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Determination of the Phenol and Tocopherol Content in Italian High-Quality Extra-Virgin Olive Oils by Using LC-MS and Multivariate Data Analysis

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Abstract

Italian extra-virgin olive oil market presents several kinds of products, some of which are of higher quality compared to others. Italian high-quality extra-virgin olive oils can have Protected Designation of Origin, Protected Geographical Identification and organic farming denominations. Each high-quality olive oil produced shows peculiar chemical composition and organoleptic characteristics. The present work was aimed to endorse the use of Italian high-quality extra-virgin olive oils and to promote the "Italian quality olive oil culture" to the consumers. To obtain this, task two analytical methods were developed in order to investigate the phenol and tocopherol contents in one hundred eighty-six high-quality extra-virgin olive oils by using HPLC coupled to mass spectrometry and fluorimetric detection, respectively. Olives milled to obtain these samples belong to forty-seven different cultivars. Furthermore, four different assays were used in order to evaluate antioxidant activity of all samples: TEAC, ORAC, FRAP, and DPPH, while the total phenols content was assessed by the Folin-Ciocalteu method. Finally, all these information were used to make a principal component analysis (PCA) in order to verify a possible correlation between oils belonging to the same region, or to the same cultivar, or to denomination. To the best of our knowledge, this is the first research article that reports the bioactive molecule content and a PCA statistical correlation between total phenol content, antioxidant activity (TEAC, FRAP, DPPH and ORAC), tocopherols and phenols in a large array of Italian high-quality extra-virgin olive oils (174) produced in different regions; in addition a number of 12 samples came from foreign countries (Portugal, Spain and Croatia). The gathered information attained could be useful for the generation of a database of a qualitative and quantitative profile of antioxidant molecules in extra-virgin olive.

Keywords Extra-virgin olive oils · Phenols · Tocopherols · Protected designation of origin · HPLC-MS

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Introduction

Olives and olive oil are essential components of an equilibrated dietary regimen because of their nutritional and biological value [Bendini et al. 2007]. The beneficial effects of olive oil is related to different classes of compounds, and particularly to the well-balanced acidic composition, and especially the presence of phenolic compounds (phenols and tocopherols) that exert important radical-scavenging activity [Cioffi et al. 2010].

Phenols are an important group of natural compounds, which are produced in the secondary metabolism of various plants in nature [Suárez et al. 2008]. Thousands of scientific articles have been produced on the characterization of bioactive molecules in extra-virgin olive oil (EVOO) [Pirisi et al. 1997, Gimeno et al. 2000, Ruperez et al. 2001, Bendini et al. 2003, Cardoso et al. 2005, De La Torre-Carbot et al. 2005, Gómez-Rico et al. 2008, Pinheiro-Sant'Ana et al. 2011, Motilva et al. 2013, Capriotti et al. 2014]. This product, which may be considered as a functional food, contains different classes of phenols: simple phenols, phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavonoids [Carrasco-Pancorbo et al. 2005].

In 2011, the European Food Safety Authority provided a scientific opinion on polyphenols in olive oil [EFSA 2011]. This opinion highlighted how some beneficial effects on human health (like protection of LDL particles from oxidative damage and maintenance of normal blood HDL-cholesterol concentrations) can correlates to the consumption of polyphenols present in olive oil. According to EFSA, molecules responsible of olive beneficial effects on human health are hydroxytyrosyl and oleuropein complex. The scientific substantiation of health claims is related to the consumption of 2–15 mg per day of hydroxytyrosol or oleuropein complex.

In addition to phenols, tocopherols also contribute to the remarkable stability of the oil. The vitamin E is a fat-soluble vitamin and is composed by a group of eight isomers, α-,β-,γ- and δ-tocopherols and α-,β-,γ-and δ-tocotrienols. Vitamin E is of nutritional, physiological, and analytical interest. These organic molecules are redox-type lipids and shown different antioxidant activity degree [Wang and Quinn 1999]. α-Tocopherol is reported to be the isomer with the greatest biological activity [Hosomi et al. 1997; Brigelius-Flohé et al. 2002] because it acts as peroxyl radical scavenger and protects polyunsaturated fatty acids [Wang and Quinn 1999; Niki 2014; Traber and Atkinson 2007]. The high-antioxidant activity of α-tocopherol refers to its reaction speed with peroxy radical. This vitamin E isomer reacts with peroxy radical faster than the other tocopherols and 1000 times faster than polyunsaturated fatty acids [Buettner 1993]. Thanks to this antioxidant activity, tocopherols prolong the shelf lives of many foods containing fat or oil. Moreover, together with phenols, vitamin E is a very promising oil stabilizing agent during frying, thanks to its capacity to reduce acrolein and acrylamide production [Sordini et al. 2019].

According to a recent EFSA scientific opinion [EFSA 2015] an average requirements and a population reference intake cannot be set for α-tocopherol, but an adequate intake for this molecule for adults could be 11–13 mg/day. Moreover, it was estimated that a daily α-tocopherol good absorption (ca. 75%) can be associated with intake of fat dietary sources [EFSA 2015]. Vegetable oils, cereals (especially wheat germ oil), some fatty fishes, seeds, and nuts represent the principal sources of vitamin E. Each food source is characterized by different ratios of the eight form of vitamin E, and α- and γ-tocopherols are the more abundant [Belitz et al. 2009]. α-Tocopherol is the main isomer present in extra-virgin olive oil, with a concentration ranging from 100 to 760 mg/Kg [Špika et al. 2015; Di Serio et al. 2016; Kalogeropoulos and Tsimidou 2014; Alves et al. 2019]. The reported great variability in Vitamin E concentration can be attributed to many factors as: cultivar, quality, territory, climate, production techniques, storage conditions, and olive maturity index [Iqdiam et al. 2018; Olmo-García et al. 2018].

Italian extra-virgin olive oil market presents several kinds of products, some of which are of higher quality compare to others. Italian high-quality extra-virgin olive oils can have Protected Designation of Origin (PDO) and Protected Geographical Identification (PGI) and organic farming denominations. These can be obtained milling monovarietal olives or different olives cultivars together (blend). European Union awards PDO brand to all the foodstuffs with peculiar qualitative characteristics. These characteristics depend exclusively on the territory in which they were produced. Atmospheric conditions together with production techniques allow obtaining an imitable product outside a specific production area. According to the European Regulation of 2012 [EU Regulation 2012], Italy shows the presence of forty-two PDO extra-virgin olive oils from eighteen regions. Then, except to Piedmont and Valle D'Aosta, all Italian regions have at least one PDO extra-virgin olive oil. Considering both the Italian geographical variability (like climate and territory) and the different production techniques, each PDO oil produced shows peculiar chemical composition and organoleptic characteristics. European Union recognizes to all the foodstuffs another brand: the PGI (Protected Geographical Identification). This kind of mark is less restrictive than PDO. PGI denomination is attributed to all the products whose characteristics and qualities depend on a specific geographical origin. In the European Regulation of 2012 [EU Regulation 2012], PGI Italian extra-virgin olive oils are four belonging to Sicily, Tuscany, Calabria, and Marche regions.

After a careful literature survey, many research articles, regarding the antioxidant molecules in extra-virgin olive oils, can be found even though applied to a limited number of

samples. Alves and collaborators evaluated the contribution of chemical compounds to the composition of forty extra-virgin olive oils produced in different regions of Mediterranean [Alves et al. 2019]. Eight monovarietal extra-virgin olive oils were analyzed by Olmo-Garcia and coworkers in order to evaluate the effects of technological parameters on minor component composition [Olmo-García et al. 2018]. Phenols profile was elucidated to investigate how oil quality can be influenced by harvesting period on fifty-four extra-virgin olive oils belonging to four cultivars in two crop years [Piscopo et al. 2018]. Bioactive molecules content of seventy-six samples was used to make a relationship between quality and price in the olive oil market [Fiorini et al. 2018]. Tsimidou et al. (2019) quantified total hydroxytyrosol and tyrosol content in thirty Italian extra-virgin olive oils using five different HPLC methods and ^{1}HNMR one. Two recent published research articles [Fanali et al. 2018; Klikarová et al. 2019] reported the bioactive molecules content in Italian mono- and multivarietal and PDO extra-virgin olive oils, in a limited samples set (32 samples).

The present work, which is a part of a more extended project, was aimed to endorse the use of Italian high-quality extra-virgin olive oils and to promote the "Italian quality olive oil culture" to the consumers. For this purpose, 186 high-quality extra-virgin olive oil samples were analyzed. Olives milled to obtain these samples belong to forty-seven different cultivars. To obtain this task, a miniaturized phenols extraction method was validated. The procedure adopted for phenols extraction taking into account a previously validated procedure [Ricciutelli et al. 2017] but with lower amount of both sample and solvent. This kind of procedure was needed considering the high amount of samples analyzed (186 high-quality extra-virgin olive oil samples) in order to minimize solvent volumes and extraction time. Moreover, two analytical methods were developed in order to investigate the phenol and tocopherol contents in extra-virgin olive oils by using HPLC coupled to mass spectrometry and fluorimetric detection, respectively. Furthermore, four different assays were used in order to evaluate antioxidant activity of all samples: Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), and dyphenyl-1-picrylhydrazyl (DPPH) radical scavenging, while the total phenols content was assessed by the Folin-Ciocalteau method. Finally, all these information were used to make a principal component analysis (PCA) in order to verify a possible correlation between oils belonging to the same region, or to the same cultivar, or to denomination. The gathered information attained could be useful for the generation of a database of a qualitative and quantitative profile of antioxidant molecules in PDO, PGI, organic farming, and monovarietal and blend Italian extra-virgin olive oils obtained from different cultivars harvested in different Italian regions from 2017 to 2018.

To the best of our knowledge, this is the first research article that reports the bioactive molecule content and a PCA statistical correlation between total phenol content, antioxidant activity (TEAC, FRAP, DPPH and ORAC), tocopherols, and phenols in a large array of Italian high-quality extra-virgin olive oils (174) produced in different regions; in addition a number of 12 samples came from foreign countries (Portugal, Spain, and Croatia).

Experimental Conditions

Materials

The α, β, γ, and δ-tocopherol and α-tocotrieol standards were purchased by Extrasynthese (Genay Cedex, France) and stored at -20 °C. Commercial phenolic standards such as Trolox (6-Hydroxy-2,5,7,8 tetramethylchroman-2 carboxylic acid, gallic acid, ferulic acid, caffeic acid, verbascoside, hydroxytyrosol, tyrosol, oleuropein, luteolin, luteolin-7-*O*-glucoside, apigenin, and ethyl gallate) were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). The reagents as TPTZ (2,4,6-tripyridyl-s-triazine), $FeCl_3$*$6H_2O$, sodium acetate, glacial acetic acid, fluorescein, 2,20-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium carbonate, Folin-Ciocalteau reagent, potassium persulfate, ABTS (2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic) diammonium salt), and DPPH (2,2 – dyphenyl-1-picrylhydrazyl) were purchased from Merck KGaA (Darmstadt, Germany).

Acetonitrile, methanol, ethanol, formic acid, isopropanol, and *n*-hexane used for chromatographic analysis were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). Water (resistivity above 18 MΩ cm) was obtained from a Milli-Q SP Reagent Water System Merck Life Science (Merck KGaA, Darmstadt, Germany).

Samples

One hundred seventy-four Italian extra-virgin olive oil samples were collected from the productive campaign 2017–2018 in various Italian oil mills sited in Garda area (Lombardy, Trentino, Veneto), Liguria, Emilia-Romagna, Tuscany, Umbria, Marche, Lazio, Abruzzo, Sardinia, Campania, Apulia, Calabria, and Sicily. Moreover some European extra-virgin olive oils were collected: 2 from Portugal, 7 from Spain, and 3 from Croatia. All the samples are listed in Table 1. The oils were poured into glass amber vials and stored at -20 °C, each sample was thawed and analyzed on the same day.

Table 1 High-quality extra-virgin olive oils analyzed (PDO: Protected Designation of Origin; PGI: Protected Geographical Identification; Bio: organic farming; MV: monovarietal)

Country	Denomination	Label	*Cultivar*, year (n. samples)
Apulia	PDO	Terra di Bari	*Coratina*, 2017 (2)
	Bio		*Leccino,* 2017 (1)
			Picholine, 2017 (1)
	MV		*Coratina*, 2017 (5), 2018 (3)
			Ogliarola, 2017 (2), 2018 (3)
			Peranzana, 2017 (2)
			Frantoio, 2017 (1)
	Blend		*Coratina-Nocellara del belice,* 2017 (1)
Sicily	PDO	Monti Iblei Gulfi	*Tonda Iblea*, 2017 (2)
		Valle del Belice	*Nocellara del Belice*, 2017 (1)
		Val di Mazara	*Nocellara del Belice-Biancolilla*, 2017 (1)
			Biancolilla, 2017 (1)
			Nocellara del Belice, 2017 (1)
	PGI		*Cerasuola-Nocellara del Belice-Biancolilla*, 2017 (1)
	Bio		*Nocellara Etenea-Nocellara Messinese,* 2017 (1)
	MV		*Nocellara Messinese*, 2017 (1)
			Nocellara Etnea, 2017 (2)
			Nocellara del Belice, 2017 (2)
			Biancolilla, 2017 (1)
			Cerasuola, 207 (2)
	Blend		*Nocellara del Belice-Biancolilla-Gioconda,* 2017 (1)
			Nocellara Etnea-Tonda Iblea-Biancolilla, 2017 (1)
Tuscany	PDO	Chianti classico	*Leccino-Moraiolo-Frantoio-Pendolino-Morchiaio*, 2017 (1)
			Leccino-Moraiolo-Frantoio-Pendolino, 2017 (2)
			Correggiolo-Leccino-Frantoiano, 2017 (1)
			Moraiolo-Leccino, 2017 (1)
			Frantoio, 2017 (2)
		Seggiano	*Olivastra Saggianese*, 2017 (3)
		Terre di Siena	*Leccino-Moraiolo-Frantoio-Pendolino*, 2017 (1)
	PGI		*Frantoio*, 2017 (1)
			Leccino-Moraiolo-Frantoio, 2017 (4)
			Leccino-Moraiolo-Frantoio-Maurino, 2017 (1)
			Leccino-Frantoio, 2017 (1)
			Leccino-Moraiolo-Frantoio-Correggiolo, 2017 (1)
			Correggiolo-Pendolino-Maurino-Leccio del Corno, 2017 (1)
	Bio		*Leccino-Moraiolo-Frantoio*, 2017 (5)
			Leccino-Moraiolo-Frantoio-Pendolino, 2017 (3)
			Frantoio-Moraiolo-OlivastraSeggianese-Leccino, 2017 (1)
	Blend		*Frantoio-Moraiolo-Leccino*, 2017 (3)
			Leccino-Moraiolo-Frantoio-Pendolino, 2017 (3)
	MV		*Leccino*, 2017 (2)
			Maurino, 2017 (1)
			Pendolino, 2017 (3)
			Frantoio, 2017 (7)
			Moraiolo, 2017 (4)
			Coratina, 2017 (1)
			Arancino, 2017 (1)
			Leccio del Corno, 2017 (3)

Table 1 (continued)

Country	Denomination	Label	*Cultivar*, year (n. samples)
			Nocellara, 2017 (2)
			Raggiolo, 2017 (2)
Liguria	MV		*Taggiasca*, 2017 (2)
Emilia Romagna	MV		*Correggiolo,* 2017 (1)
Marche	PGI		*Leccino,* 2017 (1)
	Bio		*Ascolana,* 2017 (1)
			Raggiola, 2017 (1)
	MV		*Orbetana,* 2017 (1)
			Tenera Ascolana, 2017 (1)
			Raggiola, 2017 (1)
	Blend		*Leccino-Frantoio-Carboncello-Pendolino-Rosciola-Lea-Moraiolo,* 2017 (1)
Campania	PDO	Colline Salernitane	*Carpellese-Frantoio-Rotondella,* 2017 (1)
		Cilento	*Salella,* 2017 (1)
	MV		*Ravece*, 2017 (1)
			Cammarotana, 2017 (1)
			Ortice, 2017 (1)
	Blend		*Carpellese-Frantoio-Rotondella,* 2017 (1)
Abruzzo	PDO	Colline Teatine	*Gentile di Chieti-Intosso-Leccino,* 2017 (1)
	MV		*Oliva Grossa,* 2017 (1)
			Dritta, 2017 (2)
			Intosso, 2017 (1)
	Blend		*Gentile di Chieti-Intosso-Leccino,* 2017 (1)
			Dritta-Tortiglione, 2017 (2)
Garda area	PDO	Garda	*Casaliva-Leccino-Moraiolo-Pendolino-Frantoio*, 2017 (1)
		Garda Trentino	*Casaliva*, 2017 (1)
		Veneto Valpolicella	*Grignano*, 2018 (1)
			Grignano-Favarolo-Pendolino-Trepp, 2017 (1)
	MV		*Casaliva*, 2017 (2)
			Coratina, 2017 (1)
			Grignano, 2017 (1)
	Blend		*Casaliva-Frantoio-Leccino*, 2017 (2)
Calabria	Bio		*Ottobratica-Sinopolese*, 2017 (1)
	Blend		*Ottobratica-Sinopolese*, 2017 (2)
	MV		*Nocellara del Belice*, 2017 (1)
			Ottobratica, 2017 (3)
			Carolea, 2018 (1)
Lazio	PDO	Tuscia	*Canino*, 2017 (2), 2018 (1)
		Lazio	*Itrana*, 2018 (1)
	MV		*Itrana*, 2017 (2), 2018 (1)
			Canino, 2018 (2)
			Frantoio, 2018 (1)
	Blend		*Caninese- Frantoio-Maurino*, 2017 (3)
			Caninese-Frantoio-Leccino, 2017 (1)
Sardinia	PDO	Sardegna	*Bosana-Semidana*, 2017 (1)
			Bosana, 2017 (1)
	Blend		*Bosana-Semidana*, 2017 (1)

Table 1 (continued)

Country	Denomination	Label	*Cultivar*, year (n. samples)
	MV		*Semidana*, 2017 (1)
			Bosana, 2017 (2), 2018 (1)
			Nera di Oliena, 2018 (1)
Umbria	PDO	Umbria Colli Orvietani	*Leccino-Frantoio-Moraiolo*, 2017 (1)
		Umbria Colli Assisi-Spoleto	*Leccino-Frantoio-Moraiolo*, 2017 (1)
	Blend		*Leccino, Frantoio, Moraiolo*, 2017 (4)
	MV		*Coratina*, 2017 (1)
			Frantoio, 2017 (1)
			Moraiolo, 2017 (1)
Portugal	PDO	Portugal	*Cobrançoga*, 2017 (1)
			Madural, 2017 (1)
Spain	PDO	Priego de Cordoba	*Hojiblanca*, 2017 (1)
	MV		*Arbequina,* 2017 (2)
			Hojiblanca, 2017 (1)
			Arbosana, 2017 (1)
			Picual, 2017 (2)
Croatia	MV		*Frantoio*, 2017 (1)
	Blend		*Busa-Carbonazza-Bianchinera-Rosignola-Morasola,* 2017 (2)

Phenols Extraction

Phenolic compounds extraction was needed before HPLC-PDA/MS analysis, for the total phenolic content and antioxidant activity evaluation [Ricciutelli et al. 2017]. 1 mL of oil diluted in 1 mL of n-hexane has been placed in a centrifuge tube and homogenized. The sample was extracted with 1 mL of methanol/water (3:2, *v/v*), shaken for 5 min and extracted for 2 min in an ultrasonic bath. Then was centrifuged for 10 min at 3000 rpm. The aqueous phase (1 mL) was recovered and washed with 1 mL of *n*-hexane. Before HPLC injection, 20 μL of internal standard (I.S.) ethyl gallate (10 mg in 10 mL of methanol) were added to each extract. While for total phenolic content and antioxidant activity evaluation, the final solution was dissolved in 1 mL of initizal MeOH/H_2O (3:2, *v/v*) before the analysis.

Phenols Determination by HPLC-PDA/MS

HPLC analysis were performed using a Shimadzu (Kyoto, Japan) series instrument, composed of binary solvent pumps LC-20 AD, a SPD-M20A photodiode array detector (PDA), and a LCMS-2020 mass spectrometer detector (MS). MS detector was equipped with an electrospray ionization (ESI) source operating in negative ionization (NI) mode and single quadrupole MS.

The separation was achieved on an Ascentis Express C18 (150 × 4.6 mm, 2.7 μm) analytical column (Merck Life Science, Merck KGaA, Darmstadt, Germany). The mobile phase for HPLC-PDA/ESI-MS analyses was water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) working in the gradient mode 0 min, 10%; 4 min, 35%B; 12 min, 47%B; 12.5 min, 60%; 16 min, 75%; and 21 min, 100% B with a flow rate 1.0 mL/min. All analyses were performed at room temperature of the column and the injection volume was 5 μL. PDA was monitored at a wavelength of 280 nm. The ESI ion source conditions were follows: DL (desolvation line) temperature, 280 °C; nebulizing gas (nitrogen) flow, 1.5 L/min; drying gas (nitrogen) flow rate, 5 L/min; and heat block temperature, 300 °C. Mass scan range was set in the range of m/z 100–800; event time was 0.5 s. SIM (single ion monitoring) mode was used for phenol quantification: gallic acid (170 *m/z*), hydroxytyrosol (154 *m/z*), tyrosol (138 *m/z*), oleuropein aglycone (378 *m/z*), ligstroside aglycone (362 *m/z*), oleocanthal (304 *m/z*), oleacein (320 *m/z*), apigenin (270 *m/z*), and luteolin (286 *m/z*),

Tocopherols Determination by HPLC-FLD

Extra-virgin olive oil samples were analyzed without any pretreatment. In order to quantify α, β, and γ-tocopherol and α-tocotrienol in a single analysis, each sample was diluted with *n*-hexane (1:10, 1:15 or 1:30, *v/v*) before NP-HPLC analysis. Each oil was analyzed in triplicate.

HPLC analyses were carried out using a Shimadzu Nexera-X2 system (Shimadzu, Milan, Italy), including a CBM-20A controller, two LC-30 AD dual-plunger parallel-flow pumps, a DGU-20A_{SR} on-line degasser, an autosampler SIL-30 AC, a

CTO-20 AC column oven, and a RF-20AXS fluorescence detector with cell capacity 12 μL, xenon lamp, and wavelength range 200–750 nm. Data acquisition was performed by the LCMSsolution Ver. 5.85 software (Shimadzu).

Tocopherols analyses were carried out on an Ascentis Si, 250 × 4.6 mm I.D. with particle size of 5 μm (Merck KGaA, Darmstadt, Germany) column. The injection volume was 5 μL. Analyses were carried out in isocratic mode: *n*-hexane and isopropanol (99:1, *v/v*). Flow rate was 1.7 mL/min. Column oven was set at 25 °C. Data were acquired using a fluorimetric detector at 290 nm for the excitation wavelengthof and 330 for emission.

Sample Extraction Methods Validation

Recovery of phenols was determined by carrying out the extraction procedure on a sample of soybean oil. Soybean oil used for extraction recovery test was previously analyzed in our laboratory.

The extraction procedure was carried out after addition of known amounts of luteolin, oleuropein, apigenin, tyrosol, and hydroxytyrosol on the samples chosen for the extraction methods validation. Each fortification was carried out on three different samples, and every extract was analyzed in triplicate. Recovery was calculated using the following formula:

Recovery % = [(Conc. Sample Fortified − Conc. Sample Unfortfied)/Fortification]*100.

RP- and NP-HPLC Methods Validation

To quantify the polyphenols and tocopherols content in the one hundred eighty-six high-quality extra-virgin olive oil samples calibration curves have been constructed by using each single available standard. For quantification of polyphenols, five different concentrations of each component, in the range between 100 and 0.1 mg/L, prepared by diluting a stock solution of about 1000 mg/L, using methanol as a solvent, were analyzed for five consecutive times by RP-HPLC under the same chromatographic conditions optimized for the samples. Before injection, 20 μL of internal standard (I.S.) ethyl gallate (10 mg/10 mL) was added to 1 mL of each standard solution.

To quantify tocopherols, five different concentrations of each component, in the range between 5 and 0.005 mg/L, prepared by diluting a stock solution of about 100 mg/L, using n-hexane as a solvent, were analyzed for five consecutive times by NP-HPLC under the same chromatographic conditions optimized for the samples.

The validation process of the RP-HPLC and NP-HPLC methods were carried out following EURACHEM guidelines [Eurachem]. The instrumental intraday and interday repeatability and the recovery were calculated on six replicated injections at one concentration level for the analytes (luteolin, oleuropein, apigenin, tyrosol, and hydroxytyrosol: 1 mg/L; α-, β-, γ-, and δ-tocopherols and α-tocotrienol: 0.01 mg/L). Limit of detection (LOD) and limit of quantification (LOQ) values were also calculated.

Total Phenolic Content Determination

Determination of total polyphenol content of the olive oil extracts was determined by the Folin-Ciocalteau colorimetric method, using the same method reported by Fanali and coworkers (Fanali et al. 2018). Briefly 1580 μL of a methanol/water (50:50, *v*/v) mixture and 100 μL of Folin-Ciocalteu were added to 20 μL of sample extracts. Then 300 μL of sodium carbonate solution (20% *w/v*) was added, mixed, and incubated for 2 h in a dark environment at room temperature. The absorbance reading was 765 nm. The results were expressed as mg of gallic acid equivalents (GAE)/g of sample. Total phenolic content was determined from the gallic acid calibration curve (0,05–1,6 mg/ml).

Antioxidant Activity Evaluation

DPPH [2,2-Diphenyl-1-Picrylhydrazyl] Assay

The DPPH assay was performed according to the method described by Fanali and coworkers (Fanali et al. 2018). Briefly, 20 μL of diluted sample and 180 μL of ethanolic solution of DPPH (0.1 mM) were placed in the well of the microplate. After 20 min of incubation, in the dark at room temperature, the absorbance was detected at 518 nm. The antioxidant capacity of sample was determined by a calibration curve ($R^2 = 0.995$), using Trolox as reference compound (20–200 μM).

ABTS• + [2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulphonic Acid)] Assay

Free radical scavenging activity of oil extract was evaluated by colorimetric assay according to a previously described method (Fanali et al. 2018). Briefly, a volume of 10 μL of each diluted sample was mixed with 190 μL of ABTS• + working solution prepared according to Fanali et al. (2018) in a 96-multiwell plate (Greiner Bio-one, Germany). The absorbance was recorded at 734 nm after 20 min. A calibration curve was prepared with Trolox as a concentration 50–700 μM. Results were expressed as μmol Trolox equivalent (TE) per g of sample.

Oxygen Radical Absorbance Capacity Assay (ORAC-Fluorescein)

The ORAC assay was performed as described by Fanali and coworkers (Fanali et al. 2018). Fluorescence was read with an excitation wavelength of 485 nm and an emission wavelength

of 520 nm. A fluorescein stock solution was prepared according to Fanali et al. (2018). Briefly, 120 μL of Fluorescein and 20 μL of diluted extract solutions were mixed and incubated for 15 min at 37 °C. 60 μL AAPH solution (12 mM, final concentration) were then added, and the volume of the final reaction mixture was 200 μL in well. The microplate was immediately placed in the reader and the fluorescence decay was measured every minute for 90 cycles in a 96-well cell culture plate (Greiner Bio-one, Germany) using a multifunctional microplate reader (Infinite®, 200 PRO multimode reader, Tecan, IT). The microplate was automatically shaken prior each reading. Elaboration data was made according to the procedure already reported by Fanali et al. (2018). The activity of the sample was expressed as μmol of Trolox Equivalents (TE) per gram of oil. The antioxidant capacity of sample was determined by a calibration curve, using Trolox as reference compound (0–100 μM).

The Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP assay was carried out according to the procedure described in by Fanali et al. (2018). Before the analyses, oil extracts were diluted 10 times. The fresh working FRAP reagent was prepared daily following the procedure already reported by Fanali et al. (2018). 10 μL of diluted sample solution was added to 190 μL of FRAP reagent. The absorbance was measured after 6 min at room temperature at wavelength of 593 nm.The antioxidant capacity of sample was determined by a calibration curve, using Trolox as reference compound (25–500 μM). Results were expressed in μmoles of TE/ g of FW.

Data Processing

All experimental measurements were performed in triplicate, but average values were computed and used for data analysis. The obtained data matrix contained as many rows as EVOO samples (185) and as many columns as variables (18) i.e., phenols, tocopherols, tocotrienol, Tyrosol, Luteolin, FOLIN, DPPH, ORAC, etc.

First of all, Principal Component Analysis (PCA) was performed on the whole data matrix as a multivariate display methods in order to visualize the data structure; in this case, the use of autoscaling prior multivariate data processing was necessary to uniform variable scale.

Subsequently, Linear Discriminant Analysis (LDA) was applied as a linear classification technique in order to classify the oil samples on the basis of their geographical provenience. In particular, for classification analysis, only the 4 Italian Regions having a number of samples sufficient to fully represent the variability of the class were considered: the Apulia EVOO samples scribed to class 1, the Sicily EVOO samples to class 2, the Tuscany ones to class 3, and the Lazio samples to class 4.

LDA (Massart, Vandeginste, Buydens, De Jong, Lewi & Smeyers-Verbeke, 1998) is a probabilistic classification technique, which searches for directions (canonical variables) with maximum separation among categories; the first canonical variable is the direction of maximum ratio between inter-class and intra-class variances.

Classification results were validated both in cross validation using 5 cancellation groups and using an external test test (20% of the total samples randomly selected).

Results and Discussion

The identification and quantification of phenols (phenolic acids, phenolic alcohols, flavones, secoiroids) in Italian and European extra-virgin olive oils was attained by means of RP-HPLC in combination with PDA and MS detection. When possible, the identification of compounds was confirmed by comparison with standard materials. Tocopherols were analyzed by NP-HPLC coupled with a fluorimetric detector. Normal-phase systems show elution of homologs in order of increasing polarity with separation based on methyl substituents on the chromanol moiety. Peak identification was obtained by comparing the retention time of the sample with a standard solution.

The validation process of the two methods employed provided the results shown in Table 2 for LOD and LOQ, both for phenols and tocopherols. LOD and LOQ values in the low milligrams per liter range attested the applicability of the two methods to the analysis of polyphenols and tocopherols in trace level in foodstuffs. Good linearity was obtained for all the analytes, as confirmed by the correlation coefficient R^2, ranging from 0.993 to 0.998. Concerning the intraday repeatability and intralaboratory reproducibility, coefficient of variation (CV) values of < 7.5% demonstrated good precision at the concentration level tested. Concerning recovery, values ranging from 86 to 95% were obtained for all the analytes at the concentration level tested, thus demonstrating good accuracy [Hokanson 1994].

All the samples analyzed in this work were subjected to solvent extraction prior to RP-HPLC analyses of phenolic fraction, total phenolic determination, and antioxidant activity evaluation (as described in the experimental section), except for tocopherols analysis. Due to the high number of EVOO samples (186), a miniaturized phenols extraction method was validated. The procedure adopted for phenols extraction taking into account a previously validated procedure [Ricciutelli et al. 2017], but with lower amount of both sample and solvent. This kind of procedure was needed in order to minimize solvent volumes and extraction time. With respect to Ricciutelli et al. [2017] this new miniaturized phenols

Table 2 Regression equations, correlation coefficients (R^2), LOD, LOQ, recoveries, retention times, λmax, and MS values for each bioactive molecule

Compound	$R_t \pm sd$	Area (RSD %)	Regression line	R^2	LOD (mg/L)	LOQ (mg/L)	Recovery (%)
α-tocopherol	3.50 ± 0.014	2.30	y = 5E+06x - 675,531	0.993	0.001	0.003	82.8
α-tocotrienol	3.78 ± 0.019	3.08	y = 3E+06x - 547,890	0.992	0.001	0.003	77.5
β-tocopherol	4.87 ± 0.039	3.23	y = 5E+06x - 736,705	0.994	0.002	0.004	79.6
γ-tocoferol	5.21 ± 0.090	1.22	y = 5E+06x - 1E+06	0.995	0.001	0.002	80.1
δ-tocopherol	7.22 ± 0.095	3.45	y = 7E+06x - 705,487	0.998	0.002	0.004	90.6
Gallic acid	2.31 ± 0.089	2.67	y = 0,0009x + 0,0007	0.993	0.039	0.092	89.4
Hydroxytyrosol	3.27 ± 0.097	2.80	y = 0,0014x + 0,0039	0.992	0.043	0.0092	87.3
Tyrosol	4.16 ± 0.149	3.21	y = 3E-05x - 0,0001	0.975	0.045	0.096	85.2
Caffeic acid	4.62 ± 0.170	2.52	y = 0,0031x + 0,0061	0.996	0.037	0.090	78.9
Verbascoside	5.28 ± 0.124	2.49	y = 0,0043x - 0,0007	0.993	0.035	0.79	90.2
Luteolin 7-*O*-glucoside	5.42 ± 0.138	2.63	y = 0,006x + 0,0103	0.995	0.028	0.050	82.8
Ferulic acid	5.94 ± 0.110	2.81	y = 0,0029x + 0,0008	0.995	0.036	0.089	75.2
Oleuropein	6.61 ± 0.170	1.54	y = 0,0087x + 0,0048	0.994	0.046	0.098	80.1
Luteolin	8.50 ± 0.060	2.17	y = 0,0089x + 0,022	0.994	0.037	0.083	91.0
Apigenin	10.18 ± 0.115	2.51	y = 0,0127x + 0,063	0.993	0.026	0.078	84.2

extraction procedure employed one fifth of both sample and extraction solvent. This means that, for the analysis of 186 samples, with at least three replicated extraction for sample, a total amount of solvent of 500 mL is needed.

Recovery of phenols was determined carrying out the extraction procedure on a sample of soybean oil. Each sample was fortified of known amount of hydroxytyrosol, tyrosol, oleuropein, luteolin, and apigenin, as described in the experimental section. In all cases, fortification was performed on 3 samples and each extract was analyzed in triplicate. Recovery values for extraction ranged from 75 to 91%, thus demonstrating good accuracy.

As already reported by Pascale et al. (2018), quantification of secoridoid derivatives in olive oils is difficult due to the lack of commercial standard materials. The above-mentioned molecules are present in the samples of our interest in different isomeric forms, this makes difficult both the chromatographic separation and quantification. According to Pascale et al. (2018) it was decided to detect and quantify them with negative polarity and oleuropein calabration curve, respectively. Quantification of all the oleuropein aglycone isomers, oleocanthal, and oleacin were approximated considering the extract ion chromatograms corresponding to their molecular ions and then comparing it to oleuropein. As previously reported by Pascale et al. (2018), this can be possible because the negative charge of oleuropein formed during the ESI process is similar or identical to the oleuropein aglycone isomers, oleocanthal, and oleacin portion. Other phenols were easily quantified with their specific calibration curves.

As reported in Table 3 the most representative phenols for all the samples analyzed were oleacin, oleocanthal, oleuropein aglycone complex, and ligstroside aglycone according to literature data [Ricciutelli et al. 2017; Savarese et al. 2007]. Table 4 reports the content of bioactive molecules present in Italian extra-virgin olive oils divided by selected regions. Considering oleuropein aglycone and ligstroside aglycone,

Table 3 Content (mg/Kg) of bioactive molecules, total phenolic content, and antioxidant activity of all the Italian EVOO samples analyzed

Compound	min	max	average
α-tocopherol	76.4	376.3	159.7
α-tocotrienol	1.3	6.7	3.0
β-tocopherol	1.1	9.2	2.7
γ-tocopherol	1.1	25.1	7.8
Gallic acid	< LOD	40.9	6.2
Hydroxytyrosol	3.6	299.5	64.4
Tyrosol	42.9	297.6	114.0
Oleacin[a]	15.2	762.9	351.1
Oleuropein aglycone[a]	51.6	3482.4	928.6
Oleocanthal[a]	< LOD	407.9	153.5
Luteolin	4.0	149.0	62.3
Ligstroside aglycone[b]	17.6	1495.5	410.0
Apigenin	< LOD	38.8	6.9
Total phenolic content			
mg GAE/g PF	0.05	0.70	0.33
Antioxidant activity (μmol TE/g)			
TEAC	1.28	12.48	5.65
DPPH	0.21	2.72	1.16
ORAC	1.02	20.92	9.03
FRAP	0.37	3.83	1.65

Bioactive molecules were quantitatively determined based on calibration curves obtained with the correspondent standard compound: [a] oleuropein and [b] verbascoside

Table 4 Content (mg/Kg) of bioactive molecules in EVOO samples analyzed divided by selected region

		α-tocopherol	α-tocotrienol	β-tocopherol	γ-tocopherol	Gallic acid	Hydroxytyrosol
Abruzzo	*range*	106.2–213.8	3.3–5.1	4.6–7.4	3.6–18.2	2.0–9.3	22.4–95.5
	average	153.6	4.0	5.3	9.7	5.3	59.5
Apulia	*range*	86.7–245.3	1.3–5.9	1.5–5.1	2.6–24.4	<LOD-40.9	21.2–147.1
	average	152.6	3.0	2.3	8.1	9.6	69.7
Calabria	*range*	131.7–224.6	2.6–3.2	1.6–2.8	2.6–15.6	4.1–40.3	37.7–299.5
	average	174.8	2.8	2.3	9.3	12.0	147.8
Campania	*range*	104.6–245.7	3.2–6.7	2.7–7.8	4.5–24.1	<LOD-12.3	9.4–111.3
	average	181.0	4.8	4.3	12.7	6.3	72.0
Garda	*range*	84.9–189.8	1.9–2.9	1.4–3.2	3.6–11.5	0.5–9.7	21.4–46.8
	average	140.1	2.4	2.0	7.1	4.5	33.3
Lazio	*range*	138.3–255.2	2.2–3.5	1.7–3.7	3.6–12.8	<LOD-9.2	11.3–286.0
	average	192.3	3.0	2.5	6.9	3.4	67.4
Marche	*range*	143.9–248.5	3.4–4.5	2.7–6.5	7.1–25.1	2.1–14.6	31.7–166.4
	average	205.5	3.8	4.1	14.9	6.2	75.1
Sardinia	*range*	130.2–182.4	2.4–3.5	1.5–2.4	3.5–10.6	0.3–20.2	43.9–269.0
	average	161.3	3.0	1.9	5.1	8.2	99.8
Sicily	*range*	76.7–214.8	1.9–4.7	1.6–9.2	1.1–5.1	<LOD-15.5	3.6–71.7
	average	118.2	2.7	3.1	2.9	5.2	30.9
Tuscany	*range*	76.4–246.1	1.3–5.1	1.1–6.8	3.2–17.2	<LOD-25.1	18.2–120.2
	average	148.1	2.9	2.4	8.1	4.7	64.0
Umbria	*range*	126.8–221.1	2.5–3.3	1.5–2.5	5.1–14.4	2.9–6.9	50.0–84.3
	average	176.4	3.0	2.0	10.1	4.4	62.2

	Tyrosol	Oleacin[a]	Oleuropeinaglycone[a]	Oleocanthal[a]	Luteolin	Ligstrosideaglycone[b]	Apigenin
Abruzzo	53.6–152.1	180.0–641.1	562.7–1326.4	53.2–244.0	16.6–81.2	21.9–774.0	<LOD-6.2
	90.9	329.1	901.6	131.8	49.8	385.1	1.1
Apulia	53.2–167.6	83.7–660.0	491.2–3482.4	42.1–313.9	7.3–85.5	239.0–1495.5	<LOD-17.2
	107.3	246.4	1257.8	113.7	38.5	733.2	4.4
Calabria	78.3–297.6	41.8–692.2	149.6–1473.5	<LOD-282.3	23.3–107.9	47.9–587.9	1.9–27.1
	188.6	292.5	722.9	140.2	61.5	301.3	13.3
Campania	42.9–169.7	134.4–640.7	332.0–1368.5	41.8–175.9	9.2–55.4	97.6–554.0	<LOD-1.9
	96.7	310.4	816.9	96.3	28.8	286.9	0.3
Garda	44.5–175.0	175.9–389.4	292.9–1559.9	69.9–179.5	42.4–70.5	131.5–960.3	3.5–9.7
	129.3	242.7	666.7	119.0	55.7	313.4	6.9
Lazio	44.3–267.9	81.8–657.2	394.7–2083.4	26.0–239.3	18.4–114.7	84.7–453.8	0.6–38.8
	109.3	434.8	951.7	124.0	61.4	274.8	15.5
Marche	91.1–166.2	148.2–729.9	531.0–1225.6	69.9–263.4	26.1–84.8	216.2–729.7	<LOD-10.1
	125.6	460.9	871.5	175.4	63.7	438.0	2.9
Sardinia	66.8–218.7	134.8–327.7	349.3–1411.1	45.4–142.6	4.0–104.5	189.9–628.0	1.7–22.1
	110.5	206.0	1050.0	75.3	55.2	425.0	9.3
Sicily	46.9–186.6	48.8–566.1	51.6–742.3	12.0–336.6	9.8–113.5	17.6–474.5	<LOD-17.2
	99.1	281.9	320.0	136.5	51.1	174.6	4.3
Tuscany	42.9–218.9	108.0–762.9	84.7–3200.9	50.6–407.9	5.4–149.0	94.2–1156.3	LOD-18.2
	108.3	434.9	1100.4	190.5	73.0	456.2	6.3
Umbria	110.4–203.9	189.3–480.1	590.1–1182.5	98.2–236.4	45.9–105.2	197.3–782.6	2.3–14.1
	141.3	346.4	863.0	157.8	69.3	418.0	6.9

Bioactive molecules were quantitatively determined based on calibration curves obtained with the correspondent standard compound: [a] oleuropein and [b] verbascoside

phenols content in Sicilian extra-virgin olive oils was lower compares to other regions. Apulian and Tuscanian oils exhibited a higher mean concentration value of phenols. Phenols Apulian oils were characterized by a high-oleuropein aglycone complex concentration (1257.8 mg/Kg), if compared to average value for Italian oils (928.6 mg/Kg). Furthermore, it can be deduced that high-phenol concentration of Tuscanian oils respect to the Italians one can be attributed to a high content of oleacin, oleocanthal, and oleuropein aglycone complex. On the contrary, Sicilian oils exhibited a lower concentration for all the bioactive molecules analyzed. Calabrian oils showed higher concentration values for gallic acid, tyrosol, and hydroxytyrosol, and lower concentration values for oleacin, ligstroside aglycone, and oleuropein aglycone complex. This can due to oleuropein aglycone complex hydrolysis and subsequent increase of tyrosol and hydroxytyrosol.

As can be seen in Table 3, α-tocopherol was the vitamin E isomer most representative for all the oils analyzed, followed by γ-tocopherol according to literature data [Špika et al. 2015; Di Serio et al. 2016; Kalogeropoulos and Tsimidou 2014; Alves et al. 2019]. The mean content of α-tocopherol for most of the region considered was approximately comparable to the mean content of all samples reported in Table 3. Sicilian oils showed a lower mean content of α-tocopherol; on the contrary, oils from Marche, Lazio, and Campania regions exhibited a higher mean concentration value. Moreover, oils from Marche, Abruzzo, and Campania regions showed higher concentration values for all the Vitamin E isomers quantified respect to the mean concentration value of Italian oils.

Although there is this variability between different regions, all the samples analyzed showed a good oleuropein complex and hydroxytyrosol content. A daily consumption between 20 and 30 g of extra-virgin olive oils analyzed in this work ensures a daily intake of about 8–16 mg of oleuropein complex and hydroxytyrosol, as recommended by EFSA guidelines [EFSA 2011]. Moreover, high-quality extra-virgin olive oils, that have been analyzed, represent a good fat dietary sources of α-tocopherol. So daily consumption of ca., 20–30 g, of these oils insures a 30–45% of an adequate intake of α-tocopherol (11–13 mg/day) [EFSA 2015].

Results obtained for extra-virgin olive oils produced in Croatia, Spain, and Portugal are shown in Table S1. For all the samples analyzed, α-tocopherol content was higher than Italian oils (Croatia 234.6 mg/Kg, Portugal 261.5 mg/Kg, Spain 201.2 mg/Kg). On the contrary, concentration of phenols, oleuropein aglycone complex in particular, was lower than Italian oils (Croatia 677.8 mg/Kg, Portugal 510.1 mg/Kg, and Spain 746.8 mg/Kg). Spanish oils showed comparable phenols mean content to Italian oils except to oleuropein aglycone complex, ligstroside aglycone and hydroxytyrosol. All these differences can be attributed to the geographical variability (like climate and territory), cultivars, and different production techniques.

Multivariate Data Analysis

Since the display of 186 samples on the same score plot was rather confusing and difficult to interpret (see Fig. S1 in supplementary material), the Principal Component Analysis was performed on the entire data set, but the samples were then projected in two blocks, obtaining two different score plots (Figs. 1 a and b). Fig. 2 shows the loading plot on the plane PC1 versus PC2. Looking at the score plots, it can be noted that the extra-virgin olive oils differ according to their geographical origin. The clusters are not clearly separated but oil samples from the same Italian Region form areas of higher density; it should be considered that all the samples are high-quality EVOOs with a minimal inter-variability. In Fig. 1a it is possible to recognize a cluster for the Sicilian oils, all of which at negative value on PC1 and PC2 (except 2 samples). In Fig. 2b, the clusters of the Garda, Sardinia, Spain, Umbria, and Croatia oils are clearly visible; the samples from Abruzzo, Marche, and Campania are more dispersed but all fall at positive values of PC2.

From the observation of the loading plot in Fig. 2, it can be seen that FRAP, FOLIN, TEAC, DPPH, and ORAC are variables highly correlated and at positive values along PC1 as also the two variables Ligstroside aglicone and Oleuropein aglicone that overlap but at more negative value of PC2. Tocopherols and tocotrienols instead constitute a group of four correlated variables at positive values along PC2. Therefore, the oil samples at positive values of PC1 are characterized by a greater antioxidant power, and oils with positive values of PC2 will be characterized by a higher content of tocopherols and tocotrienols. The other variables are arranged around the origin of the axes, most of them (DCOaglycone, DCLaglycone, Hydroxytyrosol, etc...) to positive values of PC1 and only 2 variables (Tyrosol, Apigenin) to negative values both of PC1 and PC2.

Combining the information of the loadings with that of the scores in Fig. 1a, it can be deduced that the values of FRAP, FOLIN, TEAC, DPPH, and ORAC are higher in the EVOO of Tuscany. Tuscan oils show an overall higher content of antioxidant compounds. The Apulian and Lazio oils are dispersed around the origin of the axes, while the Sicilian ones, that are mostly found to negative values of PC1, are characterized by a lower content of antioxidant compounds. Looking at Fig. 1b, it is possible to see how the samples of Abruzzo and Marche, followed by those of Campania, are in the positive quadrant on PC1-PC2 and therefore have a higher content of antioxidant molecules. The oils of Garda and Liguria have lower contents of tocopherols and tocotrienols, they fall in the lower left quadrant, with negative values of both PC1 and PC2. The oils of Sardinia are similar to those of the Liguria but with slightly higher FRAP, FOLIN, TEAC, DPPH, and ORAC values.

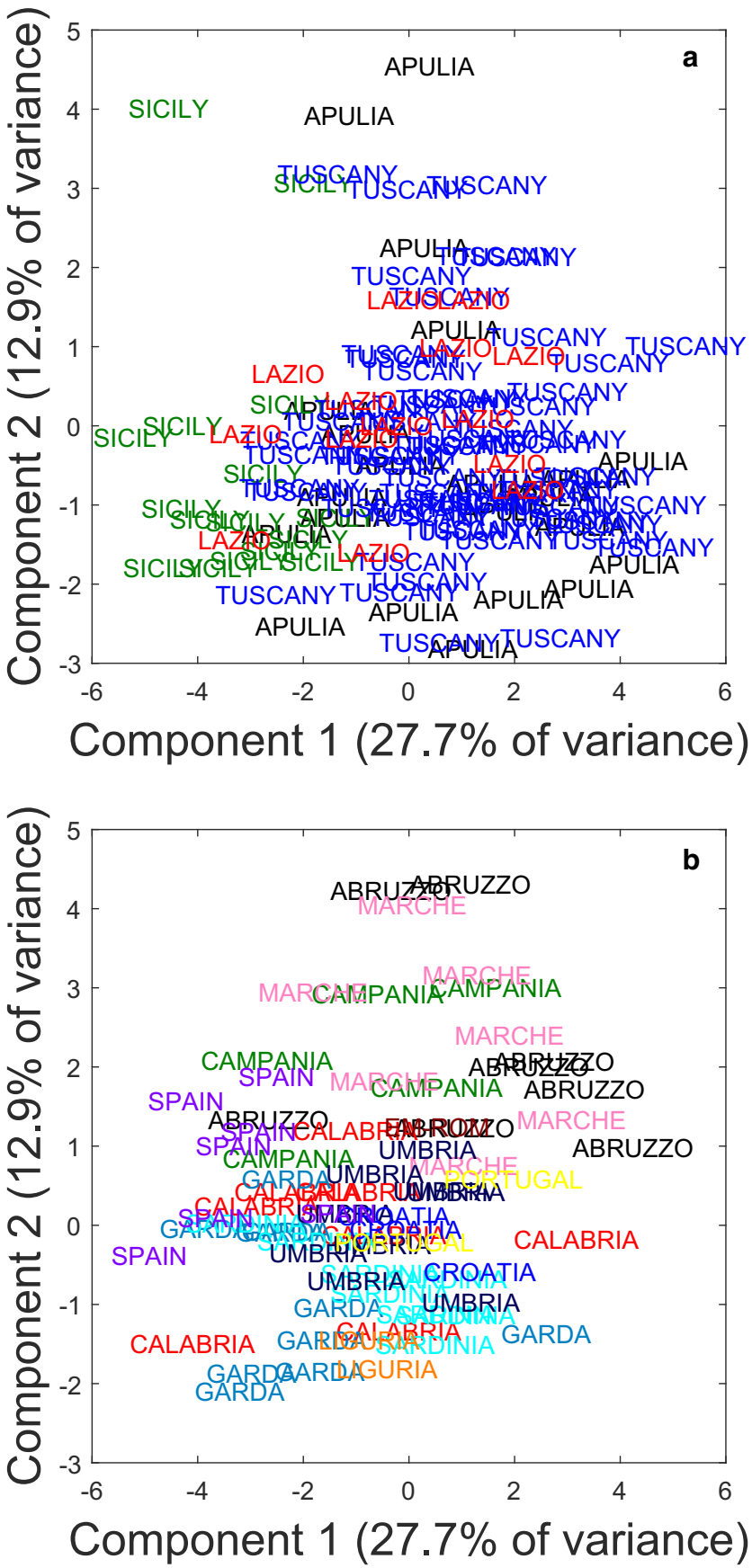

Fig. 1 **a**. Score plot of the first 115 EVOO samples in the orthogonal space PC1-PC2 computed with all the 186 samples. **b**. Score plot of the last 70 EVOO samples in the orthogonal space PC1-PC2 computed with all the 186 samples

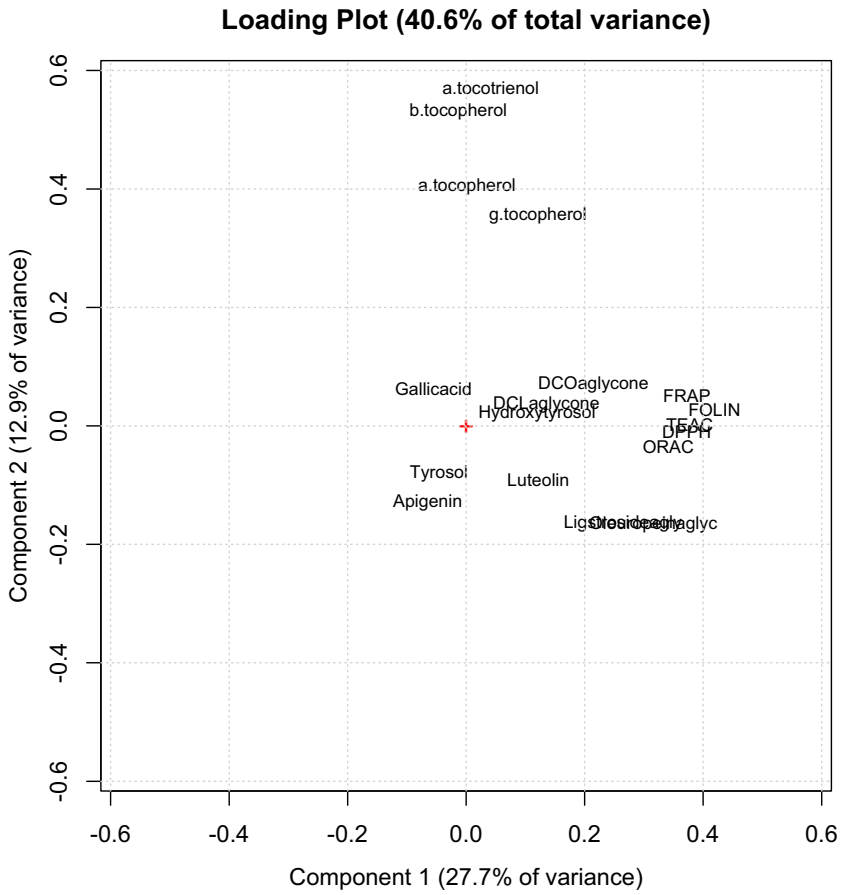

Fig. 2 Loading plot in the orthogonal space PC1-PC2

Classification Analysis

Linear Discriminant Analysis (LDA) was applied as a classification technique in order to discriminate the EVOO samples on the basis of the Geographical Origin. Only the 4 Italian regions having a number of samples sufficient to represent the variability of the regional production were considered:

1 Apulia (22 samples), **2** Sicily (16 samples), **3** Tuscany (63 samples), and **4** Lazio (14).

The LDA results show the ability of antioxidant molecules to classify extra-virgin olive oils on the basis of their geographical origin. The mean rate of test set samples correctly predicted was about 70%.

In particular, the LDA results showed the best prediction ability for category 2 (Sicily) with a classification rate of 93% and prediction rate of 85% followed by category 3 (Tuscany): classification and prediction rates of 90% and 70%, respectively. Classification and prediction abilities for the remaining 2 categories (Apulia and Lazio) were lower.

These results demonstrate that there are differences regarding the contents of bioactive antioxidant molecules among Italian extra-virgin olive oils belonging to different geographical regions, but these differences are not always marked and significant and this is probably due to the cold extraction that keeps antioxidants unaltered.

Conclusions

The present work aimed to evaluate the bioactive content in a large array of Italian high-quality extra-virgin olive oils produced in different regions, in addition to few selected samples coming from foreign countries (Portugal, Spain, and Croatia). Quantitative data demonstrated that all of them have exceeded the nutritional claims as reported by EFSA Journal, 2011 and 2015). The assessment of a potential correlation of the phenol and tocopherol content and the antioxidant assays within the extra-virgin olive oils investigated results highlighted some differences between the different geographical regions, even though not always marked and significant, probably due to the cold extraction that keeps antioxidants unaltered. The "comprehensive" gathered information attained could be useful for the generation of a database of a qualitative and quantitative profile of antioxidant molecules in PDO, PGI, organic farming, and monovarietal and blend Italian extra-virgin olive oils obtained from different cultivars.

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Author's Contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Filippo Mandolfino, Fabio Salafia, and Alessandra Vilmercati.

Conceptualization: Marina Russo, Chiara Fanali, and Francesco Cacciola; Statistical analysis: Monica Casale; Writing – original draft preparation – review and editing: Marina Russo, Francesco Cacciola, Laura Dugo, Francesca Rigano, and Monica Casale; Supervision: Laura De Gara, Paola Dugo, and Luigi Mondello. The first draft of the manuscript was written by Marina Russo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest Marina Russo declares that she has no conflict of interest. Francesco Cacciola declares that he has no conflict of interest. Filippo Mandolfino declares that he has no conflict of interest. Fabio Salafia declares that he has no conflict of interest. Alessandra Vilmercati declares that she has no conflict of interest. Chiara Fanali declares that she has no conflict of interest. Monica Casale declares that she has no conflict of interest. Laura De Gara declares that she has no conflict of interest. Paola Dugo declares that she has no conflict of interest. Luigi Mondello declares that he has no conflict of interest. Francesca Rigano declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human or animal subjects.

Informed Consent Informed consent not applicable

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