Study on the composition and quality of several sicilian EVOOs (harvesting year 2015)

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Extra virgin olive oil (EVOO) is one of the most representative food of the Mediterranean diet for its high nutritional value and beneficial effects on human health due to the presence of fatty acids and minor components. In this study, the content in fatty acids, sterols, triterpenic dialcohols and squalene was analyzed in several EVOOs of different Sicilian varieties, produced during harvesting year 2015. The results showed that the quantitative composition in fatty acids of EVOO samples was in accordance with European Union legislation. Instead, the total sterol content was above the limit (≥ 1000 mg/kg), except for seven samples belonging to *Nocellara del Belice* and *Verdello* varieties, from different areas of Sicily. Therefore, the control of these components is important to guarantee the composition and nutritional values of EVOOs and particularly to confirm the quality of local oleic production.

Keywords: EVOO, fatty acids, phytosterols, triterpenic dialcohols, squalene

1. INTRODUCTION

Extra virgin olive oil (EVOO) is one of the most representative food of Mediterranean diet [1,2] for its high nutritional value. The beneficial effects of EVOO on human health are related to its characteristic composition in fatty acids and minor components (1-2%), such as squalene and phytosterols, aldehydes, polyphenols, pigments, vitamins, minerals, etc. [3-7].

Fatty acids are identified as saturated (SFA), mono-unsaturated (MUFA), poly-unsaturated (PUFA) and *trans* fatty acids (TFA). The predominant fatty acids present in vegetable oils and fats are saturated and unsaturated compounds with straight aliphatic chains and, generally, most of all they have an even number of carbon atoms, from 16 to 18, with a single carboxyl group. Among the latter, the unsaturated are classified as ω-9 considered not-essential for humans, and the ω-3 and ω–6 as essential fatty acids [8, 9]. In general, the composition in fatty acids and a higher degree of unsaturation of the latter define the characteristics of olive oils and they are important for benefic effects as the reduction of cardiovascular risk, coronary disease, etc. [10].

Phytosterols derived directly from squalene, are principal compounds of the unsaponifiable matter of vegetable oils. They are precursors of steroidal hormones, structurally related to cholesterol, but differ from the latter for the side chain; in fact, for the chemical structure, it is possible to divide phytosterols with a double bond typically between C-5 and C-6 of the sterol moiety, whereas phytostanols are saturated with this bond [11]. The sterol fraction of EVOOs is important for different biological effects such as anti-inflammatory and antibacterial activity, cancer prevention, reduction of plasma cholesterol and low-density lipoprotein (LDL) [12-15]. In this fraction, there are also the triterpenic dialcohols, considered within the sterol profile to characterize olive oils variety [16, 17].

Squalene, instead, is an intermediate compound in the biosynthesis of sterols and triterpenoids and represents the major olive oil hydrocarbon (more than 90%) and it accounts for more than 50% of the unsaponifiable fraction of the oil [18]. Squalene is regarded as being partially responsible for the beneficial effects of olive oil, particularly chemo-preventive activity [19, 20]. Its content depends on the olive cultivar [21], and the oil extraction technology [22], but it could be reduced dramatically during the process of refining [23].
The content of fatty acids and phytosterols represents an important parameter of oil identity [24], while squalene is useful to evaluate quality and stability; the profile of these compounds, in fact, is used to detect adulteration of olive oil with other edible fats and oils.
The European Union and International Olive Oil Council have fixed in EU Regulations 1348/2013 and 1830/2015 [25, 26], the limits for fatty acids, total sterol content, some individual sterols and triterpenic dialcohols in olive oil and olive-pomace oil to define the composition of EVOOs.
In this study, the composition in fatty acids, sterols, triterpenic dialcohols and squalene was analyzed in several Sicilian EVOOs from the most important cultivars of this region, to guarantee the nutritional values and quality of olive production.

2. EXPERIMENTAL PART

2.1 SAMPLING

This study was carried out on 43 samples of extra virgin olive oil (EVOOs) obtained by olive fruits harvested at the optimum ripening, during the crop year 2015, from different areas of Sicily (Palermo, Trapani, Messina and Catania). EVOOs samples were monovarietal (*Nocellara etnea, Nocellara messinese, Minuta, Nocellara del Belice, Verdello, Biancolilla, Uovo di Piccione, Ogliarola messinese, Sanbenedettese*) and the most used multivarietal oils of this area (*Biancolilla/ Nocellara del Belice, Ogliarola messinese/Biancolilla* and *Nocellara del Belice/Biancolilla/Ogliarola messinese)* (Table I). These samples were classified to the "extra virgin" category, according to the physical-chemical parameters, regulated by EU Regulation 1348/2013 [25].
For each sample, 500 mL were stored in dark bottles without leaving space on the neck, at a temperature of 4°C for the analysis of fatty acids, phytosterols, triterpenic dialcohols and squalene.

2.2 FATTY ACIDS ANALYSIS

Fatty acids methyl esters (FAMEs) analysis was performed according to EU Regulation 1833/2015 [27]. It consists of the hydrolysis of triacylglycerides and cold transesterification with a methanol KOH solution; the methyl esters were prepared by vigorously shaking solution of the oil in heptane (0.1 g in 2 mL) with 0.2 mL of the methanolic KOH solution. The resulting solution was then injected into a gas chromatograph DANI MASTER GC-FID (Milan, Italy), equipped with a fused silica capillary column Phenomenex Zebron ZB-WAX (polar phase in polyethylene glycol) with a length of 30 m, internal diameter of 0.25 mm and film thickness of 0.25 µm. Helium was used as a carrier gas at a column flow rate of 1.2 ml/min, with a split ratio of 1:100. The temperature of the injector (split/splitless) and detector was of 220°C and 240°C, respectively. The oven was programmed as follows: initial temperature at 130°C, final temperature at 200°C (10 min) with an increase of 3°C/min. The fatty acid methyl esters were identified by comparing the retention times with those of standard compounds. The relative percentage area of the fatty acids was obtained using the following relationship: $\%FAX = [AX/AT] \times 100$, where FAX stands for fatty acids to quantify, AX is the area of the methyl-esters and AT is the total area of the identified peaks in the chromatogram.

2.3. PHYTOSTEROLS ANALYSIS

The EVOOs extraction for analysis of phytosterols and triterpenic dialcohols was conducted according to the official method reported in EU Regulation 1348/2013 [25].
EVOOs samples were submitted to saponification with an ethanol potassium hydroxide solution, previous addition of an internal standard (5α-cholestan-3β-ol) for sterols quantification. The unsaponifiable matter was extracted with diethyl ether and treated on a silica gel plate chromatography to separate the fraction constituted by sterols and triterpenic dialcohols. This fraction was derivatized to obtain the corresponding trimethylsilyl ethers and subsequently ana-

Table I - EVOO samples for different variety and zone of Sicily

Variety	Zone	Samples
Nocellara etnea	Catania	(n=3)
Nocellara messinese	Messina	(n=3)
Minuta	Messina	(n=4)
Nocellara del Belice	Messina	(n=1)
	Agrigento	(n=1)
	Trapani	(n=6)
	Palermo	(n=1)
Verdello	Messina	(n=3)
Biancolilla	Messina	(n=3)
Uovo di Piccione	Messina	(n=3)
Ogliarola messinese	Messina	(n=3)
Sanbenedettese	Messina	(n=3)
* Biancolilla 75% - Nocellara del Belice 25%	Messina	(n=3)
* Ogliarola messinese 60% - Biancolilla 40%	Messina	(n=3)
* Nocellara del Belice 50% - Biancolilla 25% - Ogliarola messinese 25%	Trapani	(n=3)

(*) Blended oils in various proportions.

lyzed by gas chromatography, using a chromatograph DANI MASTER GC, equipped with a capillary column (ZB-1 Phenomenex: 15 m × 0.25 mm, 0.25 µm film thickness) and a flame ionization detector. The injector was operated in split mode (ratio 1:50) and the injection volume was 1 µl. The operating conditions were as follow: carrier gas: helium at 1 ml/min; injector and detector temperature: 290°C; column temperature was programmed to 240°C for 5 min and then ramped up at 2°C/min to 290°C for 5 min.

The phytosterols and triterpenic dialcohols were identified by using commercial standard obtained from Sigma Aldrich (St Louis, Mo, USA). According to EU Regulation 1348/2013 [25], the apparent β-sitosterol was calculated as the sum of Δ-5,23-stigmastadienol, clerosterol, β-sitosterol, sitostanol, Δ-5-avenasterol and Δ-5,24-stigmastadienol. The relative amount of each phytosterol and triterpenic dialcohol was expressed as a percentage of total sterols, while the total sterols were expressed as sum of single phytosterols (mg/kg).

2.4 SQUALENE ANALYSIS

For the analysis of squalene, EVOOs samples (~0.12 g) were submitted to an extraction method using a single-step SPE, previous dissolution in 0.6 mL of *n*-hexane and loading in Supelco Discovery DSC-Si Silica column (500 mg) (Supelco, Milan, Italy). For the chromatographic analysis, an Acquity UPLC® Waters liquid chromatography system was used equipped with a photodiode array detector ACQ-PDA and an Acquity UPLC® Waters BEH C18 column of 1.7 µm (2.1 × 50 mm), protected by 0.2 µm stainless steel In-Line Filter with a Holder Waters. The chromatographic analysis was carried out at 40°C, using a mobile phase composed of acetonitrile/acetone (60:40 v/v) under isocratic condition, with injection volume of 2 µL and flow rate 0.8 mL/min, at 217 nm wavelength [28].

3. RESULTS AND DISCUSSIONS

The results obtained showed the quantitative composition of EVOO samples.

Relating to fatty acids, in the EVOOs analyzed the most representative were oleic, linoleic, palmitic and stearic. Instead, palmitoleic, margaric, margaroleic, linolenic, arachidic and gadoleic acids were found in small quantities; miristic acid was 0.01% in all samples, while behenic and lignoceric were less than 0.07% (Table II). All fatty acids detected were within the limits established by EU Regulation 1830/2015 [25]: miristic ≤ 0.03%; linolenic ≤ 1.00%; arachidic ≤ 0.60%; gadoleic ≤ 0.40%; behenic ≤ 0.20%; lignoceric ≤ 0.20%; palmitic: 7.50-20.00%; palmitoleic: 0.30-3.50%; margaric and margaroleic ≤ 0.30%; stearic: 0.50-5.00%; oleic: 55.00-83.00%; linoleic: 2.50-21.00%.

From this analysis, some varietal differences can be

Table II - Fatty acids % (mean ± standard deviation) in different EVOOs samples

	Nocellara etnea	Nocellara messinese	Nocellara del Belice	Minuta	Verdello	Biancolilla	Uovo di Piccione	Ogliarola messinese	Sanbenedettese	*Biancolilla/ Nocellara del Belice	*Ogliarola messinese/ Biancolilla	*Nocellara Belice/ Biancolilla/ Ogliarola messinese
Palmitic C16:0	12.81±0.11	14.32±0.96	12.00±0.94	15.56±0.93	12.17±2.40	13.09±1.25	10.50±0.75	11.05±0.51	15.41±0.36	13.10±1.58	13.16±0.95	13.34±1.16
Palmitoleic C16:1	1.03±0.07	1.28±0.42	0.82±0.15	1.55±0.15	0.75±0.26	1.20±0.35	0.40±0.20	0.63±0.15	1.78±0.18	1.00±0.28	0.90±0.18	1.13±0.40
Margaric C17:0	0.15±0.01	0.17±0.07	0.06±0.05	0.12±0.03	0.11±0.04	0.10±0.03	0.03±0.04	0.06±0.05	0.16±0.04	0.13±0.04	0.19±0.06	0.09±0.04
Margaroleic C17:1	0.27±0.01	0.20±0.05	0.10±0.07	0.22±0.04	0.15±0.01	0.20±0.03	0.05±0.01	0.10±0.02	0.27±0.02	0.23±0.04	0.23±0.04	0.16±0.05
Stearic C18:0	2.70±0.47	3.04±0.27	3.02±0.51	2.54±0.66	2.74±0.34	2.00±0.42	2.74±0.32	2.22±0.41	2.41±0.33	2.53±0.33	3.55±0.57	2.70±0.25
Oleic C18:1	72.92±0.53	71.81±3.58	75.28±1.34	66.61±1.38	73.50±0.26	72.20±2.75	75.72±1.58	76.31±1.62	70.75±1.97	72.94±3.73	74.02±2.43	73.24±1.40
Linoleic C18:2	8.46±0.05	7.56±2.71	7.22±1.62	12.00±0.92	9.14±3.27	9.90±2.36	9.40±1.59	7.00±1.64	7.61±2.10	8.47±2.28	6.26±1.82	7.70±1.95
Linolenic C18:3	0.78±0.04	0.73±0.21	0.66±0.07	0.70±0.12	0.73±0.04	0.60±0.05	0.50±0.15	0.75±0.09	0.76±0.18	0.71±0.01	0.82±0.11	0.84±0.15
Arachidic C20:0	0.49±0.01	0.54±0.04	0.47±0.06	0.36±0.06	0.37±0.05	0.32±0.06	0.30±0.04	0.43±0.06	0.49±0.10	0.46±0.08	0.54±0.03	0.48±0.10
Gadoleic C20:1	0.36±0.01	0.29±0.02	0.28±0.05	0.25±0.04	0.33±0.04	0.20±0.06	0.18±0.02	0.39±0.05	0.30±0.07	0.32±0.03	0.31±0.05	0.27±0.09
Behenic C22:0	0.02±0.00	0.02±0.01	0.05±0.03	0.03±0.02	0.02±0.01	0.06±0.02	0.07±0.01	0.02±0.00	0.02±0.01	0.04±0.02	0.03±0.01	0.02±0.01
Lignoceric C24:0	0.03±0.00	0.02±0.01	0.04±0.01	0.03±0.01	0.02±0.00	0.05±0.02	0.06±0.02	0.03±0.01	0.03±0.00	0.04±0.02	0.02±0.01	0.02±0.00
C18:1/C18:2	8.63	9.50	10.43	5.55	8.04	7.30	8.06	10.90	9.30	8.61	11.82	9.51
MUFA/SFA	4.60	4.06	4.89	3.68	4.85	4.73	5.58	5.60	3.95	4.57	4.31	4.49
MUFA/PUFA	8.08	8.88	9.70	5.41	7.57	7.04	7.72	9.99	8.73	8.12	10.66	8.76

(*) Blended oils in various proportions.
MUFA: monounsaturated fatty acids sum; SFA: saturated fatty acids sum; PUFA: polyunsaturated fatty acids sum.

Table III - Phytosterols and triterpenic dialcohols (mean ± standard deviation) in different EVOOs samples.

Variety	Cholesterol (%)	Brassicasterol (%)	Campesterol (%)	Stigmasterol (%)	Apparent β-Sitosterol (%)	Δ-7-stigmastenol (%)	Total sterols (mg/kg)	Erythrodiol+Uvaol (%)
Nocellara Etnea	0.13±0.02	0.08±0.03	2.70±0.03	1.25±0.76	95.29±0.91	0.32±0.09	1459.0±229.1	0.57±0.04
Nocellara messinese	0.12±0.06	0.05±0.06	2.97±1.13	1.15±0.70	90.72±7.36	0.31±0.03	1328.3±210.7	0.88±0.23
Minuta	0.25±0.01	0.09±0.03	3.24±0.22	1.51±0.61	93.41±1.05	0.41±0.15	1132.5±103.4	0.72±0.28
Nocellara del Belice	0.26±0.13	0.06±0.02	3.45±0.37	1.20±0.24	92.55±2.34	0.26±0.09	954.2±227.8	1.08±0.43
Verdello	0.28±0.04	0.24±0.30	2.98±0.51	0.90±0.32	93.23±0.62	0.45±0.21	1063.3±324.7	1.04±0.52
Biancolilla	0.23±0.09	0.10±0.02	2.59±0.75	1.16±0.25	92.86±1.27	0.18±0.09	1354±172.1	0.37±0.09
Uovo di Piccione	0.27±0.04	0.03±0.02	4.04±0.43	0.82±0.25	92.00±0.79	0.20±0.07	1114±232.5	0.95±1.32
Ogliarola messinese	0.19±0.05	0.06±0.03	3.06±0.57	0.77±0.30	95.30±0.95	0.12±0.06	1156±249.3	0.65±0.21
Sanbenedettese	0.16±0.09	0.01±0.02	3.23±0.36	1.25±0.54	94.73±1.16	0.21±0.11	1238±237.8	0.67±0.39
Biancolilla /Nocellara del Belice	0.15±0.04	0.05±0.04	2.71±0.66	1.63±0.78	93.39±0.21	0.26±0.12	1245.5±186.0	0.87±0.04
Ogliarola messinese /Biancolilla	0.17±0.00	0.04±0.01	3.43±0.05	2.13±0.65	93.27±0.54	0.17±0.03	1566.5±498.5	1.41±1.34
Nocellara del Belice / Biancolilla /Ogliarola messinese	0.12±0.04	0.02±0.01	2.49±0.41	0.84±0.19	95.89±1.08	0.24±0.10	1326±156.4	1.65±0.25

observed according to the fatty acids profile. The *Minuta* variety, particularly, presented the lower content of oleic acid (66.61%) and the higher of linoleic acid (12.00%); on the contrary, *Nocellara del Belice* and *Ogliarola messinese* varieties showed higher percentages of oleic acid (75.28% and 76.31% respectively) and lowest of linoleic acid (7.22% and 7.00% respectively). *Uovo di Piccione* variety had the lowest amount of palmitic, palmitoleic, margaric, margaroleic, linolenic, arachidic and gadoleic and the highest amount of behenic and lignoceric acids, while *Biancolilla* contained the lowest stearic level. From the multivarieties, instead, *Ogliarola messinese/Biancolilla* presented the higher values of oleic acid (74.02%) and the lowest of linoleic (6.26%).

Considering the ratio oleic/linoleic acids, the parameter used to evaluate the stability of oils, characterize cultivar and oil mixture, samples of monovarietal oils *Ogliarola messinese* and *Nocellara del Belice* and multivarietal oil of *Ogliarola messinese/Biancolilla* showed the higher values corresponding to the greatest oxidative stability (10.90, 10.43 and 11.82 respectively). Instead, the lowest oleic/linoleic ratio found in *Minuta* oils (5.55) indicated a less oxidative stability, as previously described by other authors for this variety [29]. These data were confirmed also by MUFA/SFA and MUFA/PUFA ratios: monovarietal *Ogliarola messinese* and *Nocellara del Belice* and multivarietal oil *Ogliarola messinese/Biancolilla* showed the higher value, while *Minuta* the lowest, confirming their different oxidative stability (Table II). Most in general, the multivariety, *Ogliarola messinese/Biancolilla* showed the highest ratio oleic/linoleic acids and MUFA/PUFA among of all samples analyzed.

Also, these parameters, however, are affected also by environmental factors such as rainfall and geographical origin [17].

In Table III the main phytosterols and triterpenic dialcohols are reported. The values of single sterols were within the limit established by the EU Regulation 1348/2013 [25]: cholesterol (≤ 0.5%), brassicasterol (≤ 0.1%), campesterol (≤ 4.0%), stigmasterol (< camp.), apparent β-sitosterol (≥ 93.0%), Δ-7-stigmastenol (≤ 0.5%). Instead, the total sterol content was above the limit (≥ 1000 mg/kg), except for samples belonging to *Nocellara del Belice* and *Verdello* varieties, coming from different areas of Sicily (Table III). However, to better understand these data, the values of single sterols were compared among them and the only significant difference was the highest concentration of cholesterol and lower β-sitosterol in the *Nocellara del Belice* and *Verdello* EVOO samples (Table IV). These differences were particularly due to the intrinsic feature correlated to the variety, as documented by other authors for the *Benizal* and *Cornicabra* varieties from Spain [30] and *Khashabi* variety from Syria [31]. However, it could be influenced also by climatic changes and different pedoclimatic conditions.

The triterpenic dialcohols, expressed as sum of uvaol

Table IV - Cholesterol and β-sitosterol concentrations (mg/kg) in EVOOs samples with lower total phytosterols content

EVOOs Variety	Zone	Cholesterol (mg/kg)	β-sitosterol (mg/kg)	Total sterol (mg/kg)
Nocellara del Belice	ME	2.31±0.65	750±5.2	852±7.9
	AG	2.28±0.52	908±8.1	1038±9.1
	TP	2.52±0.42	696±4.5	917±7.3
	TP	1.24±0.31	1034±8.6	1245±9.8
	PA	1.10±0.28	755±6.0	890±7.2
	TP	1.90±0.30	1209±18.3	1372±15.2
	TP	3.04±0.74	721±4.9	859±7.0
	TP	3.45±0.63	706±4.5	850±6.2
	TP	2.91±0.68	527±3.2	632±4.3
Verdello	ME	2.83±0.56	786±4.2	903±6.8
	ME	2.36±0.43	1230±9.0	1437±11.5
	ME	2.90±0.37	765±5.1	850±5.8

and erythrodiol, were found within the limit established by EU (≤ 4.5%), with a range of 0.37-2.35%. From all samples analyzed, the higher levels of triterpenic dialcohols were observed in multivarietal *Ogliarola messinese/Biancolilla* and *Nocellara del Belice/ Biancolilla/Ogliarola messinese,* followed by monovarietal EVOOs *Nocellara del Belice* and *Verdello* with a lower total sterol content (Table III).
Lastly, considering the analysis of squalene, analyzed EVOO samples showed a similar content, with a range of 1525-3461 mg/kg (Table V). For squalene, there is no specific limit; however, the International Olive Council reported values in EVOOs among 150-800 mg/kg [32], but higher concentrations of squalene in EVVOs were reported, for example, 4240 mg/kg [33] and 3900-9600 mg/kg [34].

Table V - Content of squalene (Mean ± standard deviation) in different Sicilian EVOOs samples.

Variety	Squalene (mg/kg)
Nocellara Etnea	3323.75 ± 65.23
Nocellara messinese	2785.85 ± 46.91
Minuta	1525.06 ± 87.92
Nocellara del Belice	2499.27 ± 44.78
Verdello	2619.19 ± 15.25
Biancolilla	2135.63 ± 76.34
Uovo di Piccione	3461.99 ± 94.77
Ogliarola messinese	1943.49 ± 55.83
Sanbenedettese	2694.7 ± 94.53
Biancolilla/Nocellara del Belice	2827.07 ± 22.44
Ogliarola messinese/Biancolilla	2769.46 ± 76.16
Nocellara del Belice/ Biancolilla/Ogliarola messinese	1611.01 ± 36.25

The analysis of all these components studied in Sicilian EVOOs, and particularly in samples with a lower total sterols content, showed no significant correlations among them. Although squalene is the precursor in biosynthesis of steroids and triterpenoids, an increase of this compound was not observed in samples with a decreased total content of sterols. Instead, the higher content of cholesterol observed in these *Nocellara del Belice* and *Verdello* EVOO samples was not correlated to the composition in fatty acids, although it is characteristic of each olive oil variety [35] and specific for cultivars grown within a well-limited geographical region [36].

4. CONCLUSIONS

Data of this study on fatty acid composition, sterol profile and squalene content were particularly correlated to variety, but could be affected also by several factors in addition to variety [37-39], like the ripening cycle of the fruit, cultivar, oil extraction, refining procedures and storage conditions [40-42] and by agronomic and climatic conditions [43, 44, 37]. In fact, these differences in Sicilian *Nocellara del Belice* and *Verdello* samples were observed for the first time in EVOOs of the 2015 harvesting period, and, to verify the influence of the different pedoclimatic conditions and climatic changes of these last years, further analysis shall be carried out on samples from the next olive production.
The monitoring on the content of these components is indeed important to characterize EVOOs of different variety, sustain and increase the local production in the oleic sector, defining the optimal harvest time for olive oils [40] and guaranteeing the nutritional value of oils against the risk of adulteration.

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