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Antioxidant potential of extra virgin olive oils extracted from three different varieties cultivated in the Italian province of Reggio Calabria

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Summary

In this study, the physicochemical properties and bioactive compounds of olive oils from cultivars "Roggianella", "Sinopolese" and "Ottobratica", grown in the province of Reggio Calabria (Italy) have been evaluated.

Polyphenols are a large family of compounds found in fruits and vegetables, which exhibit strong antioxidant activity by scavenging different families of Reactive Oxygen Species (ROS).

Dialdehydic form decarboxymethyl oleuropein aglycon, hydroxytyrosol acetate, dialdehydic form oleuropein aglycon, pinoresinol, 1-acetoxypinoresinol, tyrosol and vanillic acid were the main phenolic compounds in all samples analyzed. Pinoresinol was the most abundant compound in the lignin fraction. In all oil samples analyzed the highest antioxidant capacity was attributed to Roggianella oil (36.85 % I of DPPH and 4.07 % I of ABTS) compared to Ottobratica (27.37 % I of DPPH and 2.52 % I of ABTS) and Sinopolese (18.33 % I of DPPH and 1.72 % I of ABTS). The main characteristics of the Roggianella cultivar were a very high concentration of total phenols (530 mg/kg of gallic acid) and α-tocopherol (211 mg/kg).

Keywords*: Olea europaea*; extravirgin olive oil; phenolic compounds; antioxidant activity.

Introduction

The olive tree (*Olea europaea* L.) is one of the most important crops in Mediterranean countries, especially Italy, Spain and Greece. Extra virgin olive oil shows very interesting nutritional and sensorial properties (Visioli and Galli, 1998; Huang et al., 2008; Urpi-Sarda et al., 2012).

Extra virgin olive oil resists oxidation due to its composition. Apart from the saponifiable fraction, composed mainly of triglycerides, with a low polyunsaturated fatty acid content, it contains a group of antioxidant molecules made up mainly of polyphenols and tocopherols (Coetesi et al., 1995; Tsimidou, 1998; Visioli and Galli, 1998; Esti et al., 1998; Velasco and Dobarganes, 2002; Lavelli and Bondesan, 2005; Bendini et al., 2007; Sicari et al., 2009; Sicari et al., 2010; Giuffrè et al., 2010).

The polar phenolic molecules of virgin olive oil belong to different classes: phenolic acids, phenolic alcohols, lignans, secoiridoids and flavonoids (Angerosa and Di Giovacchino, 1996; Brenes et al., 1999; Owen et al., 2000a; Owen et al., 2000b; Gomez-Alonzo et al., 2002; Lavelli and Bondesan, 2005).

Of the antioxidant compounds, it is also important to consider the tocopherols. These are compounds whose concentration strongly depends on the variety of the plant. Usually, values found in extra virgin olive oil range from 100 mg to 300 mg/kg.

The concentration of phenols in virgin olive oil is strongly affected by agronomic factors, such as the area of origin, the cultivar, the stage of fruit ripening and also by several agronomic procedures and the technological, operative conditions of the oil extraction process (Morellò et al., 2004; Bruni et al., 1994; Servili et al., 2004; Cerretani et al., 2005; Inglese et al., 2009; Suthawan and Alyson, 2012; Di Vaio et al., 2013).

For this reason, differences in the chemical make-up of the oil are inevitable, especially as regards the minor polar compounds (antioxidants).

Phenolic compounds are responsible for the bitterness and pungency of extra virgin olive oil (Angerosa and Di Giovacchino, 1996; Andrewes et al., 2003; Kalua et al., 2005; Kalua et al., 2006). The dialdehydic form decarboxymethyl ligustroside aglycon (p-HPEA-EDA) is the compound which is most responsible for the pungent taste (Beauchamp et al., 2005; Kalua et al., 2005).

These compounds are related to the health benefits associated with the consumption of olive oil (Visioli and Galli, 1995; Visioli et al., 1995; Visioli et al., 1998; Petroni et al., 1994; Manna et al., 1999; Tuck and Hayball, 2002; Psaltopoulou et al., 2004; Franconi et al., 2006).

In the present work, the chemical-physical properties, tocopherols, total phenols and the phenolic fraction of three Italian cultivar extra virgin olive oils has been evaluated.

Materials and methods

Plant material and growing areas selected

The study was carried out on the three different cultivars (Ottobratica, Sinopolese and Roggianella), grown in the experimental orchards belonging to the Calabrian regional government, located near Gioia Tauro in the province of Reggio Calabria, Italy.

The agronomic processes carried out were the same for each cultivar: fertilizing, spraying for parasites, and pruning. The olives were harvested by hand in mid-October 2015 directly from the plant, and immediately taken to the laboratory, where the oil was extracted within 12 hours of harvesting, using a laboratory "Mini 30" made by Agrimec (Firenze, Italy).

After crushing and malaxing of the olive paste, oil extraction was performed at room temperature by means of a pressure system. The olive paste was mixed for 30 min, and then a pressure of 200 atm was applied for 40 min. The oil was separated from the water (by centrifuge), then filtered through a paper at room temperature and analyzed immediately.

Reagents

Gallic acid (GA), p-hydroxyphenyl-ethanol, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, o-coumaric acid, apigenin, luteolin and oleuropein were from Extrasynthese (France). α, β and γ-tocopherol was from Sigma-Aldrich (Italy). All reagents used of HPLC or analytical grade from Carlo Erba (Milan, Italy).

Peroxide, free acidity and Rancimat test

Quality parameters (peroxide value, acidity value and UV absorption characteristic) were evaluated following the methodology proposed by Reg. (CEE) 2568/91, and later modifications of the Reg. (CEE)

2003. Oxidative stability was carried out using a Rancimat apparatus. The temperature was maintained at 110 °C during analysis, and airflow was 12 l/h. The analyses were carried out in triplicate.

Tocopherol analysis

The tocopherol contents were estimated with the IUPAC 2.432 method (IUPAC, 1987). Analytical HPLC was conducted using a Knauer liquid chromatography (Asi Advanced Scientific Instruments, Berlin - Germany) and a UV-VIS a diode array detector (DAD). A normal phase column Lichrosphere Si60 (250 mm length, 4.6 mm i.d. and 5 µm particle size) was used with an injection volume of 20 µl and a flow rate of 1.0 ml/min. The absorbance was measured at 295 nm. The results were expressed as mg of tocopherol per kg of oil. Tocopherols were quantified by an external standard method.

Determination of total phenols and phenol content by HPLC

The total phenols were determined using the Folin Ciocalteu's reagent method (SINGLETON and ROSSI, 1965). Briefly, 10 ml of sample were taken from the solution and placed into a flask of 100 ml, to which were added 50 ml of ultrapure water, 5 ml of Folin Ciocalteu's reagent, 20 ml of Na_2CO_3 (15% in water) and finally made up to 100 ml with ultrapure water. The flask was left in the dark for 2 hours and then the solution was subjected to spectrophotometric analysis at 750 nm, with a Perkin Elmer apparatus (Model Lambda 2).

Phenolic compounds in different olive varieties were determined by HPLC (PIRISI et al., 2000). The HPLC analysis was performed using a Knauer instrument (Asi Advanced Scientific Instruments, Berlin - Germany), and a UV-VIS diode array detector (DAD).

The column used was a Nova-Pak C-18 with the following features: 300 mm length, 3.9 mm i.d., and 4 µm particle size (Waters – Italy). The injection volume was 10 µl of the phenolic solution. Two mobile phases were used, the first (A) water/acetic acid (98:2, v/v) and the second (B) methanol/acetonitrile (1:1, v/v), with a flow rate of 1 ml/min.

The gradient used was as follows: 0-25 min 95% A/5% B; 25-35 min 70% A/30% B, 35-40 min 60% A/40% B, 40-50 min 52% A/48% B; 50-55 min 30% A/70% B; 55-60 min 0% A/100% B; 60-65 min 95% A/5% B. The monitoring wavelength was 280-320 nm. The identification of each compound was based on a combination of retention time and spectral matching.

Antioxidant activity

Total antioxidant activity was estimated by ABTS (radical scavenging) and DPPH (Free radical scavenging activity). The radical scavenging capacity of the samples for the ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate) radical cation was determined as described by RE et al. (1999). ABTS (7 mM) and $K_2S_2O_8$ (140 mM) were mixed and stored in the dark at room temperature for 16 h before use. The mixture was diluted (1:80) with ethanol to give an absorbance at 734 nm using a spectrophotometer. For each olive oil sample, a diluted methanol solution of the sample (100 µl) was allowed to react with fresh ABTS solution (900 µl), and then the absorbance was measured 6 min after initial mixing. All measurements were performed in triplicate.

Radical scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by BLOIS (1958), with some modifications: for each olive oil, 1.5 ml was mixed with 1.5 ml of a 0.2 mM methanolic DPPH solution. After an incubation period of 30 min at 25 °C, the absorbance at 515 nm was measured. The free radical-scavenging activity of each solution was then calculated as percentage inhibition. All measurements were performed in triplicate.

Statistical analysis

All parameters analyzed were carried out in triplicate. The results were reported as mean values of three repetitions and standard deviation. The data were subjected to analysis of variance (one-way ANOVA) using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). Separation of the means was obtained using Tukey's test, and significant difference was defined as ($P \leq 0.05$).

Results

The mean values obtained for the different parameters analyzed in the fruit are shown in Tab. 1 and 2. The analyzed olive oils (Tab. 1) showed good values for the regulated physicochemical parameters evaluated. Free acidity is measured as free fatty acids expressed as a percent of oleic acid and should be ≤ 0.8%; peroxides are expressed as milliequivalent of free oxygen per kilogram of oil ≤ 20 meq O_2/kg. The quality parameters did not exceed the limits established for the best commercial quality olive oil, designated as "extra virgin olive oil category", by CEE (1991/2568) and later modifications of the CEE (2003).

The lowest values of acidity were those of the cultivar Sinopolese (0.28%), while those of Ottobratica and Roggianella were 0.31 and 0.44%, respectively.

As shown in Tab. 1, the oils showed peroxide values of 3.85, 4.32 and 5.03 meq O_2/kg of oil in Roggianella, Ottobratica and Sinopolese, respectively.

The amounts of total phenols in analyzed oils show significant differences between different cultivars (Tab. 1). The highest content of

Tab. 1: Levels of total phenols, tocopherols, antioxidant activity and Rancimat test of extra virgin olive oil from three calabrian cultivars

	Sinopolese	Roggianella	Ottobratica	Sign. P*<0.05*
Acidity (AV) (% oleic acid)	0.28±0.03^b	0.44±0.01^a	0.31±0.12^b	**
Peroxide (PV) (m Eq O_2/kg)	5.03±0.14^b	3.85±0.56^a	4.32±0.15^b	**
Total phenols (mg/kg of gallic acid)	367.43±7.07^b	530.14±8.41^a	417.74±5.68^c	**
α-tocopherol (mg/kg)	145.21±4.5^b	235.51±7.67^a	177.59±8.04^b	*
(β + γ) tocopherols (mg/kg)	29.44^b	51.26^a	37.16^c	**
Total tocopherols (mg/kg)	174.65	286.77	214.75	
DPPH° (% I)	18.33±2.83^a	36.85±3.25^c	27.37±2.09^b	**
ABTS° (% I)	1.72±0.03^b	4.07±0.14^a	2.52±0.02^b	*
RANCIMAT 110 °C (h)	18.76	27.45	21.33	**

Data are expressed as mean ± SD from three replications. Values having different superscripts are significantly ($p < 0.05$)

Tab. 2: Phenolic compounds in extra virgin olive oil obtained from the cultivars Sinopolese, Roggianella and Ottobratica

Compounds	Sinopolese mg/kg	Roggianella mg/kg	Ottobratica mg/kg	Sign. $p<0.05$
vanillic acid	8.44±0.71^{b}	7.05±0.37^{b}	14.17±0.89^{a}	**
4-hydroxybenzoic acid	0.91±0.17^{b}	0.65±0.04^{b}	1.76±0.25^{a}	**
p-coumaric acid	7.27±0.25^{b}	7.18±0.40^{b}	14.39±2.30^{a}	**
o-coumaric acid	1.24±0.15^{b}	2.48±0.38^{a}	0.65±0.07^{b}	**
ferulic acid	2.51±0.40^{b}	0.48±0.15^{c}	4.60±0.60^{a}	**
cinnamic acid	11.62±0.94^{b}	19.79±1.64^{a}	14.35±1.31^{b}	**
hydroxytyrosol (3,4-dihydroxyphenyl-ethanol)	2.57±0.47^{a}	3.03±0.34^{a}	3.28±0.22^{a}	n.s.
tyrosol (p-hydroxyphenyl-ethanol)	12.95±0.95^{b}	10.62±0.39^{c}	15.39±1.09^{a}	**
(+) 1-acetoxypinoresinol	18.14±0.23^{b}	24.45±0.56^{a}	23.02±1.41^{c}	**
(+) pinoresinol	58.87±2.32^{b}	51.31±0.79^{c}	88.49±3.55^{a}	**
dialdehydic form decarboxymethyl oleuropein aglycon (3,4 DHPEA-EDA)	50.43±2.51^{b}	48.91±2.41^{b}	58.72±2.23^{a}	**
dialdehydic form decarboxymethyl ligustroside aglycon (p-HPEA-EDA)	7.46±0.34^{b}	8.57±0.25^{a}	7.98±0.14^{b}	**
hydroxytyrosol acetate	21.36±0.50^{b}	32.99±1.23^{a}	7.31±1.03^{c}	**
oleuropein aglycon	0.21±0.03^{c}	0.72±0.10^{a}	0.53±0.01^{b}	**
dialdehydic form oleuropein aglycon	8.20±0.33^{b}	20.53±0.55^{a}	4.77±0.19^{c}	**
dialdehydic form ligstroside aglycon	1.78±0.12^{a}	1.93±0.14^{a}	1.56±0.28^{a}	n.s.
apigenin	0.90±0.13^{b}	1.97±0.18^{a}	0.24±0.03^{c}	**
luteolin	1.54±0.51^{b}	2.74±0.19^{a}	1.13±0.25^{b}	**
Phenolic acids (mg/kg)	31.99	37.63	49.92	
Phenolic alcohols (mg/kg)	15.52	13.65	18.67	
Lignans (mg/kg)	77.02	75.76	112.31	
Secoiridoids (mg/kg)	89.44	113.65	80.87	
Flavonoids (mg/kg)	2.44	4.71	1.37	
Total poliphenols HPLC (mg/kg)	216.41	245.40	263.14	

Data are expressed as mean ± SD from three replications. Values having different superscripts are significantly ($p < 0.05$) different

this fraction was detected in Roggianella oil (530 mg/kg), while the lowest value was detected in Sinopolese (367 mg/kg). The results are in agreement with other Mediterranean oil olives (Franconi et al., 2006; Esti et al., 1998).

The α-tocopherol content had a mean value of 145.21 mg/kg, 235.51 mg/kg and 177.59 mg/kg for Sinopolese, Roggianella and Ottobratica, respectively (Tab. 1). These results are similar to those described by Aguilera et al. (2005); Allalout et al. (2009); Escuderos et al. (2009); Nakbi et al. (2010).

Tab. 1 also shows the values obtained from the Rancimat test, expressed in hours. The longest time before induction was for the Roggianella cultivar (27.45 h), followed by Ottobratica and Sinopolese (21.33 h and 18.76 h, respectively). These results agree with those from the literature (Aparicio et al., 1999; Baccouri et al., 2008; Dabbou et al., 2010).

Tab. 1 shows the antioxidant activity determined by DPPH and ABTS assay. A positive correlation was observed between the antioxidant activity and the concentration of total polyphenols and α-tocopherol. The extra virgin olive oil from the Roggianella cultivar had a higher antioxidant activity than the oils from Ottobratica and Sinopolese cultivars.

Five classes of compounds were identified by phenolic HPLC analysis: phenolic acids, phenolic alcohols, lignans, secoiridoids and flavonoids (Montedoro, 1972; Montedoro et al., 1992; Mateos et al., 2001) (Tab. 2). ANOVA analysis showed significant differences between the phenolic contents of different cultivars (Tab. 2).

The major phenolic compounds (Tab. 2 and Fig. 1) identified in the olive oils were secoiridoids (dialdehydic form decarboxymethyl oleuropein aglycon (3.4-DHPEA-EDA) and lignans (pinoresinol, 1-acetoxypinoresinol). The secoiridoidic derivatives of oleuropein and ligstroside have been described previously (Montedoro et al., 1993; Owen et al., 2000b).

The major phenolic acids were vanillic acid, 4-hydroxybenzoic acid, p-coumaric acid p-coumaric acid, ferulic acid and cinnamic acid. The concentration of total phenolic acids was 49.92 mg/kg in Ottobratica oil, and 37.63 and 31.99 mg/kg in Roggianella and Sinopolese, respectively.

Of the phenolic acids, cinnamic acid showed the greatest concentration in all samples analyzed, while vanillic acid and p-coumaric acid show the highest concentration in oil from Ottobratica cultivar. The concentration of these compounds is largely affected by agronomic and technological conditions of virgin olive oil production (Servili and Montedoro, 2002).

The main phenolic alcohols were hydroxytyrosol (3.4-DHPEA) and tyrosol (p-HPEA) as reported in the literature by Selvaggini et al. (2006) and Bendini et al. (2007). Their concentration is generally low in freshly pressed oil, but this increases with oil storage due to the effect of hydrolysis of secoiridoid compounds (Montedoro et al.,

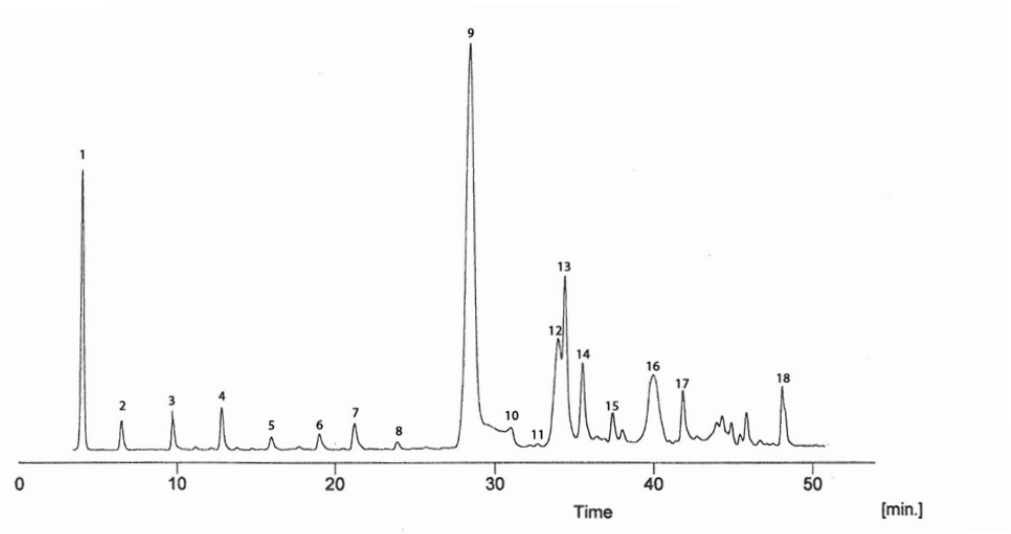

Fig. 1: Gallic acid (std) (1), hydroxytyrosol (2), tyrosol (3), vanillic acid (4), 4-hydroxybenzoic acid (5), p-coumaric acid (6), ferulic acid (7), o-coumaric acid (8), dialdehydic form decarboxymethyl oleuropein aglycon (9), oleuropein aglycon (10), cinnamic acid (11), dialdehydic form decarboxymethyl ligustroside aglycon (12), (+) pinoresinol (13), (+) 1-acetopinoresinol (14), luteolin (15), dialdehydic form oleuropein aglycon (16), apigenin (17), hydroxytyrosol acetate, dialdehydic form ligstroside aglycon (18).

1992). In this current study the hydroxytyrosol content was 2.57, 3.03 and 3.28 mg/kg for Sinopolese, Roggianella and Ottobratica, respectively (Tab. 2). The tyrosol content ranged from 10.62 mg/kg in Roggianella to 15.39 mg/kg in Ottobratica, with an intermediate value in Sinopolese (12.95 mg/kg).

The content of 3.4-DHPEA-EDA ranged from 48.91 mg/kg in Roggianella oil to 58.72 mg/kg in Ottobratica oil. Sinopolese oil shows an intermediate content of 50.43 mg/kg.

Total secoiridoids are present in a higher concentration in oil from Roggianella, with a value of 113.65 mg/kg. Lower values were obtained in Sinopolese and Ottobratica: 89.44 and 80.87 mg/kg, respectively.

Olive oil from Roggianella cultivar had a high concentration of hydroxytyrosol acetate (32.99 mg/kg) compared to Sinopolese and much higher than Ottobratica (21.36 and 7.31 mg/kg), respectively.

Gordon et al. (2001) and Ammendola et al. (2011), have shown that hydroxytyrosol acetate is a more powerful antioxidant than α-tocopherol or hydroxytyrosol, the latter being less soluble in a lipid substrate. Studies on olive polyphenols have revealed the importance of the lipophilicity of antioxidants on cell uptake and membrane crossing, and on the substrate to be protected (membrane constituents or LDL) (Grasso et al., 2007).

Pinoresinol and 1-acetoxypinoresinol are major component of the phenolic fraction of olive oil (Owen et al., 2000b). They are common components of the lignan fraction. The content of pinoresinol was 51.31, 58.87 and 88.49 mg/kg in Roggianella, Sinopolese and Ottobratica, respectively (Tab. 2). The content of 1-acetoxypinoresinol ranged from 18.14 mg/kg in Sinopolese to 24.45 mg/kg in Roggianella. Ottobratica oil had an intermediate concentration 23.02 mg/kg. Lignans are an important part of the phenolic fraction of olive oil because they may play a major role in the health promoting effect of the Mediterranean diet (Setchell et al., 1981; Axelson et al., 1982; Owen, 2000b).

The flavonoids analyzed included luteolin and apigenin. In all oils analyzed, the greatest concentration was in oil from the Roggianella cultivars (1.97 mg/kg of apigenin and 2.74 mg/kg of luteolin). Rovelli et al., (1998), reported that flavonoids such as luteolin and apigenin were phenolic components of virgin olive oil. These results confirm this chemical class as a minor constituent of the phenolic fraction as previously described for other virgin olive oils (Garcia et al., 2002; Mulinacci et al., 2005).

Discussion

The concentration of phenolic compounds is an important factor when evaluating the quality of virgin olive oil because of their involvement in its resistance to oxidation, and its sharp bitter taste (Andrewes et al., 2003; Esti et al., 1998).

The variability of the minor fractions of olive oil cannot always be correlated to definite factors (Fiorino and Nizzi Grifi, 1991), which causes difficulty in reliably identifying the genetic and geographic origin of any single extravergin olive oil.

One of the factory that greatly influence the composition of the minor fractions, in particular the phenolic fraction, is the fruit's degree of ripeness. This means that all the many factors which influence fruit ripening will each have an effect on the quality on the oil (Inglese et al., 2009; Fiorino and Nizzi Grifi, 1991).

The various cultivars may show different reactions to environmental and agronomic factors. This means that environmental factors, which vary from year to year, can have a notable effect on the composition of the oil from any particular cultivar (Lo Curto et al., 2001; Fiorino and Ottanelli, 2004; Lombardo et al., 2008; Tura et al., 2007; Salvador et al., 2001; Sweeney et al., 2002; D'Imperio et al., 2007).

Thus, although the three cultivars considered in the present work were all found in the same geographical area, and had undergone the same agronomic processes, each of them had, no doubt, reacted differently to the same environment.

This interaction between cultivar and environment will have caused differences in the antioxidant content of the olive oils studied.

As it is widely reported in the literature, the phenolic compounds content varies according to degree of ripeness (Fiorino et al., 1991; Youssef et al., 2009; Sicari et al., 2009; Giuffrè et al., 2010).

Olives which are not completely ripe give an oil with a higher content of phenolic compounds compared to oil from fruit which have reached full ripeness. Since trees of the Sinopolese and Ottobratica cultivars grow very tall, a uniform harvest was not possible. Often, the first fruit that fall, when using a mechanical vibrator, are those which are ripest and grow on the outermost branches of the tree. On the other hand, the small size of Roggianella trees allowed a harvest from each part of the tree, including less mature fruit.

The present work has not considered the olive maturity index to avoid creating an overly complex model. The aim of the present work was to study the antioxidant profile of extra virgin olive oil extracted

from three different cultivars.
Of the three cultivars, Roggianella showed the greatest oxidative stability. This may be explained by the fact that the secoiridoids are present in greater quantity in oil from Roggianella than in oil from the other two cultivars. Furthermore, the tocopherol content (α, β e γ), is also higher in oil from Roggianella compared to oil from the other cultivars.
This work confirmed that cultivar is the most important factor influencing the antioxidant profile of the olive oil.

Conclusions

This research confirmed that oxidative stability in virgin olive oil is correlated mainly to polyphenols and tocopherols.
The quality of extra virgin olive oil is linked to its phenolic content. Phenolic compounds influence not only its shelf-life but also its health and sensorial properties.
The cultivar used to produce virgin olive oil is an important factor in determining its content of antioxidant molecules. From the study of the data obtained in this work, it can be noted that Roggianella, Ottobratica and Sinopolese varieties all produced high quality olive oil.
Roggianella oil showed a higher antioxidant activity compared to Ottobratica and Sinopolese oils.
In particular, the extra virgin olive oil extracted from Roggianella cultivar olives had a higher content of hydroxytyrosol acetate, dialdehydic form oleuropein aglycon and of α-tocopherol, all compounds with a high antioxidant capacity.

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