olive fruit. This effect may be explained by modification of activity of enzymes responsible for phenolic synthesis, as L-phenylalanine ammonia-lyase, the activity of which increases with water stress [28, 58, 59]. Nevertheless, this effect could also be due to different water percentage in the olive paste, which imply different concentration of hydrophilic phenols according to their partition coefficient [60] and/or different efficacy to release these compounds during crushing and malaxation [61].

Major results support that increased amount of water produces oils with lower phenolic content [59, 62]. Nevertheless, some authors reported no effect, or even an increase in phenolic compounds, particularly during the first stages of fruit ripening in irrigated trees compared to rain fed ones. This effect may be related to genetic or other agronomic aspects [61, 63].

Relatively recent research [64] supports that the concentration of secoiridoids increases with water stress. Servili et al. [28] also reported an increase in aglycon derivatives of oleuropein and a decrease in tyrosol in water-stressed trees.

Also during extraction, the amounts of chlorophylls and carotenoids decrease maybe due to an effect on pigment biosynthesis in the olive fruit or to a loss of these pigments during oil extraction [65]. A decrease in the concentration of lignans in oils originated from drought-stressed trees has also been reported [61].

2.1.4 Sanitary state of drupes

One of the principal olive pests in the Mediterranean basin is olive fly (*Bactrocera olea*). This insect has a detrimental influence on VOO quality because it affects the main quality parameters (acidity, peroxide value, UV absorbance and organoleptic quality), and negatively alters the chemical composition of the oil (sterols, phenols, fatty acids and volatile fraction) [36, 66–69]. Gómez-Caravaca et al. [70] observed a decrease in phenols, *o*-diphenols, and, in particular, in some secoiridoid derivatives in cases of strong attack of this pest. They related the increase in PPO activity to the entrance of oxygen from the exit hole made by fly larvae, which enhances phenolics oxidation. As the degree of infestation increases, chlorophylls and carotenoids decrease has also been reported [69].

2.1.5 Alternate bearing in olive trees

Olive tree is characterized by a very marked alternate bearing; this phenomenon is due to the competition between fruits in the earliest stages of growth for assimilates and water [71] and their inhibitory effect on floral induction [72] apparently mediated by gibberellic acid [73]. Both factors cause competition between vegetative and reproductive organs during 'on' year (high-fruiting season) leading to a reduced vegetative growth during this year, which entails a reduction in blooming and consequent fructification in the next year.

Oil composition changes depend on the production year – i.e. the concentration of phenolic compounds in VOO is higher during 'off' years (low fruiting seasons) [74].

2.2 Technological factors

Research has also been focused on the effect of different variables involved in the extraction procedure (e.g. crushing, malaxation time and temperature, volume of water added) on the phenolic composition of VOO [57, 75–77]. The conclusion is that the phenolic composition of VOO is highly affected by endogenous enzymes of olive fruit, which are liberated in the paste during crushing and malaxation.

In fact, during crushing, oleuropein, demethyloleuropein and ligstroside present in the fruit are hydrolysed by action of endogenous β-glucosidases leading to the formation of secoiridoids aglycons such as 3,4-DHPEA-EDA, *p*-HPEA-EDA, ligstroside aglycone (*p*-HPEA-EA) and 3,4-DHPEA-EA [2, 78–80]. It is admitted that mechanical crushers are more effective in the extraction of phenolic compounds than traditional stone mills [81]. Crushing results in the formation of olive paste subjected to malaxation, during which a

decrease of phenolic compounds in oil (mainly oleuropein and hydroxytyrosol derivative 3,4-DHPEA-EDA and 3,4-DHPEA-EA) occurs owing to activation of PPO and POD by fruit disruption [25, 80]. This decrease is more relevant with long malaxation at high temperature [80]. In addition, partitioning of phenolic compounds in the different phases of the paste (oil, water and solids) as a function of their affinities towards each phase, which is related to the relative polarities of the compounds and phases ratio, is involved in the decline of phenolics in oil [82].

On the other hand, Gómez-Rico et al. [80] showed that the concentration of tyrosol derivative (*p*-HPEA-EDA) increased by increasing malaxation time and temperature; whereas the concentrations of verbascoside and flavonoids (as rutin, luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside) did not change significantly.

The application of new techniques for olive oil processing such as regulation of averaged concentration of O_2 by creating an N_2 ambient during malaxation [25, 83, 84], and oil extraction after removal of stones (stones include high concentration of β-glucosidases) [85] can increase VOO phenolic amount by controlling oxidative reactions.

The extraction system also plays a key role in the phenolic composition of VOO regarding the quantity of water added to the paste. This water dilutes the phenolic compounds and helps to their elimination in the aqueous phase. Accordingly, the pressure system that does not require water addition shows higher concentration of phenolic compounds than the three-phases centrifugation system, which requires warm water addition [76]. However, the two-phases centrifugation system, characterized by no water addition and short processing time, and the three-phases centrifugation, with low water addition and short processing time, improve the oil phenolic composition in comparison with pressure system [57] and traditional three-phase system [57, 76, 86, 87].

During oil storage, the enzymatic activity continues in the cloudy phase leading to a decrease in the phenolic composition of the VOO, mainly of secoiridoids aglycons [88].

At the end of the process, the filtration step applied in some olive-oil mills can influence the phenolic composition of VOO. In fact, filtration eliminates water from the oil and, consequently, eliminates the major quantity of hydroxytyrosol dissolved in the aqueous phase or diminishes its activity [89]. However, the reduction of water may increase the apparent phenolic content because it permits a higher availability of the phenolic compounds that remain in the oil, which can be more easily extracted by a methanol–water mixture [89].

3 Genetic variability of antioxidants of VOO

The *O. europaea* L. species is possibly of hybrid origin [90, 91]. The huge diversity described in this specie and cytogenetic studies allow concluding that it is an allopolyploid [91]; i.e. the designated species proceeds from a cross between different species of the genre *Olea* with 11 or 12 chromosomes and subsequent duplication [92].

The olive tree is a preferably allogamous plant in which the pollen of the given variety takes more time to reach the micropile than the pollen of other varieties [93]. As reported by Angiolillo et al. [94] and Rallo et al. [95], the species of such pollination type (outcrossing) show high levels of heterozygosity and DNA polymorphism among individuals. This heterozygosity is represented by high variation among cultivars concerning several characteristics of olive tree, olive fruit and corresponding olive-oil composition leading to the probable differentiation of more than 1200 varieties cultivated worldwide [96]. Accordingly, variation in the phenolic composition of VOO has been reported both in the cultivated (*O. europaea* subsp. *europaea* var. *europaea*) and in the wild (*O. europaea* subsp. *europaea* var. *sylvestris*) forms of the olive tree.

3.1 Genetic variability between cultivars

Phenolic composition of olive fruit and the corresponding VOO is qualitatively affected by the cultivar [55, 97–99]. To date, various studies have been conducted with the aim of describing the variability of phenolic profiles in the different olive cultivars [12, 19, 100].

The total phenolic content, determined photometrically, shows a drastic variation among cultivars [101–103]. Indeed, a study carried out since 1992 till 1998 on 28 cultivars grown in the germplasm bank of Cataluña confirmed that the cultivar is the determinant factor in the variation of total phenols (63%), although the year of production represented 9%, the interaction cultivar*year of production represented 10%, and the experimental error 18% [104]. A similar study, conducted simultaneously on 24 cultivars grown in the Olive World Germplasm Bank of Córdoba, showed that variation of total phenols due to cultivar may reach 78% and the effect of year of production is only 0.07%, with a range of variation in total phenols content from 121 mg of caffeic acid/kg of oil (in 'Nevadillo de Santisteban del Puerto') to 1240 mg of caffeic acid/kg of oil (in 'Chetoui') [105].

The phenolic profile determined by HPLC analysis demonstrated that oleuropein is present in the fruit of almost all olive cultivars with concentrations between 35 and 2400 mg/kg of fruit [12], while demethyloleuropein and verbascoside are cultivar-dependent [10, 18, 42]. Demethyloleuropein, a degradation product of oleuropein, as some investigators claim [9, 18], could be used as a cultivar marker as it was present in two ('Coratina' and 'Leccino') out of eight Italian cultivars as reported by Esti et al. [19], and in only 2 ('Cailletier' and 'L11') out of 11 French cultivars, as reported by Amiot et al. [18]. With regard to verbascoside, Gómez-Rico et al. [43] noted an inverse relationship between the amount of this phenol and that of oleuropein. They found that 'Arbequina', which had the lowest content of oleuropein, had the highest content of verbascoside; 'Picual' and

'Hojiblanca' which had the highest content of oleuropein, had the lowest content of verbascoside.

In VOO, the concentration of hydrophilic phenols is influenced by the cultivar, although the phenolic profile is almost the same [78, 85]. It is accepted that secoiridoid derivatives of oleuropein and ligstroside are often the main phenolic compounds in fresh VOO [47, 106–108]. In fact, secoiridoid derivatives of hydroxytyrosol, especially 3,4-DHPEA-EDA, are the major phenolic compounds encountered in the cultivars 'Arbequina', 'Cornicabra', 'Picolimón' and 'Picual', with values ranging from 105.0 to 1113.2 mg of caffeic acid/kg of VOO; however, the secoiridoid derivatives of tyrosol (mainly *p*-HPEA-EDA) are major phenolic compounds, of 'Morisca' and 'Picudo' with values ranging from 54.8 to 769.6 mg of caffeic acid/kg of oil [43].

Lignans presented also high variation between cultivars [47, 109], although with some divergence in the results maybe due to different agronomic factors (production area, ripening degree, etc.). While Brenes et al. [47, 98] detected pinoresinol and acetoxypinoresinol in all cultivars under study ('Arbequina', 'Empeltre', 'Picual', 'Picudo' and 'Jarduo'), although in a very low concentration in the last three cultivars, Oliveras-López et al. [109] did not detect pinoresinol or acetoxypinoresinol in some 'Picual' oils. High concentration of acetoxypinoresinol in 'Arbequina' oils was detected in both studies. Moreover, Brenes et al. [47] showed that the sum of the two lignans (pinoresinol and acetoxypinoresinol) may constitute the major phenolic fraction of 'Arbequina' and 'Empeltre' oils. On the other hand, Brenes et al. [98] detected simple phenols such as hydroxytyrosol, tyrosol, vanillic acid, *p*-coumaric acid and ferulic acid in all Spanish cultivars ('Picual', 'Picudo', 'Hojiblanca', 'Arbequina', 'Jarduo', 'Blanqueta', 'Empeltre', 'Villalonga', 'Cornicabra', 'Cornezuelo', 'Lechín', 'Manzanilla' and 'Verdial'); however, caffeic, syringic and homovanillic acids, detected in other oils, were not detected in any cultivar in this study. Besides, Gómez-Rico et al. [43], working with oils from six Spanish cultivars ('Arbequina', 'Cornicabra', 'Morisca', 'Picolimon', 'Picudo' and 'Picual'), did not note significant differences in simple phenolic compounds between cultivars, except higher content of both hydroxytyrosol in 'Picudo' oil and ferulic acid in 'Arbequina' oil. However, Oliveras-López et al. [109], detected amounts of hydroxytyrosol significantly higher in 'Picual' than in 'Arbequina', 'Picuda' and 'Taggiasca' oils.

Regarding flavonoids composition, Gómez-Rico et al. [43] showed that rutin and luteolin 7-*O*-glucoside are major flavonoids in all studied cultivars ('Arbequina', 'Cornicabra', 'Morisca', 'Picolimon', 'Picudo' and 'Picual'). Independently of harvest time, they did not found significant differences in rutin composition among cultivars, but the significantly higher amounts of luteolin 7-*O*-glucoside in 'Picual' oils permitted differentiation from other cultivar oils. Higher amounts of quercetin 3-*O*-rutinoside and apigenin 7-*O*-glucoside enabled discrimination of 'Cornicabra' oils, and the highest amounts of apigenin 7-*O*-glucoside made possible discrimination between 'Picual' and 'Cornicabra' oils.

Anthocyanins could also be a parameter of discrimination between cultivars at black ripening stages as their content ranged between 1050 mg/kg of fresh weight in 'Morisca' fruits and 3240 mg/kg of fresh weight in 'Cornicabra' olive oils, as reported by Gómez-Rico et al. [43], who, after application of principal component analysis, also reported that oleuropein is the most useful variable for olive cultivar classification representing 82.1% of variance, followed by apigenin 7-*O*-glucoside (11.8%), hydroxytyrosol and cyanidin 3-*O*-rutinoside (6.1%).

3.2 Genetic variability in wild olive trees

The second subspecie of olive tree is wild olive (*O. europaea* subsp. *europaea* var. *sylvestris*), found in big populations in the Mediterranean basin and well documented by authors such as Baldoni et al. [110]. One of the most noticeable differences between cultivated and wild olive trees is higher volume and more oiliness of fruit in the former.

Wild olive populations are very heterogeneous [111] and they show several interesting agronomic characters (resistance to climatic conditions, to pests and diseases, etc.) [112]. Interfertility of cultivated olive tree/wild olive tree and segregation of characters by sexual reproduction make the latter a valuable source of material for cross breeding programs and a material to define new varieties with specific characters as adaptation to given climates and higher quality oils [112, 113].

Few studies on the phenolic composition of wild olive oils have been reported so far. Baccouri et al. [113], while evaluating seven oil samples from seven different oleasters (wild olive trees) under similar agronomic, environmental and technological conditions, observed high genotype effect on the total phenols and *o*-diphenols amounts with values ranging between 182 and 430 mg expressed as caffeic acid/kg of oil for total phenols and 105–217.6 mg of caffeic acid/kg of oil for *o*-diphenols. In addition, they noticed high variation of tocopherols content between samples, although α-, β-, γ- and δ-tocopherols were detected in all oils in different amounts, but maintaining α-tocopherol as major component. The HPLC total tocopherols values ranged between 310 and 780 mg of tocopherols/kg of oil among genotypes.

3.3 Genetic variability in segregated population

Due to the high level of heterozygosity in *O. europaea* species, cross breeding is considered the best strategy to be adopted by breeding programs to generate new cultivars responding to the objectives of these programs [114]. In fact, in such cases, any cross combination provides segregated populations with a wide range of variation for any characteristics similar to, or even slightly larger than that of the parent cultivars [115–117]. This tendency has been observed in several

Table 1. Studies on variability of phenolic compounds related to VOO in germplasm banks (GB), field trials (FT), segregating progenies (SP), wild olive (WO) and samples from orchard, industries or market (SO)

Phenolic compounds	Plant material	References	Observations
3,4-DHPEA *p*-HPEA Vanillic acid Total phenoles Tocopherols Total carotenoids Total chlorophylls	18 (Italy/FT)	[118]	Variability according to harvest time and cultivation zone
Total phenols Total tocopherols	2 (Italy/FT)	[53]	Interaction environmental conditions vs. cultivars
3,4-DHPEA *p*-HPEA 3,4-DHPEA-EDA *p*-HPEA-EDA 3,4 DHPEA-EA	5 (Italy/FT)	[78]	Significant effect of cultivar on hydrophilic phenols
3,4-DHPEA *p*-HPEA 3,4-DHPEA-EDA *p*-HPEA-EDA 3,4 DHPEA-EA Vanillic acid Caffeic acid Total phenols *p*-HPEA-ester	2 (Italy/SO)	[86]	Effect of extraction system Two phases systems yield higher phenols content than three phases
Total phenols	3 (Italy/SO)	[119]	No correlation total phenols vs. added leaves
Total phenols	2 (Italy/SO)	[120]	High influence of crushing on total phenols
Total phenols	1 (Italy/SO)	[121]	High effect of malaxation on total phenols content
Total phenols	1 (Italy/SO)	[122]	Influence of extraction systems on total phenols content
Total phenols α-Tocopherols	1 (Spain/SO)	[123]	Great effect of production system but not of production year
Tocopherols Total phenols Secoiridoid derivatives Carotenoids Chlorophylls Xanthophylls	3 (Italy/SO)	[124]	Significant influence of malaxation temperature
Carotenoids Chlorophylls Total phenols *o*-diphenols	1 (Spain/SO)	[75]	Significant effect of extraction systems and crop year
Carotenoids Chlorophylls Total phenols	4 (Spain/FT)	[57]	Significant variations among olive varieties
Total phenols *o*-diphenols Simple phenols Secoiridoid derivatives Lignan derivatives	7 (Italy/SO)	[125]	Significant effect of cultivar, degree of ripeness, production and extraction technologies
Chlorophylls Carotenoids Total phenols α-Tocopherols	1 (Italy/SO)	[126]	Significant effect of storage conditions

(*Continues*)

Table 1. (Continued)

Phenolic compounds	Plant material	References	Observations
3,4-DHPEA	6 (Spain/FT)	[43]	High variability among cultivars
p-HPEA			Significant influence of harvest time
3,4-DHPEA-EDA			
p-HPEA-EDA			
3,4-DHPEA-EA			
p-HPEA-EA			
Total phenols	1 (Spain/FT)	[59]	Great effect of water availability
Total phenols	1 (Spain/FT)	[127]	Great effect of fruit ripening
Carotenoids			Great effect of water availability
Chlorophylls			
Tocopherols			
Total phenols	3 (Italy/SO)	[128]	Great effect of fruit ripening
o-diphenols			
Total phenols	2 (Croatia/FT)	[129]	Significant effect of ripening and cultivar, with more pronounced effect of ripening
o-diphenols	1 (Italy/FT)		
Chlorophyll			
Tocopherols	2 (Tunisia/FT)	[102]	High variability among cultivars
Total phenols	3 (Italy/FT)		
Antioxidant compounds	5 (Spain/SO)	[130]	High variability between cultivars
	1 (Israel/SO)		
	3 (Italy/SO)		
Total phenols	6 (Tunisia/FT)	[131]	High variability among cultivars
α-Tocopherols			
Pigments			
3,4-DHPEA	13 (Spain/FT)	[98]	Hydroxytyrosol, tyrosol, and luteolin increased during ripening; glucoside aglycons decreased
p-HPEA			No clear tendency in the remaining traits
Vanillic acid			
p-coumaric acid			
Ferulic acid			
Vanillin			
Apigenin			
Luteolin			
3,4-DHPEA-EDA			
p-HPEA-EDA			
3,4-DHPEA-EA			
p-HPEA-EA			
Total phenols	2 (Spain/SO)	[132]	Effect of cultivar, ripening index and crop season
Chlorophylls	2 (Italy/SO)	[133]	Differences between Spanish cultivars and foreign cultivars
Carotenoids	1 (Greece/SO)		
Total phenols	2 (Spain/SO)	[134]	Important effect of cultivar and crop season
o-Diphenols			50% of oxidative stability is contributed by phenols
Tocopherols			
Chlorophyls			
Carotenoids			
Total phenols	2 (Tunisia/SO)	[101]	Significant effect of cultivars and environments
Chlorophylls			
β-Carotene			
o-diphenols			
Secoiridoids	4 (Spain/SO)	[109]	Quantitative variations among cultivars
Lignans	2 (Italy/SO)		
Simple phenols			
Flavonoids			

(*Continues*)

Table 1. (Continued)

Phenolic compounds	Plant material	References	Observations
Total phenols Tocopherols Chlorophylls Carotenoids	7 (Tunisia/WO)	[113]	High variability among cultivars
Pigments Phenolic profile	5 (Tunisia/WO)	[135]	Oleaster's oils different from European and Tunisian oils
Total phenols o-diphenols Pigments	7 (Tunisia/WO)	[136]	High effect of harvesting time
α-Tocpherols Catorenoids Chlorphylls Total phenols	6 (Tunisia/SP)	[116]	High variability in SP, with some values higher than genitors
3,4-DHPEA *p*-HPEA Cinnamic acids Oleuropein Total Phenols	50 (Italy/SP)	[117]	High variability among genotypes
Total phenols Chlorophylls	2 (Tunisia/SP)	[137]	High variability among genotypes
Total phenols Chlorophylls Carotenoids	2 (Tunisia/SP)	[138]	Significant differences among the two new cultivars
Total phenols *o*-Diphenols Hydroxytyrosol Tyrosol Hydroxytyrosol aglycones Tyrosol aglycones Tocopherols Chlorophylls Carotenoids	1 (Italy/SP)	[139]	Significant effect of cultivation zone on the evaluated new cultivar
3,4-DHPEA *p*-HPEA EA EA glucoside EA glucoside derivatives Deacetoxyoleurop-ein aglycone 3,4-DHPEA-EA Secoiridoid derivatives Luteolin Apigenin	10 (Italy/GB)	[106]	High variability among SP
Total phenols	28 (Spain/GB)	[104]	Drastic variability among cultivars
Total phenols	24 (Spain/GB)	[105]	Drastic variability en total phenols content among analysed cultivars

characters as oil content, productivity, fruit retention force and oil composition.

In summary, the breeding process consists of the realization of the crosses among the most outstanding cultivars, plantation and growth of the seedlings and systematic evaluation of the obtained genotypes for interesting agronomic and oleic characters. The final objective is selection of the outstanding genotypes.

Although the presence of high amount of phenols in VOO (higher than 400 mg/kg) is a desirable character for high-quality olive oils, only few studies have been reported on evaluation of phenolic compounds of descendants obtained by cross breeding. At this concern, Dabbou et al. [116] reported significant differences in total phenols between descendants obtained by crossings between 'Chemlali' and other autochthonous and foreign varieties. Total phenols

concentration ranged between 53.20 and 290 mg expressed as gallic acid/kg of oil. Also, they reported significant differences among descendants in terms of α-tocopherols (values ranging between 226.42 and 599.68 mg/kg of oil), carotenes (values between 5.34 and 16.5 mg/kg of oil), and chlorophylls (values between 9.13 and 34.04 mg/kg of oil).

Other evaluations of phenolic compounds in fruit from selections obtained by breeding since 1971, by Professor Bellini (Department of Horticulture of the University of Florence), also showed high variability of concentration in these compounds among the different genotypes in the same cultivation regions and at the same ripening stages [117]. They reported high variation between selections in total phenols amounts in the different regions: in the Metapponto region, the total phenols amount ranged between 17 and 95.9 μg expressed as gallic acid/mg fruit extract during September and between 16 and 61 μg gallic acid/mg fruit extract during October. In the Rossano region, the values ranged between 26.5 and 60.9 μg gallic acid/mg fruit extract in September and 32.8 and 56.4 μg gallic acid/mg fruit extract in October. In the Spoleto region, the total phenols amount among genotypes ranged between 60 and 94 μg gallic acid/mg fruit extract in September and 44.3 and 94 μg gallic acid/mg fruit extract in October. Finally, they noted high variation among genotypes of oleuropein/cinnamic acid ratio, which permitted to classify genotypes in three groups according to oleuropein content, which was lower than, similar to, or higher than that of cinnamic acids.

A summary examination of the revised articles related to the factors affecting phenolic composition in VOO is illustrated in Table 1.

4 Final considerations

VOO is increasingly economically important mainly due to its natural origin and to the worldwide awareness of its beneficial composition rich in antioxidants. In fact, VOO contains several groups of phenolic compounds such as tocopherols, pigments, phenolic alcohols and acids, etc.; these compounds are common also in other vegetable products. On the contrary, a number of coumarin-like compounds known as secoiridoids are exclusive to the *O. europaea* species. In this paper, we focused on the biosynthesis of all phenolic groups of the olive fruit and the corresponding VOO considering all agronomic factors such as cultivar, harvesting time, cultivation zone, water availability, sanitary status of the fruits and alternate bearing and processing factors such as crushing, paste malaxation, phases separation, that may affect these biosynthesis pathways, with a special focus on the genetic factor due to the high heterozygosity among this species for all studied parameters.

Increased interest in phenolic compounds due to their beneficial effect on VOO quality and their progressive use as nutraceuticals and other specific uses will probably forward research into new olive cultivars with higher content of phenolic compounds by classical breeding or by cysgenic transformation in the near future. The initial steps in the breeding process for higher phenols content are to study the genetic variability among the existing cultivars and to determine the best ripening index for better comparison between genotypes; however, published information related to these topics is still scarce and very controversial.

Therefore, more studies on genetic variability of phenolic compounds among olive germplasm banks in all producing countries are needed, which should be supported by international and local scientific and managerial cooperation.

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