Chemical and sensory differences between high price and low price extra virgin olive oils

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Abstract

The aim of the study was to identify new potential chemical markers of EVOO quality by using a multicomponent analysis approach. Sixty-six EVOOs were purchased from the Italian market and classified according to their price as low price EVOOs (LEVOOs) and high price EVOOs (HEVOOs) costing 3.60-5.90 euro/L and 7.49-29.80 euro/L respectively. Several parameters strictly related to olive oil quality have been investigated, as volatile substances, polar phenolic substances, antioxidant activity, fatty acid composition, and α-tocopherol. Significant differences in terms of chemical composition have been highlighted for the first time between the two EVOOs classes investigated. Among the most interesting outcomes, R ratio (free tyrosol and hydroxytyrosol over total free and bound forms), measuring the extent of secoiridoids hydrolysis, resulted to be significantly higher in LEVOOs than in HEVOOs. Other key differences were found in the volatile substances composition and in the stearic acid percentage; the latter resulted to be significantly higher in LEVOOs than in HEVOOs (P = 0.00013). Sensory analysis was also performed on EVOOs in order to integrate the chemical characterization thus giving a comprehensive picture of the overall quality of these oils.

Keywords

Quality marker

Polyphenols

Volatile substances

Sensory analysis

Authentication

Multicomponent analysis

1. Introduction

Currently, global olive oil supply is still concentrated in few traditional producer countries, which are also the largest consumers. Considering the mean of last three years (2014-2016) of olive oil production, the European Union (EU) is confirmed as the main olive oil market with 2,021,800 tons followed by Syria, Turkey, Tunisia, and Morocco. The top contributors in the EU result to be Spain and Italy, respectively with 60% and 20% of the total production. Furthermore, EU also represents the principal olive oil consumer with 1,759,200 tons, followed by USA, Turkey, Syria and Morocco (International Olive Oil Council (IOOC), 2015a). As such, world olive oil market is extremely complex and constantly evolving. Extra virgin olive oil (EVOO) is the top product among the olive oils. It is obtained through physical means by mechanical or direct pressing of the olives. It is not subjected to any treatment except washing, decantation, centrifugation and filtration. According to the European laws and regulations (European Commission, 2008; IOOC, 2015b) EVOO must comply with strict chemical and organoleptic parameters of quality. The final quality of the product results in special sensorial properties and in chemical features that have been intensively investigated by several scientists. Some parameters, like oil acidity or oil peroxide values, only help to do an initial screening permitting to discard oils that do not fulfil the legal limits for EVOO. They do not guarantee, however, for the quality of the oil, also because these parameters have low values even if the oil has undergone a fraudulent refining process. Instead, other parameters like phenolic or volatiles substances are much more meaningful when dealing with the assessment of the quality, but they are not regulated by legislation, there are no minimum values established for their total contents or for some individual substances. European Food Safety Authority (EFSA), anyhow, opens the possibility, for the producers or industry, of using the claim "olive oil polyphenols contribute to the protection of blood lipids from oxidative stress'' when the product contains at least 5 mg of "hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol)" per 20 g of olive oil (European Commission, 2012). By and large, the EVOO quality has a very broad range, as can be expected if considering the influence of three main factors: agronomic (state of olive grove and olive cultivars, the growing area, the fruit ripening, the cultivation techniques, the water resources, the fertilization, and the soil

management), technological (e.g. extraction system and malaxation conditions), and environmental (e.g. temperature and light during storage of olive oil) (Jolayemi, Tokatli, & Ozen, 2016; Fregapane, Gomez-Rico, Inarejos, & Salvador, 2013). To maximize the oil quality taking into account the numerous factors influencing the EVOO quality, the production costs are very high; in fact, even if EVOO has always a relatively high price, high quality EVOO, niche products, e.g. some monovarietal EVOOs, will always have much higher prices as compared to oils produced in large scale industrial plants, due to the handwork required, to the limited availability of raw material and to many other conditions required. Thus, even if EVOO retail price depends also on the market demand and can change one year from another, if a same period and a same market are considered, there will always be differences in terms of chemical composition, quality and also price between niche products and industrial products. Oil price plays an important role in the exchange relationship between the retailer and consumer. It represents one of the most determinant variables in the decision to purchase the product (Martínez, Aragonés, & Poole, 2002; Scarpa, & Del Giudice, 2004). Research focused on the consumer perception of olive oil quality revealed that consumers consider high price as an indicator of quality, often purchasing the most expensive EVOO on sale (Di Vita, D'Amico, La Via, & Caniglia, 2013).

In this contest, the present study started from the assumption that high price means high quality and low price means low quality; even if this could be not true for all of the cases, this assumption should work on the average of a high number of samples investigated. Thus for the first time it was aimed by the present study to assess the chemical and sensory differences between EVOOs found on the Italian market at different price, low price extra virgin olive oil (LEVOO) priced between 3.60 and 5.90 euro/L, on average 4.90 euro/L and high price extra virgin olive oil (HEVOO), priced between 7.49 and 29.80 euro/L and an average price of 15.30 euro/L. The focus was mainly on parameters that are in strict relation with the oil quality: volatile substances, polar phenolic substances, tocopherol, antioxidant activity, and fatty acid composition. Also samples of another commercial

olive oil category, known as “olive oil (OO): oil consisting of a blend of refined olive oil and virgin olive oil” (IOOC, 2015b), were included in the study, in order to have a reference for the very lowest quality of olive oil samples commercially available in retail. The outcome of this multicomponent approach could highlight possible new markers of EVOO quality and may help to define the relationship between quality and price in the olive oil market.

2. Materials and methods

2.1. Reagents and standards

The analytical standards of hydroxytyrosol, tyrosol, vanillic acid, oleuropein, luteolin and apigenin were purchased from Extrasynthese (Genay, France). *p*-Coumaric acid, ferulic acid, pinoresinol, syringic acid, α-tocopherol, luminol, lucigenin, DPPH, ABTS, Trolox, xanthine, xanthine oxidase and Folin Ciocalteu reagent were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade methanol, hexane and isopropanol (IPA) were purchased from Sigma-Aldrich (Milan, Italy). Water (resistivity above 18MΩ*cm) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All the solvents and solutions were filtered through a 0.45μm PTFE filter from Supelco (Bellefonte, PA, USA) before use.

2.2. Sampling

Sixty-six bottles of commercial EVOOs and ten of OOs were purchased from local shops and supermarkets during the period September 2015-September 2016 in the area around Macerata, in Marche Region (Italy) and stored away from light. EVOOs belonged to two price classes: low (LEVOOs), costing from 3.60 to 5.90 euro/L (15 samples) and high (HEVOO), from 7.49 to 29.80 euro/L (51 samples). OOs price ranged between 3.59 and 5.59 euro/L. The oils from the three classes (HEVOOs, LEVOOs and OOs, representing 67%, 20% and 13% respectively, of the total number of samples analysed), had close suggested expiring date, as resulted from the oils label.

2.3. Fatty acid composition

Fatty acid methyl esters were obtained by reacting 5 mg of the oil dissolved in hexane (1 mL) with 2N potassium hydroxide in methanol (0.1 mL) and then analysed by gas chromatography coupled with flame ionization detection under reported conditions (Venditti et al., 2017).

2.4. Volatile substances

An aliquot of 1.5 g of oil was weighted in a screw cap vial with pierceable septum, a small stirring magnet was added and the sample was conditioned at 40 °C for 10 min stirring at 300 rpm. A solid-phase microextraction fibre coated with 50/30 μm divinylbenzene/Carboxen/polydimethylsiloxane

(DVB/CAR/PDMS), 1 cm long, was then exposed to the headspace of the sample for 30 min and then the fibre was retracted and exposed in the hot gas chromatograph injector kept at 260 °C. The instrument used for the analyses is a gas chromatograph coupled with a mass spectrometer detector (Agilent 6850 GC-MSD 5973N, Agilent Technologies, Santa Clara, CA, USA) equipped with a capillary column coated with polyethylene glycol (DB-WAX, length 60 m, internal diameter 0.25 mm, film thickness 0.25 µm). The instrumental conditions applied were: splitless injection with a splitless time of 4 min, carrier gas (helium) flow was 1.2 mL min^{-1}, the initial oven temperature was 40 °C held for 4 min, then the temperature was raised to 120 °C at 2.5 °C min^{-1} and then raised to 250 °C at 15 °C min^{-1} and held for 3.33 min. The temperature of the transfer line was held at 250 °C, ion source (electron impact was at 70 eV) at 230 °C and quadrupole was at 150 °C; mass scan range was 29-400 amu. Identification of eluted molecules was performed by comparison of the experimental retention indices, calculated with reference to linear alkanes, with those reported in literature, and with comparison of the experimental mass spectra with those of the NIST 08 library.

2.5. Determination of α-tocopherol

The oil sample (100 mg) was dissolved in 5 mL of hexane and filtered through a 0.45-µm PTFE filter before HPLC-FLD (high performance liquid chromatography coupled to a fluorescence detector) analysis. The separation was performed on a Hypersil silica column (200 x 2.1 mm, 5 µm, from Thermo Fisher Scientific, Waltham, Massachusetts, United States). The mobile phase was hexane containing 0.25% IPA, at a flow rate of 0.5 mL min^{-1}. The injection volume was 10 µl. FLD was set with an excitation wavelength of 290 nm and an emission wavelength of 330 nm. For the quantification, seven standard stock solutions of α-tocopherol in hexane were prepared in the range 0.53-10.6 µg mL^{-1} and analysed to obtain the calibration curve (correlation coefficient R = 0.9992).

2.6. Assays for antioxidant activity

2.6.1. Total Antioxidant Activity (TAA)

The total antioxidant activity of different EVOOs and OOs was determined according to Pellegrini, Visioli, Buratti, & Brighenti, 2001. Briefly, this test is based on the capacity of antioxidant compounds contained in the olive oil to quench the ABTS radical cation [2,2'- azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], a blue/green chromophore that absorbs at 734 nm. The decrease of absorbance is proportional to the antioxidants present in the oil. The ABTS radical cation solution was prepared by reacting of 7 mM ABTS in water with 2.5 mM potassium sulphate; ethanol was added to reach an absorbance of 0.7 ± 0.2 at 734 nm. The TAA of oils was determined on oils diluted 1:4 with hexane (30 μl of olive oil mixed with 90 μl of hexane). Two mL of the ABTS solution was mixed with 10 μl of diluted olive oil and incubated at room temperature in the dark for 10 min and then read in a spectrophotometer at 734 nm and at 30 °C. Appropriate solvent blanks were prepared for each assay. The absorbance decrease was referred to the calibration curve obtained in the presence of known concentrations of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a vitamin E analogue, stable antioxidant widely used as an index of antioxidant activity. Data are reported as concentration of Trolox (μM).

2.6.2. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) assay

The method reported by Zullo & Ciafardini (2008) was used to perform 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. This assay determines the abilities of antioxidants to scavenge the DPPH and is evaluated by spectrophotometric technique by measuring the decrease of absorbance of DPPH solution at 517 nm. Briefly, 200 μl of olive oil is mixed with 600 μl of methanol, vortexed, then centrifuged for three minutes and the upper methanolic phase is separated. One hundred μl of this methanolic extract is mixed with 2 mL of 3 mM DPPH dissolved in methanol, and after 5 min of incubation at room temperature in a dark place the mixture absorbance was read in the spectrophotometer at 517 nm. The results were compared to the DPPH radical-scavenging activity of standard concentrations of Trolox. DPPH assay of different olive oil samples was reported as concentration (μM) of Trolox.

2.6.3. Chemiluminescence assays

The antioxidant activity of the different olive oils samples was analysed by chemiluminescence technique using two specific chemiluminogenic probes, lucigenin and luminol, that are sensitive to superoxide anion and hydrogen peroxide, respectively (Gabbianelli, Santroni, Kantar, & Falcioni, 1994; Murphy & Sies, 1990).

The signal of chemiluminescence is due to the reaction of lucigenin with superoxide anion produced by the xanthine/xanthine oxidase system and it is reduced by the presence in the assay of an antioxidant that reacts with superoxide anion. Using luminol, the signal of chemiluminescence is due to the reaction of the probe with hydrogen peroxide and the decrease of the signal in the presence of olive oil is indicating its antioxidant activity versus hydrogen peroxide.

Lucigenin-amplified chemiluminescence was measured in 1 mL of 50 mM Tris buffer pH 7.4 containing 0.1 U mL^{-1} xanthine oxidase, 150 μM lucigenin and 30 μl of olive oil. The reaction was started by injecting xanthine at a final concentration of 50 μM. The antioxidant capacity of the bioactive compounds contained in the olive oil was compared to the ng of superoxide dismutase (SOD) required for a radical-scavenging activity. Data are expressed as ng of SOD corresponding to 30 μl of olive oil requested to scavenger the superoxide production.

Luminol-amplified chemiluminescence was measured in 1 mL of 50 mM Tris buffer pH 7.4 with 0.1 mM luminol and 30 μl of olive oil. The reaction was initiated by injecting hydrogen peroxide at the final concentration of 50 mM. Chemiluminescence was measured in an Autolumat LB953 (Berthold Co. Wildbad, Germany). The antioxidant capacity of the bioactive compounds contained in the olive oil was compared to the mg of catalase (CAT) required for the radical-scavenging activity. Data are expressed as mg of CAT corresponding to 30 μl of olive oil requested to scavenger the hydrogen peroxide.

2.7. Folin-Ciocalteu assay

The analyses were performed following the procedures reported in Ricciutelli et al. (2017). Briefly, 2.5 g of oil were dissolved in 2.5 mL of hexane and then extracted three times for 20 min under magnetic stirring with 2.5 mL of methanol-water 80:20 *v/v*. The supernatants were collected, washed with 2 x 5 mL of hexane and stored in a 50 mL volumetric flask. An aliquot of 2.5 mL of FC reagent and 2.5 mL of saturated sodium carbonate solution were added and the solution was brought up to a volume of 50 mL with distilled water. After 120 min of reaction at ambient temperature in dark, the absorbance was measured at 765 nm in a UV–Vis spectrophotometer (Perkin-Elmer, Waltham, Massachusetts, United States). Total phenolics content was calculated and expressed as mg of gallic acid equivalent kg^{-1} of oil.

2.8. Quantification of olive oil polyphenols by HPLC-DAD-ESI/MS

The analyses were performed following the procedures reported in Ricciutelli et al. (2017). Briefly, 5 g of oil were dissolved in 5 mL of hexane and extracted in a separating funnel with 4 x 5 mL of methanol:water (60:40, v/v). The methanolic extracted solutions were collected, evaporated to dryness and reconstituted with 2.5 mL of HPLC-grade methanol before HPLC-DAD-ESI/MS analysis.

HPLC-DAD-ESI/MS (ion trap) studies were performed using an Agilent 1100 (Santa Clara, CA, USA) with a diode–array detector (DAD) and a mass spectrometer detector (ion trap) equipped with an electrospray ionization (ESI) source. The separation was achieved on a Synergi Polar analytical column. The mobile phase was water (A) and methanol/*i*PrOH 90:10 v/v (B) both containing 0.1% formic acid, working in the gradient mode. HPLC-DAD analysis was performed monitoring different wavelengths: 260 nm for vanillic acid, 280 nm for hydroxytyrosol, tyrosol, and secoiridoids derivatives, pinoresinol, acetoxypinoresinol and syringic acid; 310 nm for *p*-coumaric acid, 325 nm for ferulic acid, 338 nm for apigenin and 350 nm for luteolin. In HPLC-ESI–MS, ion source was operated in negative ionization (NI) mode and mass analyser in Full scan mode. Mass scan range was

set in the range of m/z 70–1100 and extract ion chromatograms (EICs) from total ion chromatogram (TIC) were used for analytes quantification.

2.9. Acidity and peroxide value

The determination of olive oil acidity (expressed as oleic acid g in 100 g olive oil) and peroxide value (expressed as milliequivalents of active oxygen per kg of oil) were carried out according to the EC Reg. n. 2568/1991 and IOOC standard methods (European Commission, 1991).

2.10. Sensory analysis

Sensory analysis was performed on HEVOOs and LEVOOs by a panel acknowledged by IOOC in 2000, and according to the procedure reported by Cecchi and Alfei (2013).

2.11. Statistical analysis

Data were subjected to statistical analysis in order to assess significant differences between the oil groups investigated by means of one-way analysis of variance (ANOVA) and Tukey's pairwise test using the software PAST (Hammer, Harper, & Ryan, 2001). Principal Component Analysis was performed by using software R (R Core Team, 2013).

3. Results and Discussion

3.1. Fatty acid composition

Fatty acid composition is a nutritional feature of undiscussed importance when dealing with the assessment of the quality of an oil. Fatty acids, in bound form of acylglycerols, represent the major fraction of an oil and the typical composition in olive oil is represented mainly by oleic acid, usually being 70-83% of the total fatty acid composition. The presence of oleic acid in such a high percentage in the fatty acid composition has made olive oil one of the key ingredients explaining the health benefits given by the Mediterranean diet. Average fatty acid compositions of high price extra virgin

olive oils (HEVOOs), low price extra virgin olive oils (LEVOOs) and olive oils (OOs) investigated are reported in **Table 1**. For all of the fatty acids considered, with the only exception of stearic acid, there are no significant differences ($P < 0.05$) between HEVOOs and LEVOOs, but only between EVOOs (both HEVOOs and LEVOOs) and OOs, as could be expected since the composition of OO, made up of virgin and refined olive oils (IOOC, 2015b), reflects mainly the composition of the large portion of refined olive oil present. Instead, the result obtained for stearic acid deserves particular interest, showing that its percent content in HEVOOs, resulting to be on average 2.32% in the different 51 samples analysed, is significantly lower ($P = 0.00013$) than the content in LEVOOs, on average 2.75% in the different 15 samples analysed. The content in OOs, on average 2.71% in the 10 different samples investigated, is not significantly different than that found in LEVOOs ($P > 0.05$). **Fig. 1** shows the percentage of stearic acid in the 76 oils samples in ascending order. The 10 oils having the highest percent content of stearic acid (higher than 2.77%) are 4 OOs and 6 LEVOOs. Instead, the 10 oils having the lowest percent content of stearic acid (below 2.137%), are 10 HEVOOs. To explain the trend found for stearic acid relative content, two main reasons could be considered: the compositional changes occurring during olives ripening, and the influence of pedoclimatic growing conditions and genetic characteristics of the cultivars, that are probably different between HEVOO and LEVOO, also taking into consideration that HEVOOs were all Italian oils, while LEVOOs were mostly oils from EU or non EU countries. Regarding fatty acid compositional changes occurring during ripening, even if not for all olive cultivars, it has been reported for several olive varieties that the percent content of stearic acid increases during ripening (Jolayemi, Tokatli, & Ozen, 2016; Salvador, Aranda, & Fregapane, 2001; Beltrán, Del Rio, Sánchez, & Martínez, 2004) and it is well known that the use of too ripen olives leads to a low quality oil. It is interesting to observe that stearic acid seems to discriminate more between HEVOOs and LEVOOs than oleic acid does, or than the ratio oleic acid / linoleic acid does, parameters which are well known to be associated with the quality of an olive oil (Rotondi, Bendini, Cerretani, Mari, Lercker, & Toschi, 2004). In fact, even if

the average percentage of oleic acid was higher in HEVOOs than in LEVOOs (and in OOs), the differences were not significant in this case ($P > 0.05$).

3.2. Volatile substances

Volatile substances are key compounds affecting the sensorial properties of any food. In EVOOs they confer green, fruity notes, mainly deriving from compounds formed in the lipoxygenase (LOX) cascade and that the consumer can appreciate particularly in fresh oils produced from healthy olives at the proper ripening degree and under proper processing conditions (Kalua, Allen, Bedgood, Bishop, Prenzler, & Robards, 2007). However, also several degradation processes (lipid auto-oxidation, aminoacid metabolism, sugar fermentation, etc.) lead to the production of volatile molecules that on the contrary can confer defects to the oil or in any case characterise an oil that is undergoing degradation (Angerosa, 2002). In the present study, volatile composition has been determined by means of HS-SPME-GC-MS. The oil classes HEVOO, LEVOO and OO have been compared in terms of relative composition of some selected volatile molecules and in terms of their relative content (**Fig. 2**, **Table 1**). Among the selected molecules some are known to be in relation with positive notes, like C6 compounds and some are known to be related with negative attributes, like ethanol (winey), acetic acid (fusty, vinegary), octane (fusty) (Angerosa, Lanza, & Marsilio, 1996). The content of volatile compounds known to characterise high quality oils were actually found in significantly higher amounts in HEVOOs as compared to LEVOOs, confirming also the initial assumption made in this study: HEVOOs are supposed to have higher quality than LEVOOs. Particularly, volatile substances found in significantly higher amount ($P < 0.05$) in HEVOOs than in LEVOOs, among those considered (**Table 1, Fig. 2**), were: 2-(*E*)-hexenal, pentene dimers, 1-penten-3-one, and 1-penten-3-ol. All of them are formed in the LOX pathway. 2-(*E*)-hexenal is generally the most abundant volatile compound found in high quality oils where it confers bitter almond and green notes. The quantity of pentene dimers has been associated with EVOOs quality and shown to correlate with specific sensorial attributes; 1-penten-3-one (together with secoiridoids), has been shown to correlate with

bitter and pungent sensations (Angerosa, Mostallino, Basti, & Vito, 2000), even if the isolated molecule resulted to give sweet and strawberries notes (Aparicio, Morales, & Alonso, 1997). Peak area percentages of the volatiles detected were also calculated to have an approximate idea of the volatile substances percentage composition. The comparison of the percentage composition also showed significant differences between the different oil classes investigated. **Fig. 2b** highlights clear differences: moving from HEVOOs to LEVOOs and then to OOs there is a gradual decrease of the percentage of compounds known to be associated with positive attributes (e.g. 2-(*E*)-hexenal) and an increase of the percentages of molecules known to be associated with negative notes (e.g. ethanol, octane, and acetic acid). It is interesting to observe that while the highest percentage of 2-(*E*)-hexenal is found in HEVOOs and the lowest percentage is found in OOs, for hexanal percentage the trend is opposite. This finding is in agreement with results reported by Morales, Luna, & Aparicio (2005), where olive oils characterised by the negative sensory note mustiness–humidity contained higher hexanal than 2-(*E*)-hexenal, while in good EVOOs the opposite was true. This could be explained considering the formation pathways of these molecules: 2-(*E*)-hexenal is mainly formed in the lipoxygenase cascade, and in general it is more abundant in good oils while its content is lower in oils produced with olives undergone degradation processes or in oils that have lessened their quality, for example during storage (Morales et al., 2005, Angerosa, 2002, Cavalli, Fernandez, Lizzani-Cuvelier, & Loiseau, 2004). Regarding hexanal, even if it is also an intermediate in the lipoxygenase cascade, it is among the main products formed during lipid autoxidation, thus its content increases during storage (Kalua et al., 2007). Furthermore, it has been reported that a decrease in 2-(*E*)-hexenal content was found during storage of oils (Cavalli et al., 2004). It is well known that ethanol, acetic acid and octane are molecules associated with undesirable processes occurring in the olives or in the oil. The result about 3-(*Z*)-hexen-1-yl acetate is unclear since this molecule is known to be formed in the LOX pathway and to give positive notes of green leaves and green banana (Kiritsakis, 1998) and thus generally associated with good oils. However, it could be plausible that this molecule could result also from a condensation between 3-(*Z*)-hexen-1-ol and acetic acid occurring during oil storage. It

has been reported in fact that 3-(*Z*)-hexen-1-ol content can increase during storage (Cavalli et al., 2004) and thus also the content of 3-(*Z*)-hexenyl acetate may increase if the content of acetic acid is also high, as can be in a low quality oil.

3.3. α-Tocopherol

Tocopherols and particularly α-tocopherol, representing about 95% of the total of tocopherols in olive oils, are very important molecules effectively inhibiting lipid oxidation in foods and biological systems (Kamal-Eldin & Appelqvist, 1996). Major dietary sources of tocopherols are vegetable oils to which they confer protection towards oxidation and nutritional value, thus contributing to the overall quality of the oil (Zhang et al., 2016). In the present study α-tocopherol was determined in the oil categories investigated; in HEVOOs the average content found was 229.25 mg kg^{-1}; in LEVOOs it was 224.06 mg kg^{-1} and in OOs it was 148.44 mg kg^{-1} (**Table 1**). Comparable levels of α-tocopherol (227.3 mg kg^{-1}) were found by Caporaso, Savarese, Paduano, Guidone, De Marco, & Sacchi (2015) in EVOOs from the Italian retail market.

In the present study, it resulted that there are no significant differences in its content between HEVOOs and LEVOOs, but only between EVOOs and OOs ($P < 0.0005$). Thus, α-tocopherol cannot be considered as discriminant marker of EVOO quality, and this is also in agreement with Inajeros-Garcia, Santacatterina, Salvador, Fregapane, & Gomez Alonso (2010) who reported that the concentration of tocopherols did not differ significantly between virgin and extra virgin olive oil and that fruit damage or extraction conditions responsible for the sensory defects did not significantly affect the α-tocopherol content in the oil.

The average content of α-tocopherol found in OOs is 65% of that found in EVOOs in the present study. The reduction of α-tocopherol in OOs with respect to EVOO is about ten times lower than that found for hydrophilic polyphenols, whose content in OOs results to be 6.8% as compared to their content in EVOOs. In fact it has been reported that olive oil hydrophilic phenolic substances are almost completely lost during oil refining (García, Ruiz-Méndez, Romero, & Brenes, 2006), and thus

their low content found in olive oils derive mostly from the very little fraction of virgin olive oil mixed with the refined olive oil. Instead tocopherols get lost only to a partial extent during oil refining (Ergönül & Köseoğlu, 2014) and thus their content remains significant also in OOs.

3.4. Antioxidant activity

The total antioxidant activity (TAA) is an important parameter correlated with the presence in olive oils of phenolic compounds able to scavenge the radical cations. The DPPH assay considers the resistance of olive oil compounds to the peroxyl radical action. Our results demonstrated that both TAA and DPPH assays parameters increase in the order OO<LEVOO<HEVOO (Table 1). These results are in agreement with the total phenolics content found in the different olive oil classes as resulted from the Folin-Ciocalteu test. HEVOOs gave significantly higher response than LEVOOs and OOs with the exception of DPPH assay where the difference between HEVOOs and LEVOOs is not significant ($P > 0.05$). In each of the three methods, LEVOOs gave significantly higher response than OOs. The results obtained with chemiluminescence tests instead have shown that there is an increase in the antioxidant activity versus superoxide and hydrogen peroxide of HEVOO and LEVOO with respect to OO but there is not significant difference between LEVOO and HEVOO. This behaviour maybe partially related with the α-tocopherol content measured in the three olive oil categories demonstrating that is the α-tocopherol the main antioxidant compound contained in the oil samples able to scavenge the superoxide and peroxide radicals. These outcomes are in agreement with previous studies where chemiluminescence assay was able to measure antioxidant capacity in synthetic vitamin E analogous (i.e. Trolox, nitroxides etc.) (Gabbianelli, Falcioni, Lupidi, Greci, & Damiani, 2004).

3.5. Polyphenols

In the present study major hydrophilic olive oil polyphenols have been quantified in HEVOOs, LEVOOs, and OOs by HPLC-DAD/MS (Ricciutelli et al., 2017). The results obtained are reported

in **Table 1.** OOs contained on average 22 mg kg^{-1} of total hydrophilic polyphenols: 6.8% of the total average amount found in EVOOs (323 mg kg^{-1}). The total average content of hydrophilic phenolic substances was 341 mg kg^{-1} in HEVOOs and it was significantly lower in LEVOOs (263 mg kg^{-1}). Among the 51 HEVOO samples analysed, 36 complied with the concentration required to acknowledge the health claim (European Commission, 2012); while within LEVOO samples, 6 out of 15 samples complied with the required concentration (250 mg kg^{-1}). The percentage of samples having the level of polyphenols sufficient to acknowledge the health claim is higher as compared to the percentage reported by Caporaso et al. (2015). However, besides a certain degree of variability that can be expected due to several reasons (production year, characteristics of the specific market area, origin of the samples and also the analytical method used), the difference can be explained also by considering that in the mentioned study, most of the oils investigated were from EU countries and only a small percentage of the samples was labelled as 100% Italian, while in the present study a much higher percentage of oils was 100% Italian, provided by small Italian producers, having much higher production costs but also conditions allowing to provide a much higher final quality of the product. This is also in agreement with the findings of the mentioned study (Caporaso et al. 2015), reporting that oils labelled as 100% Italian had a higher average value of polyphenols. .

Regarding the individual phenolics quantified, almost all of them resulted to be significantly higher in HEVOOs as compared to LEVOOs, with the only exceptions of *p*-coumaric acid, tyrosol and hydroxytyrosol, present in significantly higher amount in LEVOOs than in HEVOOs. The explanation for tyrosol and hydroxytyrosol can be given by their origin: they mainly derive from the hydrolysis of secoiridoid derivatives, thus the content of their free forms can be inversely related with the freshness of the oil or of the olives and thus a higher value in lower quality oils could be expected (Servili & Montedoro, 2002). This trend lead us to evaluate another parameter which could give more information than free tyrosol and free hydroxytyrosol alone, the ratio R:

$$R = \frac{(\text{free tyrosol} + \text{free hydroxytyrosol})}{(\text{free tyrosol} + \text{free hydroxytyrosol} + \text{secoiridoid derivatives})}$$

This, similarly to the oil acidity value, can be inversely related to the quality of the oil. This parameter permits a better discrimination between HEVOOs and LEVOOs. In fact, the content of tyrosol and hydroxytyrosol in HEVOO is lower than that in LEVOO with a confidence coefficient of 98.3% (P = 0.017) and 99.4% (P = 0.006), respectively, while the ratio R is lower in HEVOO than in LEVOO with a much higher confidence coefficient: 99.97% (P = 0.0003). Low values of this ratio are probably in relation with the high quality of the product. **Fig. 3** shows the analysed oil samples ordered increasingly on the base of the R values computed on each of them. If we set as threshold value R=0.0635, the oils are divided in two equal groups: the one with R value below the threshold is composed by 95% of HEVOOs and 5% of LEVOOs, the other group is composed by 40% of HEVOOs, 34% of LEVOO and 26% of OOs. To provide quantitative results on a possible prediction of quality, and to be on the safe side, the extremes of the plot can be considered: the first and the last tertiles, where the probability of finding high quality oils will be very low and very high respectively. In fact in the first tertile (corresponding to oils having R value below 0.039) only one oil is a LEVOO and the other 24 are HEVOOs, and in the last tertile (where oils have R values above 0.096) only 8 are HEVOOs (out of the 51 HEVOOs analysed), and the other 17 oils are LEVOOs and OOs. It can be concluded that an EVOO with an R value below 0.04 could be probably an oil having high quality, and an EVOO with an R value above 0.1 will be much probably an oil having low quality. Taking into consideration the study of Fregapane et al. (2013), where the extent of secoiridoid hydrolysis has been demonstrated to be associated with the freshness of the oil, our results suggest that LEVOOs could be, in many cases, old EVOOs.

The content of *p*-coumaric acid is significantly higher in LEVOOs than in HEVOOs, however, the result cannot be easily explained. It has been reported that *p*-coumaric acid content in the oil increases, for some cultivar, with the increasing malaxation temperature from 27 °C to 37 °C and to 47 °C

independently of an early harvest, a mid harvest or a late harvest of the olives (Jolayemi et al., 2016). It is well known that the increasing malaxation temperature leads to a decrease of the oil quality. Regarding the effect of ripening, it is reported that in some cases *p*-coumaric acid content increases during olive ripening and in some others it decreases (Yildirim et al., 2016), depending on the cultivar. Thus the results concerning *p*-coumaric acid content seem to be more explained by the technological process rather than the ripening degree of the olives.

3.6. Sensory analysis

Extra virgin olive oil is a food product for which not only chemical parameters but also sensory characteristics must comply with values established by the regulation (European Commission, 2008). Sensory analysis should be performed by an officially recognized panel,following specific criteria and evaluating positive and negative attributes of the oil, allowing to establish if the oil can be classified as EVOO. The results from sensory analysis performed on EVOOs investigated in the present study are reported in Table 2. Among HEVOOs, X out of Y, and among LEVOOs, X out of Y LEVOOs resulted to have some defects, and thus they actually did not comply with the quality level requested for EVOOs. The overall score given to each oil, that is a measure of all the positive attributes, was on average …for HEVOOs and …for LEVOOs and it resulted significantly higher (P <…) in HEVOOs than in LEVOOs, confirming the highest level of sensory quality of the first. It is interesting to observe that…

4. Chemometric analysis

Principal component analysis (PCA) is a useful tool for purposes of authentication or quality assessment of olive oil (Sinelli, Cosio, Gigliotti, & Casiraghi, 2007; Mildner-Szkudlarz & Jelen, 2008). The whole results obtained in the present study were summarised by means of a PCA performed with all the variables investigated in the study (**Fig. 4**). Even if with some overlappings, HEVOOs and LEVOOs occupy different regions in the principal components space, clearly showing

the difference expected also in terms of quality between them. The area occupied by LEVOOs is characterised by oils with chemical features known to characterize low quality (like higher peroxide value, higher percent acidity), but also by chemical features for the first time associated with poor quality oils, like higher stearic acid percentage and higher content of *p*-coumaric acid. R ratio (free tyrosol + free hydroxytyrosol) / (free tyrosol + free hydroxytyrosol + secoiridoid derivatives), known to be dependent on EVOO age (Servili & Montedoro, 2002), resulted also to strongly discriminate between HEVOOs and LEVOOs.

Conclusion

The present study aimed to get an overview of the chemical differences between extra virgin olive oils found on the Italian market at very different prices, assuming that the price is, on average, generally proportional to the quality; thus the final aim was to highlight possible new markers of EVOOs quality. The obtained results allowed to identify the R ratio (free tyrosol and hydroxytyrosol over total free and bound forms) as most discriminating parameter for the quality, where values above 0.1 were found for the majority of LEVOOs and OOs and values below 0.04 were found for HEVOOs (with the only exception of one LEVOO). The findings presented can provide useful tools when dealing with the assessment of the quality or authenticity of an olive oil and may contribute to define the relationship between quality and price in the olive oil market.

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