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Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey

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ABSTRACT

Despite the evident influence of the cultivar on olive oil composition, few studies have been devoted to exploring the variability of phenols in a representative number of monovarietal olive oils. In this study, oil samples from 80 cultivars selected for their impact on worldwide oil production were analyzed to compare their phenolic composition by using a method based on LC–MS/MS. Secoiridoid derivatives were the most concentrated phenols in virgin olive oil, showing high variability that was significantly due to the cultivar. Multivariate analysis allowed discrimination between four groups of cultivars through their phenolic profiles: (i) richer in aglycon isomers of oleuropein and ligstroside; (ii) richer in oleocanthal and oleacein; (iii) richer in flavonoids; and (iv) oils with balanced but reduced phenolic concentrations. Additionally, correlation analysis showed no linkage among aglycon isomers and oleocanthal/oleacein, which can be explained by the enzymatic pathways involved in the metabolism of both oleuropein and ligstroside.

1. Introduction

The olive tree, *Olea europaea* subsp. *europaea* var. *sativa*, was likely domesticated approximately 6000 years ago in the Middle East from its wild ancestor *Olea europaea* subsp. *europaea* var. *sylvestris*, (Besnard et al., 2013). Currently, more than 11 million hectares of olives are grown in 47 countries worldwide (International Olive Council, 2015). However, olive growing is still based on a vast number of traditional olive cultivars, with complex genetic relationships among them (Diez et al., 2015).

Virgin olive oil (VOO) is demonstrated to be endowed with healthy properties, thanks to its monounsaturated fatty acids profile and a plethora of multiple minor components with biological properties (Piroddi et al., 2017). VOO composition is characterized by saponifiable and unsaponifiable fractions. The saponifiable fraction represents approximately 98% of the olive components and includes triglycerides, fatty acids, phospholipids, waxes and sterol esters. The unsaponifiable fraction, approximately 2% of the total composition, encompasses a complex set of minor compounds (approximately 230 compounds) pertaining to various chemical families including aliphatic and triterpenic alcohols, sterols, hydrocarbons, phenols, tocopherols, esters, pigments, and volatile components such as aldehydes, ketones and alcohols (Servili et al., 2013). The preservation of this composition is guaranteed by the extraction process performed using physical methods at relatively low temperatures (approximately 28 °C) and without the addition of chemical solvents.

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Among the minor components of VOO, phenols are worthy of attention due to their (i) health properties (Piroddi et al., 2017); (ii) association with organoleptic attributes such as oil pungency and bitterness (Bendini et al., 2007); (iii) contribution to VOO shelf-life (Silva, Pinto, Carrola, & Paiva-Martins, 2010); (iv) uniqueness, supported by the fact that some families are exclusive of the Oleaceae family and few other dicotyledonous families (Carranco, Farrés-Cebrián, Saurina, & Núñez, 2018; Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002); Servili et al., 2016); and (v) high concentration in VOO (Servili et al., 2016). Given the great variability of phenolic families, the role of secoiridoids, conjugated forms of hydroxytyrosol and tyrosol, is notable. This group of compounds is the most concentrated in olive oil and is widely studied due to evidence of its healthy properties (Piroddi et al., 2017). The group includes oleuropein and ligstroside aglycon isomers and the decarboxymethylated dialdehyde forms of oleuropein and ligstroside aglycons, better known as oleacein (3,4-DHPEA-EDA) and oleocanthal (*p*-HPEA-EDA), respectively. Beauchamp and colleagues reported the natural non-steroidal anti-inflammatory activity of oleocanthal due to its ibuprofen-like cyclooxygenase (COX-1 and COX-2) inhibiting capacity (Beauchamp et al., 2005). Oleacein has also shown antioxidant activity similar to oleocanthal (Czerwińska, Kiss, & Naruszewicz, 2014). It appears that the healthy properties of VOO phenols are attributed individually and not to the total phenolic content (Agrawal et al., 2017; Yakhlef et al., 2018).

There is increasing social interest in VOO as a functional food; for instance, consumers are willing to pay up to 6.02 €/L more for VOO labelled with functional health claims compared to the unlabeled product (Casini, Contini, Marinelli, Romano, & Scozzafava, 2014). Numerous studies supporting the health properties of olive oil have recently pushed the European Food Safety Authority (EFSA) to approve several health claims on the commercial label of VOOs that meet specific quality requirements. The claim "olive oil phenols contribute to the protection of blood lipids from oxidative stress" can be included on the label when the VOO contains at least 5 mg as the sum of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil (EFSA, 2011).

As mentioned elsewhere, phenols also contribute to the organoleptic properties of VOO (Angerosa et al., 2004). The bitter taste is particularly related to the aglycon forms (Bendini et al., 2007), whereas the presence of oleocanthal and oleacein has been linked to the pungency of VOO, which might be described as biting tactile sensations, which are characteristic of some VOOs (Barbieri, Bendini, Valli, & Gallina Toschi, 2015).

Several studies have noted that the main factors that influence the qualitative and quantitative variability of phenolic compounds in olive oil are genotype (cultivar), climatic and agronomic conditions, edaphic factors, and the technological method applied for oil extraction. Among these factors, genotype has a preponderant influence on phenolic composition (Baiano, Terracone, Viggiani, & Del Nobile, 2013; De la Rosa, Arias-Calderón, Velasco, & León, 2016). Previous researches have been focused on a limited number of genotypes, either traditional cultivars with regional importance in terms of VOO production or new cultivars from breeding programs (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Vinha et al., 2005). Therefore, an exhaustive evaluation and classification of an extensive panel of olive cultivars according to the phenolic profiles of monovarietal oils has never been published.

In this context, this study was aimed to a) characterize the phenolic profile of a representative panel of monovarietal VOOs; b) evaluate the influence of cultivar on phenolic variability, and c) determine association patterns among cultivars according to the phenolic composition of their VOO. To accomplish these goals, we selected a set of 80 olive cultivars from 15 countries representing the main VOO producing areas worldwide. The olive cultivars were grown under the same agroclimatic conditions, and their oils were extracted by application of the same protocol to allow an unbiased characterization of the influence of genotype on the VOO phenolic profiles.

2. Material and methods

2.1. Experimental location and vegetal material

Vegetal material was collected from the World Olive Germplasm Bank of Cordoba (WOGB) (CAP-UCO-IFAPA), specifically in the collection located at the University of Cordoba (Cordoba, Spain, 37°55′56.5″ N, 4°43′13.3″ W and 173 m a.s.L.). The olive trees were planted in 2011 in a North-South orientation with 7 m between rows and 6 m between trees (238 trees ha^{-1}). This collection includes 368 olive cultivars from 22 countries, which were identified and authenticated by morphological and molecular methods, so all them are true to type (Trujillo, Ojeda, Urdiroz, & Potter, 2014).

The climate of the area where the WOGB is located is typically Mediterranean; the average annual precipitation from 2001 to 2016 was 635.6 mm, with a summer drought with < 30 mm of precipitation from June to September. The precipitation in 2014 and 2015 was 635.3 and 770 mm, respectively. The average potential evapotranspiration (ETP) from 2001 to 2016 was 1261.9 mm, while the average annual, maximum and minimum temperatures for the same period were 18.2, 47.2, and 0.0 °C, respectively (Villalobos & Testi, 2017). The collection area is characterized by vertisol soil with a texture of 41% sand, 6% silt, and 53% clay. The soil was approximately 40 cm deep, with 0.6% organic matter content. The collection was irrigated from May to September, applying 100 m^3 per ha per week (2000 m^3 of water per year) using drip irrigation. Foliar fertilization (2% potassium nitrate) was applied four times per year during November (after harvesting), March, May and September.

A set of 80 olive cultivars were selected during the 2015–2016 crop season according to their importance for the worldwide olive oil production, their geographical origin, and fruit availability (Table 1). We also studied 25 same cultivars during two consecutive seasons, 2014–2015 and 2015–2016, to estimate the reproducibility of the results. Fruit were independently collected from two olive trees per cultivar, and the VOO was extracted in each case. Therefore, each cultivar provided two independent biological samples yielding a total number of 160 samples (80 cultivars × 2 trees = 160 samples).

2.2. Sampling and VOO extraction

We manually harvested 2 kg of olive fruits from each tree by sampling all orientations within the canopy. The trees were sampled from October to December when the fruits were at a ripening index (RI) of 2.0 (yellowish-red color) according to the method proposed by the International Olive Oil Council (International Olive Council, 2011).

Monovarietal VOOs were obtained using an Abencor extraction system (MC2 Ingeniería y Sistemas, Sevilla, Spain) under optimized conditions (Peres, Martins, & Ferreira-Dias, 2014). The olives were crushed with a hammer mill equipped with a 4-mm sieve at 3000 rpm. Malaxation of olive pomace was performed at 28 °C for 30 min, and then, the biphasic system was centrifuged at 3500 rpm for 2 min. No water was added to the olive pomace at any step of the process. The VOO was decanted in graduated cylinders for approximately 8 h. Water traces were removed by filtering the samples through a cellulose filter. The samples were stored in amber glass bottles at −20 °C until analysis.

2.3. Reagents and standards

The solvents used for the analysis of phenols in VOOs were mass spectrometry (MS) grade methanol (MeOH) and *n*-hexane, both from Scharlab (Barcelona, Spain). MS-grade formic acid, also from Scharlab, was used as an ionization agent in the chromatographic mobile phases. Deionized water (18 MΩ·cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare both the aqueous

Table 1
Cultivars selected for the analysis of phenolic compounds in monovarietal VOOs.

2014/2015 crop season	2015/2016 crop season			
Alameño de Montilla	Abou Choki	Empeltre	Mission Moojeski	Verdale
Alfafara	Alameño de Montilla	Enagua de Arenas	Mixani	Verde Verdelho
Arbequina	Alfafara	Farga	Mollar de Cieza	Verdial de Huévar
Arbosana	Amygdalolia Nana	Frantoio	Moraiolo	Villalonga
Blanqueta	Arbequina	Galega Vulgar	Morisca	Zaity
Bosana	Arbosana	Gemlik	Morona	
Caballo	Ascolana Tenera	Gordal de Granada	Morrut	
Cerezuela	Azapa	Hojiblanca	Nasuhi	
Coratina	Barnea	Jabaluna	Negrillo de la Carlota	
Farga	Blanqueta	Joanenca	Ojo de Liebre	
Gordal de Granada	Bodoquera	Kalamon	Palomar	
Joanenca	Bosana	Koroneiki	Pendolino	
Kalamon	Bouteillan	Kotruvsi	Picholine Marocaine	
Koroneiki	Caballo	Kusha	Picual	
Loaime	Carolea	Lastovka	Picual de Almería	
Mastoidis	Carrasqueño de Elvas	Leccino	Picudo	
Mollar de Cieza	Cerezuela	Lechín de Sevilla	Plementa Bjelica	
Morona	Changlot Real	Levantinka	Rapasayo	
Negrillo de la Carlota	Chemlal de Kabilye	Loaime	Royal de Calatayud	
Ojo de Liebre	Chetoui	Lucio	Royal de Cazorla	
Picual de Almería	Çobrancosa	Manzanilla Cacereña	Sabatera	
Plementa Bjelica	Coratina	Manzanilla de Sevilla	Sandalio	
Royal de Cazorla	Cordovil de Serpa	Manzanilla Prieta	Sikitita	
Sabatera	Cornicabra	Mastoidis	Tanche	
Sikitita	Cornicabra de Mérida	Megaritiki	Ulliri i Bardhe i Tiranes	

mobile phase and the hydroalcoholic mixture used as extractant.

The evaluated phenols were hydroxytyrosol, oleacein (3,4-DHPEA-EDA), oleocanthal (p-HPEA-EDA), oleuropein aglycon (3,4-DHPEA-EA), ligstroside aglycon (p-HPEA-EA), luteolin and apigenin. The aglycon forms of oleuropein and ligstroside were discriminated according to their structures. Thus, it was possible to discriminate between the aldehyde open forms of oleuropein aglycon (AOleAgly, the sum of stereoisomers) and the monoaldehyde closed form of the oleuropein aglycon (MAOleAgly). By analogy, it was possible to discriminate between the aldehyde open forms of ligstroside aglycon (ALigAgly, the sum of stereoisomers) and the monoaldehyde closed form of ligstroside aglycon (MALigAgly). Standards for hydroxytyrosol, apigenin and luteolin were purchased from Extrasynthese (Genay, France). Oleacein, oleocanthal, and the aldehydic open forms of oleuropein aglycon and ligstroside aglycon were provided by Prof. Magiatis of the University of Athens (Greece) (Diamantakos et al., 2015). The monoaldehyde forms were quantified using the corresponding standards for the aldehyde open forms. Standard solutions of non-secoiridoid phenols were prepared in methanol (1 mg/mL), while secoiridoids were prepared at the same concentration in pure acetonitrile to preserve their stability and avoid undesired conversion to acetal and hemiacetal derivatives.

2.4. Sample preparation for analysis of phenolic compounds

Phenolic compounds were isolated by liquid-liquid extraction following previously published protocols (Sánchez de Medina, Priego-Capote, & Luque de Castro, 2015). For this purpose, 1 g of VOO was mixed with 2 mL *n*-hexane; then, 1 mL of 60:40 (v/v) methanol-water was added and shaken for 2 min, and the hydroalcoholic phase was separated by centrifugation. The extraction was repeated to enhance the extraction efficiency (Sánchez de Medina et al., 2017). The resulting phenolic extracts were analyzed by LC–QqQ MS/MS with three different dilution factors (1:2, 1:50 and 1:200 v/v) to encompass the concentration variability.

2.5. LC-MS/MS analysis of phenolic compounds

Analyses were performed by reversed-phase liquid chromatography followed by electrospray ionization (ESI) in negative mode and tandem mass spectrometry (MS/MS) detection. Ten μL of extract was injected in triplicate into the LC system for chromatographic separation of the target compounds using a C18 Pursuit XRs Ultra (50 × 2.0 mm i.d., 2.8 μm particle size) from Varian (Walnut Creek, CA, USA). The column compartment was kept at 30 °C. Mobile phase A was 0.1% formic acid in water, while phase B was 0.1% formic acid in MeOH. The gradient program, at a 0.4 mL/min constant flow rate, was as follows: initially, 50% phase A and 50% phase B were maintained for 0.5 min; from 0.5 to 2 min, mobile phase A was from 50 to 20%; and from min 2 to 4, mobile phase A was from 20 to 0%. This last composition was maintained for 1 min. After each analysis, the column was equilibrated for 5 min to the initial conditions.

The entire eluate was electrosprayed and monitored by MS/MS in Multiple Reaction Monitoring (MRM) mode for selective transitions from precursor to product ions for each analyte. The MRM parameters for the analysis of target phenols are listed in Supplementary Table 1. The flow rate and temperature of the drying gas (N_2) were 10 L/min and 300 °C, respectively. The nebulizer pressure was 50 psi, and the capillary voltage was 3000 V. The dwell time was set at 200 μs.

2.6. Quantitation of the target compounds and statistical analysis

Absolute quantitative analysis was performed by calibration curves obtained using refined sunflower oil spiked with the target phenols. The absence of quantifiable levels of phenols in the refined oil was checked by direct analysis with the developed method. Nine phenolic concentrations from 0.1 ng/mL to 5 μg/mL were injected in triplicate to obtain the calibration curves. The concentration of phenols in the monovarietal VOOs was determined with these models, using three replicates per sample.

ANOVA factorial analysis ($P < 0.05$) was performed to determine the influence of the independent variable (genotype) on the phenolic composition of VOO in the two crop seasons. Box-Cox transformation of the data to fit normality was applied when appropriate. Furthermore, Principal Component Analysis (PCA) was applied to identify groups of cultivars with similar phenolic profiles. The existence of significant pairwise differences among the groups formed in the PCA was evaluated with a Bonferroni post-hoc test, while Pearson correlation was used to find associations between the quantified phenols. These

Table 2
Mean, minimum and maximum concentration (expressed as mg/kg) of phenolic compounds found in VOO from the cultivars selected in the two crop seasons.

Phenol	Minimum	Maximum	Mean	SD*
2014/2015 crop season (25 cultivars)				
Hydroxytyrosol	0.46	4.89	1.68	1.08
Oleacein	59.7	866.7	364.3	201.7
AOleAgly	10.2	1545.8	283.7	425.1
MAOleAgly	39.0	1136.3	273.2	246.2
Oleocanthal	53.0	2931.1	730.9	739.1
ALigAgly	1.75	1049.7	225.1	299.0
MALigAgly	3.98	326.5	66.2	77.0
Luteolin	0.45	6.25	3.31	1.82
Apigenin	0.04	11.53	1.87	2.34
2015/2016 crop season (80 cultivars)				
Hydroxytyrosol	0.28	7.57	2.04	1.51
Oleacein	7.1	903.0	159.6	151.5
AOleAgly	4.10	3501.3	577.4	753.9
MAOleAgly	8.6	918.3	222.2	188.0
Oleocanthal	17.3	1602.3	274.1	332.0
ALigAgly	1.19	1718.2	226.1	304.5
MALigAgly	2.26	133.6	31.7	26.6
Luteolin	0.52	11.35	3.72	2.49
Apigenin	0.18	19.79	2.98	2.88

* SD: Standard deviation.

Table 3
ANOVA analysis results show the influence of genotype on the concentration of the nine phenolic compounds.

Phenol	R^2	F	*p*-value
2014/2015 crop season (25 cultivars)			
Hydroxytyrosol	0,916	11,360	< 0,0001
Apigenin	0,965	28,487	< 0,0001
Luteolin	0,962	26,065	< 0,0001
Oleocanthal	0,889	8,370	< 0,0001
Oleacein	0,754	3,185	0,003
MALigAgly	0,960	25,215	< 0,0001
ALigAgly	0,925	12,844	< 0,0001
MAOleAgly	0,894	8,764	< 0,0001
AOleAgly	0,887	8,211	< 0,0001
2015/2016 crop season (80 cultivars)			
Hydroxytyrosol	0,831	4,984	< 0,0001
Apigenin	0,948	18,585	< 0,0001
Luteolin	0,887	7,961	< 0,0001
Oleocanthal	0,924	12,270	< 0,0001
Oleacein	0,908	9,943	< 0,0001
MALigAgly	0,930	13,529	< 0,0001
ALigAgly	0,973	36,917	< 0,0001
MAOleAgly	0,957	22,405	< 0,0001
AOleAgly	0,956	21,759	< 0,0001

R^2 (determination coefficient): percentage of variability explained by the genotype in the total variance.
F ratio: variation between samples/variation within the samples.
p-value: significance level.

analyses were performed using XLSTAT software (v.2014.5.03, Addinsoft, Paris, France).

3. Results and discussion

3.1. Evaluation of the phenolic variability in monovarietal VOOs

The phenolic composition of VOO strongly depends on numerous factors, among which the cultivar (genotype) plays a key role (Baiano et al., 2013; El Riachy et al., 2011). This evidence and the absence of studies analyzing a significant number of cultivars prompted us to analyze the phenolic composition of the VOOs in a complete cultivar panel. We selected 80 olive cultivars (Table 1) according to the following criteria: a) importance in terms of VOO production, b) geographical origin, and c) fruit availability in the WOGB. Furthermore, basing on the same criteria, a subset of 25 cultivars was considered for two harvest seasons (2014/2015 and 2015/2016) to test the reproducibility of the results.

The sum of the individual concentration of phenols in the VOOs ranged from hundreds to thousands of mg/kg, as shown in Table 2, which lists the concentrations found in the two crop seasons. Supplementary Tables 2 and 3 show the concentration of each phenolic compound of monovarietal oils analyzed during two crop seasons (two individual trees for each cultivar were analyzed). The high variability in the phenolic levels of VOO can also be visualized in Supplementary Figs. 1 and 2, which illustrate the distribution of monovarietal VOOs according to the concentration of each phenol. The genetic variability of the cohort was the main factor responsible for the high variation in the concentration of phenolic compounds, given that the olive trees were grown under the same agronomical conditions and that samples were extracted by the same protocol.

The high concentration of phenolic compounds found in our monovarietal VOOs compared to other studies (Fuentes et al., 2017; Karkoula, Skantzari, Melliou, & Magiatis, 2012) might be explained by the extraction protocol. In large-scale olive oil production, water is normally added to enhance the separation of oil from olive paste. Due to the hydrophilic character of phenolic compounds, this addition can result in phenolic loses. In contrast, during the extraction of the oil samples in the Abencor system, which was used for us in the present study, no water was added so it could have preserved the phenolic compounds in the oil phase. However, our phenolic concentration levels looks to be similar with a recent research (García-Rodríguez, Belaj, Romero-Segura, Sanz, & Pérez, 2017). In agreement with previous studies (García-Rodríguez et al., 2017; Karkoula, Skantzari, Melliou, & Magiatis, 2014), secoiridoid derivatives were the most abundant phenols in all evaluated monovarietal VOOs. Secoiridoid derivatives are aglycon forms of the secoiridoid glucosides formed during oil extraction by β-glucosidase enzymatic hydrolysis of oleuropein, demethyloleuropein, and ligstroside (Servili et al., 2004). The concentration of oleocanthal, one of the most recognized phenols in VOO due to its anti-inflammatory and antioxidant properties, showed an almost 100-fold variation in the cultivar set, ranging from 17 to 1600 mg/kg (Table 2). While most cultivars showed an oleocanthal concentration close to their average value (274 mg/kg) several cultivars, such as 'Kalamon', 'Plementa Bjelica', 'Alfafara', 'Pendolino', 'Kotruvsi', 'Enagua de Arenas', 'Caballo', and 'Koroneiki', showed more than 750 mg/kg, and other 14 cultivars showed levels < 50 mg/kg (Supplementary Tables 2 and 3; Supplementary Figs. 1 and 2).

Oleacein is structurally similar to oleocanthal and is also considered to have similar pharmacological properties (Paiva-Martins et al., 2009). In this study, oleacein also showed high concentrations, with a maximum of 903 mg/kg, but they were lower than those of oleocanthal. Oleacein was found at high levels (366–900 mg/kg) in 'Pendolino', 'Blanqueta', 'Arbequina', 'Cerezuela', 'Kalamon', 'Alfafara', 'Caballo', and 'Koroneiki' and at low levels (7–50 mg/kg) in cultivars such as 'Jabaluna', 'Picual', and 'Morisca', with an average value of 364 mg/kg in the whole set (Table 2; Supplementary Tables 2 and 3; Supplementary Figs. 1 and 2). Previous studies have reported oleacein levels ranging from 100 to 400 mg/kg in monovarietal oils, while for oleocanthal, the measured levels were below 350 mg/kg (García-González, Tena, & Aparicio, 2010; Karkoula et al., 2012). Conversely, other authors provided particularly low levels of both phenols that did not exceed 30 mg/kg (Baiano et al., 2013; Ramos-Escudero, Morales, & Asuero, 2015). The variability in the concentration ranges for both phenolic compounds among studies might exist due to differences in analytical methodology, such as detection technique, quantitation strategy, or absolute or relative quantitation using non-specific standards for calibrations of the analytical equipment.

The aglycon isomers of oleuropein and ligstroside were also among

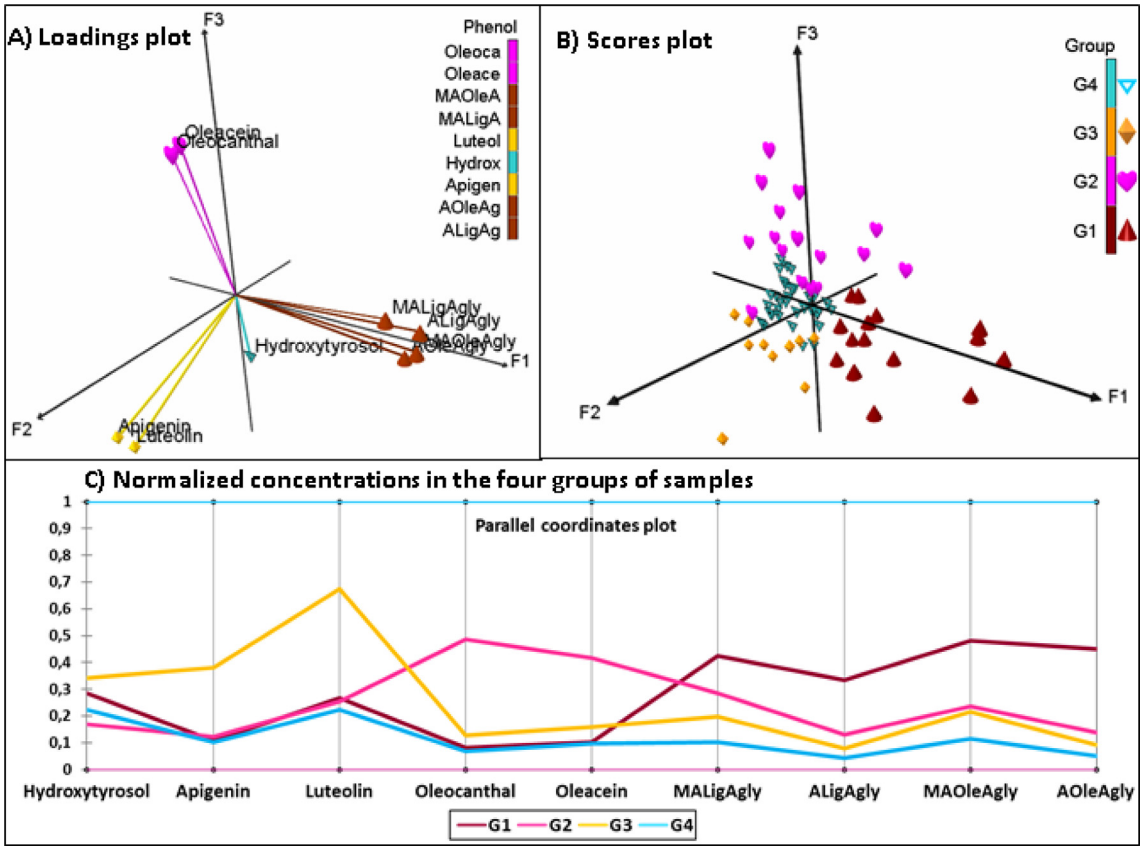

Fig. 1. Principal component analysis for the phenolic profiles of the 80 monovarietal VOOs. (A) Loadings plot. (B) Scores plot. (C) Normalized concentration profiles of the four groups of cultivars classified attending to their phenolic profile.

the most concentrated phenols in VOO. These compounds are associated with the bitter and pungent taste of olive oil (Barbieri et al., 2015). Aglycon isomers are characterized by high antioxidant activity (Taticchi, Esposto, & Servili, 2013). The concentration of aldehyde open forms of oleuropein aglycon ranged from 8 to 918 mg/kg, with an average value of 222 mg/kg. The aldehyde open forms of ligstroside aglycon were less concentrated than the analogous oleuropein isomers, ranging from 2 to 133 mg/kg, with an average value of 31 mg/kg. The monoaldehyde closed forms of oleuropein and ligstroside were quantified in a relative manner because the aldehyde forms were used as quantitation standards. Remarkably, 'Chetoui', 'Villalonga', 'Coratina', 'Zaity', and 'Cornicabra' were among the top five cultivars, with the highest content in the sum of the aglycon isomers.

Three minor phenols (hydroxytyrosol, apigenin and luteolin) were also included in the list of quantified phenols since they have been frequently analyzed in VOO for their beneficial health properties (Tuck & Hayball, 2002). Hydroxytyrosol is a simple alcohol conjugated to form oleuropein derivatives, while luteolin and apigenin are the two most representative flavonoids found in VOO. The concentration of hydroxytyrosol in the analyzed cohort ranged from 0 to 9 mg/kg, while apigenin and luteolin ranged from 0 to 20 mg/kg and from 0 to 11 mg/kg, respectively (Table 2; Supplementary Tables 2 and 3; Supplementary Figs. 1 and 2).

3.2. Influence of cultivar on phenolic profile variability of olive oil

As mentioned above, the cultivar (genotype) plays a key role in the diversity and concentration of phenolic compounds present in VOO (Baiano et al., 2013). However, neither the phenolic diversity present in the VOO nor the magnitude of the genotypic effect driving this variability has been extensively explored by analyzing a large, geographically representative set of olive cultivars. Our study provides an outstanding opportunity to shed light on these topics. To do so, we analyzed the phenolic profiles of monovarietal VOOs extracted from 80 selected cultivars growing in the same climatic conditions and subjected to the same agronomical practices. An ANOVA test was applied to test the influence of the cultivar on the phenolic compound variability. The goodness of fit statistics revealed that the percentage of the variability (R^2) explained by the genotype was highly significant (p-value < 0.001) for the nine phenolic compounds and the two consecutive crop seasons. For the first crop season (2014/2015), the percentage of variability explained by the genotype was between 75 and 96%, while for the second crop season (2015/2016), it was between 83 and 97%, respectively, for the hydroxytyrosol and aldehydic open forms of ligstroside aglycon (ALigAgly) (Table 3). Therefore, in agreement with previous studies (De la Rosa et al., 2016; Perez et al., 2014), the genotype was the main factor responsible for the variability found in the phenols analyzed in this set of cultivars.

The reproducibility of these results was further corroborated by the correlation test of phenolic concentrations between two consecutive growing seasons (Supplementary Table 4). The highest correlation was observed for apigenin and oleocanthal (p-value < 0.0001; $R = 0.90$ and $R = 0.83$, respectively), followed by MALigAgly and the sum of monitored phenols (p-value $= 0.0001$; $R = 0.78$ and $R = 0.77$, respectively). Finally, luteolin (p-value $= 0.002$; $R = 0.75$), oleacein (p-value $= 0.001$; $R = 0.72$), MAOleAgly (p-value < 0.005; $R = 0.40$), and ALigAgly (p-value < 0.005; $R = 0.39$) also provided a significant correlation coefficient between the two seasons. Although only 25 cultivars were included in this consistency test, the high correlation observed for most phenols highlighted the preponderance and stable weight of the genotype in the phenolic variability of VOOs.

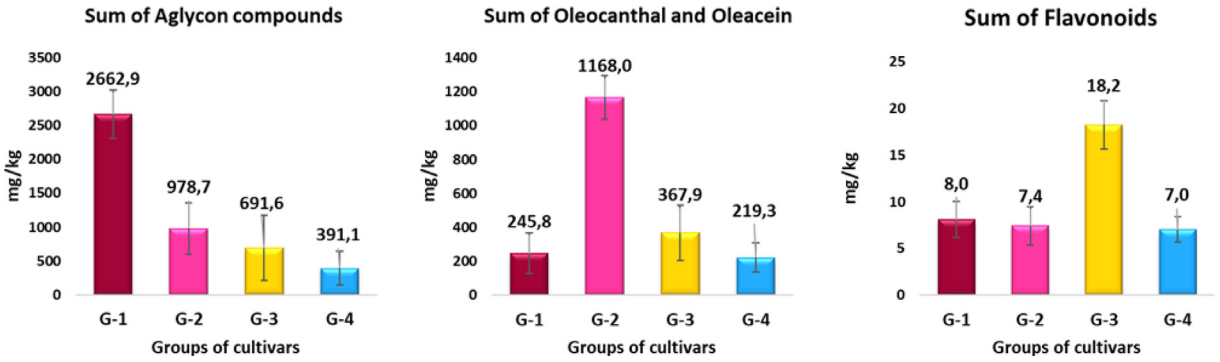

Fig. 2. Differences in the concentration of aglycon compounds, olecanthal and oleacein and flavonoids found in the four groups of monovarietal VOO classified according to the PCA.

3.3. Classification of olive cultivars attending to their VOO phenolic profiles

Once the contribution of the cultivar to the phenolic composition of monovarietal VOO was elucidated, the next step was to determine distinctive patterns in the set of cultivars according to their phenolic profiles. First, PCA was applied using the concentrations of individual phenols determined in the 80 monovarietal oils. The first three principal components (PC1, PC2 and PC3) explained 74.1% of the cumulative variability and allowed clustering of the cultivars into four main groups (G1, G2, G3 and G4), characterized by their distinctive phenolic compositions (Fig. 1). The G1 group included 18 cultivars characterized by the high concentration of oleuropein and ligstroside aglycon isomers; G2 grouped 16 cultivars with high levels of oleocanthal and oleacein; G3 clustered 10 cultivars with a high concentration of apigenin and luteolin; and finally, G4 included 36 cultivars that showed a balanced composition, with no remarkable concentration in any of the studied phenolic compounds (Supplementary Table 5). Fig. 2 illustrates differences in the concentration of these phenolic compounds in the four groups of cultivars differentiated according to the PCA. A clear difference in the concentration of the aglycon isomers of oleuropein and ligstroside and that of oleocanthal and oleacein was observed between groups G1 and G2. This difference was visualized in the MRM chromatograms obtained by analyzing monovarietal VOO from two cultivars assigned to G1 and G2 (Fig. 3). The presence of the oleuropein aglycon and ligstroside aglycon isomers within the same phenolic profile was justified because they are synthesized through the same pathway. In fact, a strong significant correlation in concentration (p-value < 0.0001 and $R = 0.87$) was found between both pairs of isomeric forms (Supplementary Fig. 3).

An ANOVA test was used to further evaluate differences between groups of cultivars according to their phenolic profiles. The test revealed highly significant differences ($p < 0.0001$) among the four groups of cultivars (Supplementary Table 6.A). Bonferroni post-hoc test detected significant differences ($p < 0.0001$) among the groups. The results of ANOVA and post-hoc tests reinforced the results of the PCA analysis (Supplementary Table 6.B).

The consistency of this classification between agronomic years was tested by applying the analyses described above to the subset of 25 cultivars in two consecutive seasons. As a result, despite climatic differences between years that might significantly affect the phenolic concentration of VOOs, more than 80% of cultivars were consistently assigned to the same PCA group in both years (Supplementary Table 7). Therefore, genotype crucially influences VOO phenolic profiles and especially the concentration of secoiridoid derivatives, as they are the most concentrated phenols in olive oil.

3.4. Influence of pathways on synthesis of secoiridoids that differentiate olive oils

Understanding the pathways leading to the biosynthesis of phenolic

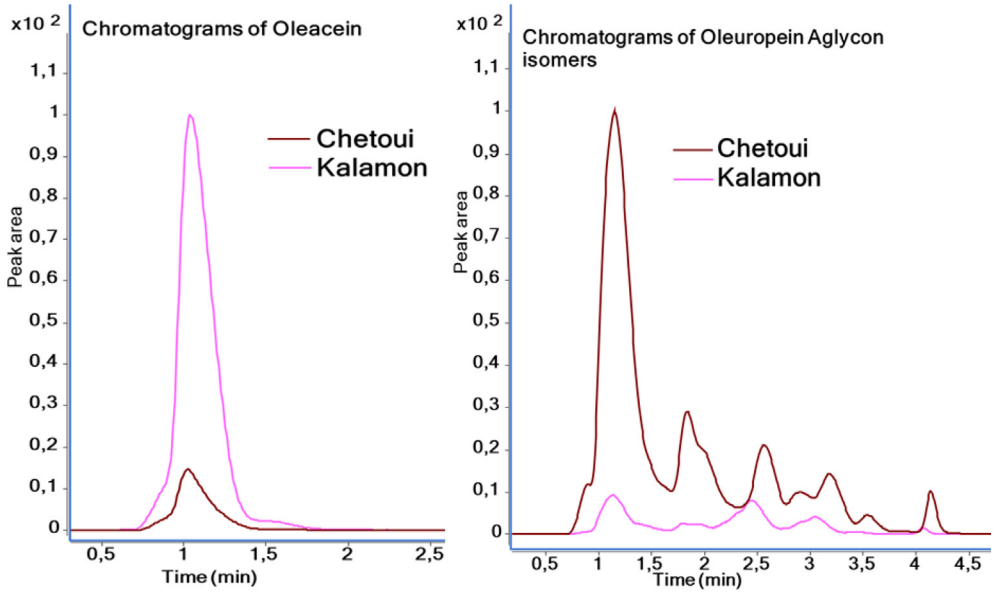

Fig. 3. Chromatograms obtained by the analysis of the VOO from two cultivars 'Chetoui' and 'Kalamon') showing differences in the concentration of oleacein and algycon isomers of oleuropein.

compounds is necessary to decipher the genetic basis of their variation. It is especially important to understand the biochemical pathways responsible for the synthesis of secoiridoid derivatives due to their high contribution to phenolic differences in monovarietal VOOs. The two main pathways proposed for the synthesis of secoiridoids in olive fruits end in the synthesis of oleuropein, although this glycoside phenol is rarely detected in VOO. The proposed pathways are mechanistically diverse and differ in the precursor of oleuropein. The first pathway, proposed by Damtoft, Franzyk, & Jensen (1993) is initiated with mevalonic acid with the formation of iridoids, and then to ligstroside, which is the precursor of oleuropein. An alternative pathway was proposed in 2002 by Ryan et al., with tyrosol as a precursor produced through the phenylpropanoid biosynthesis (Ryan, Antolovich, Herlt, Prenzler, Lavee, & Robards, 2002). In this second mechanism, tyrosol is the substrate of two synthetic pathways that led to a common final product: oleuropein. The first pathway addresses ligstroside as an intermediate, while the second transforms to the oleacein and oleuropein aglycone before final conversion into oleuropein. Both synthetic pathways occur in the first ripening stages as a common strategy to accumulate oleuropein in the fruit.

At advanced maturation and especially during olive oil extraction, enzymatic and non-enzymatic biotransformations are produced to form the secoiridoid derivatives found in VOO. The most complete biotransformation pathway was proposed by (Obied, Bedgood, Prenzler, & Robards, 2007), who explained the appearance of secoiridoid derivatives from oleuropein as a precursor. In this pathway, oleuropein is converted into several derivatives according to the involvement of esterases and β-glucosidases enzymes. Depending on the enzymatic activity, oleuropein can mainly be converted to oleuropein aglycone isomers or to oleacein via two main pathways (Supplementary Fig. 4). Thus, the formation of oleuropein aglycon isomers is catalyzed by β-glucosidase, activated during crushing and malaxation, which leads to stable isomers discriminated by functional groups: the aldehyde and monoaldehyde forms of oleuropein aglycon, the latter with the closed heterocyclic ring. The second pathway is characterized by the involvement of methylesterases to cleave the methyl group in the elenolic acid. This second pathway would be responsible for the formation of oleacein. A similar situation would occur for ligstroside to preferentially produce ligstroside aglycon isomers and oleocanthal. The technical factors of VOO extraction are critical to obtain a phenolic profile, since variation in technological factors could favor the kinetics of certain enzymatic processes.

A Pearson correlation analysis was used to find associations between the monitored phenols in the complete set of monovarietal VOOs. Several strong correlations (p-value < 0.0001 and $R < 0.69$) were found between pairs of phenolic compounds. The most interesting result was the detection of significant correlations between the isomers of oleuropein aglycon and ligstroside aglycon, and between the decarboxymethylated dialdehydic compounds (oleocanthal and oleacein) (Supplementary Table 8). No statistical association was observed between the two groups of secoiridoid derivatives, which supports the fact that aglycons and decarboxymethylated dialdehyde aglycons are produced following two independent pathways from the same initial substrates, oleuropein and ligstroside. The group of cultivars with a high content of oleocanthal and oleacein would be characterized by increased activity of demethylesterases as the key step towards the production of these two dialdehydes. Therefore, this study suggests that it is possible to breed new olive cultivars to obtain monovarietal oils enriched with certain phenols, despite the influence of agronomic and technological factors—essentially, ripening index, grinding and malaxation time and temperature. It would be promising to study the interaction between these other factors and genotype to evaluate how they can modulate variability in the phenolic profiles of VOO.

4. Conclusions

In this study, remarkable variability was found for nine phenolic compounds in the largest set of monovarietal VOOs analyzed to date. Genotype was the main factor contributing to this variability for all phenolic compounds with a percentage of total variance between 83% and 97%. The secoiridoid derivatives were the most abundant phenols of all monovarietal VOOs evaluated in this study. Various previously undistinguished olive cultivars were revealed to be very rich, interesting cultivars for certain phenolic compounds.

Multivariate analysis allowed detection of four groups of cultivars (G1, G2, G3 and G4) via their phenolic profile. G1 was characterized by a high concentration of oleuropein and ligstroside aglycon isomers and G2 by a high concentration of oleocanthal and oleacein; G3 was rich in two flavonoids (apigenin and luteolin). The last group, G4, included cultivars for VOOs that did not stand out in terms of the monitored phenols. The differences in the phenolic profiles of VOOs pertaining to G1 and G2 groups allowed detection of two independent pathways in the metabolism of oleuropein and ligstroside, through the involvement of demethylesterases and β-glucosidases.

The extensive and accurate characterization of phenolic compounds in VOO is necessary for the production of high-quality VOOs. This study opens new avenues in this research area, for example, studying the phenolic contents and their routes of production or the influence of the phenolic profiles on human health, the organoleptic features and olive oil shelf-life.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.06.002.

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