Influence of Crop Season and Cultivar on Sterol Composition of Monovarietal Olive Oils in Reggio Calabria (Italy)

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Abstract

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Sterol composition was studied for three successive crop seasons in some olive oils extracted from Italian cultivars grown in Reggio Calabria Province, Southern Italy. Three autochthonous cultivars from Calabria Region: Cassanese, Ottobratica, and Sinopolese and seven allochthonous cultivars: Coratina, Itrana, Leccino, Nocellara Messinese, Nociara, Pendolino, and Picholine were investigated. The studied olive oils showed an acceptable sterol composition in accordance with either the European Union or International Olive Oil Council regulations. The Picholine cultivar showed the highest content of total sterol, β-sitosterol, chlerosterol, campesterol, and of cholesterol. The contents of Δ^5-avenasterol, $\Delta^{5,24}$-stigmastadienol, 24-methylene-cholesterol, and Δ^7-avenasterol were high in Nociara cultivar, whereas Pendolino cultivar had the highest content of sitostanol and Sinopolese cultivar gave the highest content of stigmasterol. Results confirmed the existing significant differences ($P < 0.05$ and $P < 0.01$) in the cultivar, crop season, and crop season × cultivar.

Keywords: ANOVA; cluster analysis; harvest year; minor components; wax esters

The Mediterranean diet is based mainly on olive oil consumption. Olive oil is known for its nutritional and health benefits due to the presence of many compounds which are chemically and nutritionally interesting, among them sterols, which determine the genuineness of olive oil by their concentration and composition. The various sterol structures differ primarily in the R tail and these differences stem from only one extra methyl group (campesterol) to a more complex difference (stigmasterol) (Patel 2008). Beta-sitosterol, campesterol, and stigmasterol are structurally similar to cholesterol: they do not have methyl groups at the 4th carbon atom (4-desmethyl sterols). Stanols constitute saturated sterols, produced by hydrogenating sterols and they are less abundant in nature than sterols (Law 2000). Sterols are present in a free (80%) and esterified form and account for 30–60% of the non-saponifiable fraction of olive oil which is 0.4–0.8% of virgin olive oil (Cunha *et al.* 2006). Sterol components with the highest representation are β-sitosterol (more than 80%), campesterol (2.9–4%), and Δ^5-avenasterol (7%) (Cunha *et al* 2006; Cercaci *et al*. 2007; Martínez-Vidal *et al.* 2007). Cholesterol is also found in olive oil at a small amount of about 1–3 mg/kg (Cañabate-Díaz *et al.* 2007; Martínez-Vidal *et al*. 2007). Extra virgin olive oil contains mainly phytosterols and an insignificant amount of cholesterol (less than 0.5%).

The normal physiological process for handling dietary sterols by the gastrointestinal tract involves processing 200 mg to 500 mg of dietary cholesterol, but also 200 mg to 400 mg of phytosterols, which are a component of plant sterols (Patel 2008). Phytosterols have a positive effect on reducing total cholesterol and LDL-cholesterol in the human body blood. St-Onge *et al.* (2003) assumed that a dose of about 4 g daily would be

suitable to induce changes in total cholesterol and LDL-cholesterol concentrations. Anti-cancer, anti-inflammatory, and anti-atherogenicity activities are also benefits of these components (Berger 2004). Sterol composition in virgin olive oil extracted in the same harvesting period is not influenced significantly by different technology systems (Cercaci *et al.* 2007). The composition is influenced by other agronomic parameters like the soil type, cultivar, production techniques, oil extraction, storage conditions, and climate (Gutierréz *et al.* 1999; Salvador *et al.* 2001, 2003; D'Evoli *et al.* 2006; Cañabate-Díaz *et al.* 2007). Rivera del Álamo *et al.* (2004) demonstrated that the variation in the campesterol content in olive oil is independent of year seasons, crop area, extraction system and fruit maturity.

The aim of this paper is to study the sterol composition of pressed olive oil from autochthonous and allochthonous olives grown in Reggio Calabria (Calabria, Southern Italy). Particular emphasis is laid on the influence of crop season and cultivar on sterol composition on the basis of the European and the I.O.C. regulations.

MATERIAL AND METHODS

Material. Ten different Italian cultivars were used for this study for three successive crop seasons 2005/2006, 2006/2007, and 2007/2008: Cassanese, Coratina, Itrana, Leccino, Nocellara Messinese, Nociara, Ottobratica, Pendolino, Picholine, and Sinopolese. Olives from these cultivars are used mainly for oil extraction. Olive trees were well managed and had no nutrient deficiency or pest damage. Fifteen 25–40 years old trees per cultivar were selected and labelled in mono-cultivar groves, situated in the area of Rizziconi (South West Calabria). This area, at an altitude of 100 m a.s.l., is characterised by damp and rainy winters and hot summers. Each mono-cultivar grove was at least 3 km from the others. Olive sampling for each cultivar was conducted in biweekly intervals from October, when the fruit was 20% ripe, until fruit was no longer found on the trees. Five replicates were performed for each harvest season. Freshly and manually harvested drupes (40 kg approx. per cultivar, 2.5 kg approx. per tree) were placed in a plastic container and immediately transported to the laboratory where they were cleaned to eliminate branches and leaves and were washed with fresh water to remove dust.

Olive oil extraction. The oils were extracted using a Mini 30 laboratory mill from AGRIMEC Valpesana, Calzaiolo, S. Casciano VP (Florence, Italy), with a capacity of 40 kg. Olives were crushed with a hammer-mill. The resulting paste was mixed at a temperature between 15°C and 20°C for 35 min and pressed using a hydraulic press with a continuous increase in pressure up to 200 bar. After separation of the oil phase by centrifugation, the oil was filtered through filter paper, and it was kept in 100 ml amber glass bottles until analysis.

Determination of sterols. Sterols were determined as described in Annex V of EEC Regulation 2568/91 (EEC 1991). Olive oil (5 g) was saponified with 2M ethanolic potassium hydroxide solution, using α-cholestanol as an internal standard, after boiling; 50 ml of distilled water were added. The reaction mixture was extracted with ethyl ether three times. The three ether extracts were introduced into a separating funnel and washed with distilled water (50 ml each time) until neutral reaction. The organic extracts were dried with anhydrous sodium sulphate and filtered. These extracts were evaporated to dryness using a rotary evaporator. The remaining residue was dissolved in 2 ml of chloroform, and then the sterol fraction was separated by TLC using a plate-developing chamber, which contained hexane/diethyl ether 60:40 (v/v). After TLC separation, the silica gel plate was sprayed lightly and uniformly with 2,7-dichlorofluorescein. The sterol fraction was separated from the unsaponifiable extract by chromatography on a basic silica gel plate. The sterols recovered from the silica gel were transformed into trimethyl silyl ethers and analysed by a gas chromatograph, Model 8600 (Perkin Elmer, Waltham, USA). The working conditions were as follows: carrier gas (helium) 10 psi of pressure, auxiliary gas (hydrogen at 15 psi and air at 22 psi), split/splitless injector (operating in the split mode) temperature (280°C), flame ionisation detector (FID) temperature (290°C), oven temperature (270°C), capillary column SE 54 (30 m length × 0.32 mm *i.d.*, 0.5 μm film thickness; Mega, Milan, Italy) and an injection volume of 1 μl.

The identification of the compounds was based on a comparison of retention indices with those of standard samples and with literature data.

Chemicals and reagents. Standard sample of α-cholestanol was from Fluka (Milan, Italy), standard samples of cholesterol, campesterol, stigmasterol, β-sitosterol, and sitostanol were from Sigma-Aldrich

Table 1. Sterol composition of different cultivars (mg/kg)

	Cassanese	Coratina	Itrana	Leccino	Nociara	Ottobratica	Pendolino	Picholine	Sinopolese
Cholesterol	3 ± 0^{c}	5 ± 0^{ab}	3 ± 0^{bc}	3 ± 0^{c}	4 ± 0^{abc}	3 ± 0^{c}	4 ± 0^{abc}	5 ± 0^{a}	4 ± 0^{bc}
24-Methylene-cholesterol	0 ± 0^{d}	2 ± 0^{bc}	4 ± 0^{a}	1 ± 0^{cd}	4 ± 0^{a}	1 ± 0^{cd}	2 ± 0^{b}	1 ± 0^{cd}	1 ± 0^{cd}
Campesterol	51 ± 3^{cd}	43 ± 3de	39 ± 3^{e}	42 ± 3de	51 ± 2^{bc}	41 ± 2^{e}	38 ± 2^{e}	65 ± 3^{a}	57 ± 2^{ab}
Campestanol	2 ± 0^{ef}	2 ± 0^{cdef}	1 ± 0^{f}	3 ± 0^{abc}	4 ± 0^{ab}	2 ± 0^{cde}	3 ± 0^{bcd}	2 ± 0^{def}	3 ± 0^{abc}
Stigmasterol	13 ± 2^{c}	23 ± 2^{b}	12 ± 2^{c}	21 ± 2^{b}	16 ± 1^{c}	15 ± 1^{c}	15 ± 1^{c}	15 ± 1^{c}	27 ± 1^{a}
Δ^{7}-Campesterol	0 ± 0^{c}	1 ± 0^{bc}	0 ± 0^{c}	1 ± 0^{bc}	1 ± 0^{bc}	1 ± 0^{bc}	0 ± 0^{c}	1 ± 0^{bc}	1 ± 0^{b}
Chlerosterol	17 ± 1^{abcd}	15 ± 1^{cde}	17 ± 1^{abc}	18 ± 1^{ab}	17 ± 1^{abcd}	12 ± 1^{e}	15 ± 1^{bcd}	18 ± 1^{a}	18 ± 1^{ab}
β-Sitosterol	1458 ± 65^{bcd}	1255 ± 68d^{ef}	1223 ± 66^{def}	1261 ± 64^{cdef}	1447 ± 58^{bc}	1150 ± 57^{ef}	1116 ± 58^{f}	1651 ± 66^{a}	1576 ± 57^{ab}
Sitostanol	17 ± 2^{bcd}	21 ± 2^{ab}	12 ± 2^{cde}	12 ± 2de	16 ± 2^{bcd}	17 ± 2^{bc}	23 ± 2^{a}	10 ± 2^{e}	16 ± 2^{bcd}
Δ^{5}-Avenasterol	70 ± 6^{g}	114 ± 6de	158 ± 6^{b}	170 ± 6^{b}	202 ± 5^{a}	82 ± 5^{f}	133 ± 5^{c}	99 ± 6^{ef}	121 ± 5^{cd}
$\Delta^{5,24}$-Stigmastadienol	3 ± 1^{d}	6 ± 1^{bc}	5 ± 1^{cd}	8 ± 1^{b}	13 ± 1^{a}	3 ± 1^{d}	4 ± 1^{cd}	5 ± 1^{cd}	6 ± 1^{bc}
Δ^{7}-Stigmastenol	4 ± 1^{cd}	8 ± 1^{a}	4 ± 1^{cd}	5 ± 1^{cd}	5 ± 1^{bc}	3 ± 1^{d}	4 ± 1^{cd}	4 ± 1^{cd}	5 ± 1^{cd}
Δ^{7}-Avenasterol	3 ± 0^{d}	4 ± 1de	6 ± 0^{bc}	8 ± 0^{a}	8 ± 0^{a}	4 ± 0^{e}	5 ± 0^{cde}	5 ± 0^{bc}	5 ± 0^{bcd}
Total sterols	1641 ± 73^{b}	1498 ± 76^{bc}	1483 ± 74^{bc}	1551 ± 71^{bc}	1788 ± 65^{a}	1334 ± 63^{c}	1363 ± 65^{bc}	1880 ± 74^{a}	1840 ± 63^{a}
Campesterol/stigmasterol	4	2	3	2	3	3	3	4	2
β-Sitosterol/Δ^{5}-avenasterol	21	11	8	7	7	14	8	17	13

Values represent the mean of the three crop seasons ± standard error; lines followed by the same letter were not significantly different according to Duncan's multiple range test at $P \leq 0.05$

(Steinheim, Germany). TLC silica gel plates were from Merck S.p.A. (Milan, Italy). All other reagents were from Carlo Erba (Milan, Italy).

Statistical analysis. SPSS 15.0 software (SPSS Inc., Chicago, USA) was used to determine significant differences for all parameters (two-way ANOVA). Two effects were taken into consideration, cultivar and crop season. Duncan's test was used to determine differences between cultivars at $P \leq 0.05$. Statistica 6.0 software was used for cluster analysis, correlations between sterols and histograms.

RESULTS AND DISCUSSION

Olive oil conformity with EU and I.O.C. regulations

Two regulations, EU 61/2011 (EU 2011) and International Olive Oil IOC.T.15/NC n. 3/Rev. 4 (IOC 2009) with the same information, concerning the contents of sterol components, will be used as references for our results. Table 1 shows the composition of sterols in the different cultivars. The most important component in the ten cultivars is β-sitosterol ranging from 1116 mg/kg to 1651 mg/kg for cv. Picholine. The second component with high content was Δ^{5}-avenasterol, the highest value of this component was observed in cv. Nociara (202 mg/kg) whereas cv. Cassanese showed the lowest value; three times lower (70 mg/kg). The third component was campesterol with values ranging from 38 mg/kg in cv. Pendolino to 65 mg/kg in cv. Picholine. All cultivars showed a lower campesterol percentage of 4%, the limit value established by the regulations of the European Community (EU 2011) and of the International Olive Oil Council (IOC 2009). The results obtained for the three components with the highest content are in agreement with previous results, such as the work of Rivera del Álamo *et al.* (2004) focused on cv. Cornicabra; the results of Cercaci *et al.* (2007) for olive oil from a mixture of two cvs Leccino and Dritta and those of Cunha *et al.* (2006) for monovarietal Portuguese olive oils. Other researches also confirmed the previous

results (Parcerisa *et al.* 2000; Rui Alves *et al.* 2005; Martínez-Vidal *et al.* 2007; Temime *et al.* 2008). The minimum limit of 93%, allowed by the two regulations of the EU (2011) and IOC (2009), of the apparent sitosterol parameter which is the sum of the amount of β-sitosterol and other four adjacent phytosterols (chlerosterol, sitostanol, Δ^5-avenasterol, and $\Delta^{5,24}$-stigmastadienol), was observed in all studied cultivars. Thus, the sum of the remaining sterol compounds did not exceed 7%, confirming the authenticity of the respective oils (Sánchez Casas *et al.* 2004). The campesterol/stigmasterol ratio is an index of quality for olive oil. The highest ratio of campesterol/stigmasterol was observed in the cvs Cassanese and Picholine (4) and the lowest in cvs Coratina, Leccino, and Sinopolese (2). The highest ratio of β-sitosterol/Δ^5-avenasterol was observed in cv. Cassanese (21) and the lowest one were observed in cvs Leccino and Nociara (7). The highest value of cholesterol was 5 mg/kg (0.33%) in cv. Coratina and 5 mg/kg (0.28%) in cv. Picholine; the lowest cholesterol content was found in cv. Leccino, 3 mg/kg (0.18%). The range of cholesterol percentage (from 0.18% to 0.28%) is below the minimum limit established by the two regulations of the EU (2011) and IOC (2009). Regarding the total sterol content, the highest value was observed in Picholine cultivar, followed by Sinopolese cultivar, 1880 and 1840 mg/kg, respectively, and the lowest value 1334 mg/kg was observed in cv. Ottobratica. The values of the total sterol content of all cultivars are higher than the lower limit required by European regulation (EU 2011) and IOC (2009), which is 1000 mg/kg.

Moreover, it might be interesting to note that an average daily dose of extra virgin olive oil (40 g) of cv. Picholine contains approx. 75 mg of phytosterols (66 mg is β-sitosterol) whereas the same quantity of extra virgin olive oil of cv. Pendolino contains approx. 54 mg of phytosterols (45 mg is β-sitosterol), it means that cv. Picholine contains approx. 39% phytosterols and 47% β-sitosterol more than cv. Pendolino.

Because of no production in the two successive years (2006/2007 and 2007/2008), the cv. Nocellara Messinese was harvested just in the first studied year and is not considered in the study of different histograms and analysis of variance.

Analysis of variance

In the present paper, the analysis of variance demonstrated that the olive cultivar factor significantly influenced almost all sterols of the studied samples (Table 2). The crop season effect influenced stigmasterol ($P \leq 0.01$), β-sitosterol ($P \leq 0.01$), sitostanol, Δ^7-avenasterol, and the campesterol/stigmasterol ratio ($P \leq 0.05$). Table 2 also depicts the interaction between the two effects (cultivar × crop season) and shows insignificant differences for these following parameters: 24-methylene-cholesterol, Δ^7-stigmastenol, and β-sitosterol/Δ^5-avenasterol ratio nevertheless, it shows significant differences ($P \leq 0.05$) for these parameters: chlerosterol, sitostanol, and Δ^7-avenasterol, and highly significant differences ($P \leq 0.01$) for all the other sterols. Matos *et al.* (2007) documented that the cultivar

Table 2. The different sterols of extra virgin olive oil of cultivars grown in Reggio Calabria (Calabria, Southern Italy) with significant differences. ANOVA experiment: cultivar, crop season, cultivar × crop season

	Cholesterol	24-Methylene-cholesterol	Campesterol	Campestanol	Stigmasterol	Δ^7-Campesterol	Chlerosterol	β-Sitosterol	Sitostanol	Δ_5-Avenasterol	$\Delta^{5,24}$-Stigmastadienol	Δ^7-Stigmastenol	Δ^7-Avenasterol	β-Sitosterol apparent	Campesterol/stigmasterol	β-Sitosterol/Δ^5-avenasterol
Cultivar	ns	**	ns	*	*	ns	ns	**	**	**	**	*	**	ns	*	*
Crop season	ns	ns	ns	ns	**	ns	ns	**	*	ns	ns	ns	*	ns	*	ns
Cv. × crop season	**	ns	**	**	**	**	*	**	*	**	**	ns	*	**	**	ns

*$P \leq 0.05$; **$P \leq 0.01$; ns – not significant; each result is calculated as the mean of five different replicates for each crop season

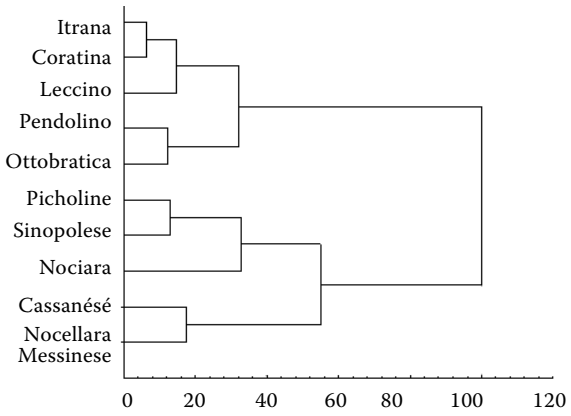

Figure 1. Dendrogram of olive oils obtained from cluster analysis (complete linkage)

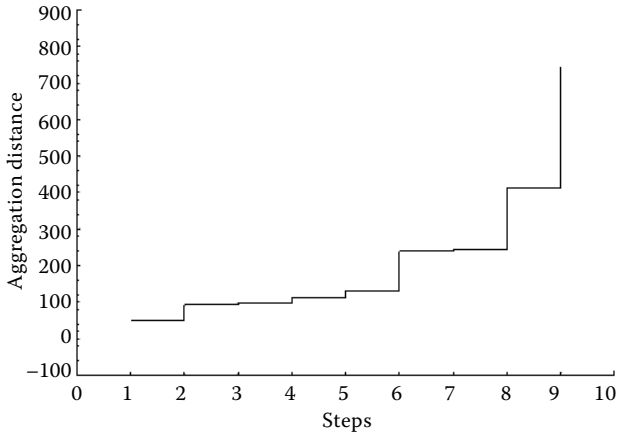

Figure 2. Aggregation distances by steps

effect influenced almost all sterol components ($P \leq 0.05$), campestanol, Δ^7-stigmastenol and stigmasterol, and highly significantly ($P \leq 0.01$) β-sitosterol, sitostanol, $\Delta^{5,24}$-stigmastadienol, Δ^7-avenasterol, campesterol, Δ^5-avenasterol, Δ^7-campesterol, 24-methylene-cholesterol, and total sterol content. Cercaci *et al.* (2007) found out that β-sitosterol and Δ^5-avenasterol are influenced by harvesting date.

Cluster analysis

The studied cultivars were submitted to a classification test using cluster analysis, based on their different content of sterol compounds. The dendrogram (Figure 1) shows the associations obtained on the basis of similarity in Euclidian distances. The aggregation distances between the classes that have been associated can also be represented by the chart as in Figure 2. The dendrogram indicates that, at a rescaled distance of 400, the cultivars are distributed in three major clusters. While, at a rescaled distance of 210, there are five groups; the first is constituted by two cvs Pendolino and Ottobratica having the two lowest sterol and β-sitosterol apparent amounts. Cvs Picholine and Sinopolese constituted the second group showing the two highest sterol contents; the third group includes cvs Leccino, Itrana, and Coratina characterised by a high Δ^5-avenasterol and a low β-sitosterol amount, the two latter cultivars are the first to be linked. The fourth group is made by cvs Cassanese and Nocellara Messinese with a high campesterol/stigmasterol ratio and low Δ^5-avenasterol content; the last group is constituted by cv. Nociara with the highest Δ^5-avenasterol content.

Variations in each crop season

The behaviour of the different cultivars for the total sterol content and for β-sitosterol content

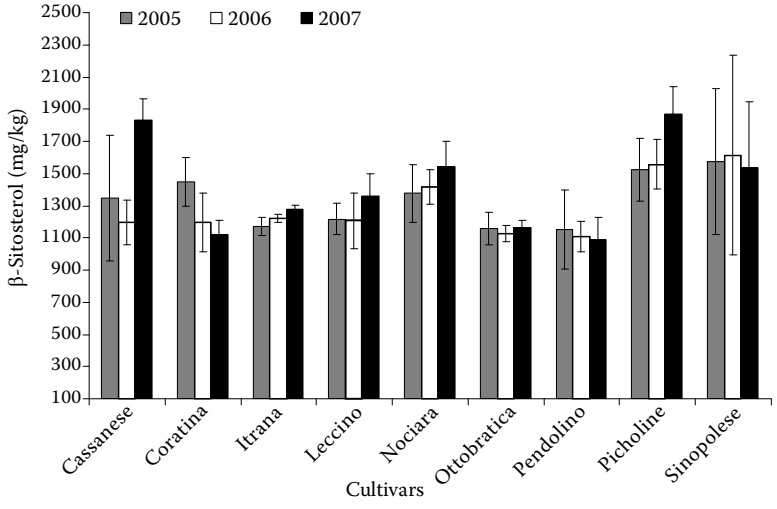

Figure 3. Variation in total sterol content for the three crop seasons 2005–2007 and for the different cultivars. The values represent the means of triplicates ± standard error

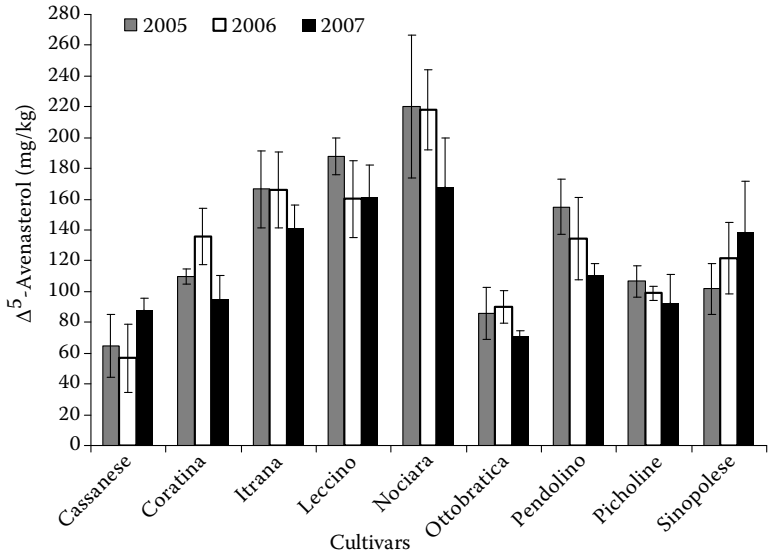

Figure 4. Variation in β-sitosterol content for the three harvest seasons 2005–2007 and for the different cultivars. The values represent the means of triplicates ± standard error

varied from one cultivar to another and it also varied from one crop season to another (Figures 3 and 4). Cultivars Cassanese (autochthonous) and Picholine (allochthonous) showed the same increasing trend during the three crop seasons, mainly in the last season, when the total sterol content increased highly, however the total sterol content in Cassanese cultivar decreased in the second crop season. The cvs Leccino, Itrana, and Nociara showed more or less the same trend. The total sterol content in cvs Sinopolese and Ottobratica, both autochthonous cultivars, was approximately constant, while cvs Pendolino and Coratina showed a decreasing trend, the opposite compared to all other cultivars.

Figure 5 shows the variation of Δ^5-avenasterol content during the three crop seasons and for the different cultivars. The increasing trend was observed only in cv. Sinopolese, unlike for cvs Pendolino and Picholine, which showed a decreasing trend. Cvs Itrana and Nociara showed constant Δ^5-avenasterol content in the first two crop seasons with a tendency to decrease in the last season unlike the Leccino cultivar, where the maximum Δ^5-avenasterol content was observed in the first crop year and constant Δ^5-avenasterol content in the second and the third crop years. The Δ^5-avenasterol content in cvs Ottobratica and Coratina showed an increasing trend from the first to the second season, then a decreasing trend from the second to the last season, the opposite was observed in cv. Cassanese.

The correlation between some sterol compounds was studied. The highest correlation was observed between β-sitosterol and campesterol content (r = 0.9394) (Figure 6). A positive correlation was also calculated between $\Delta^{5,24}$-stigmastadienol and Δ^7-avenasterol (r = 0.6970) and between 24-methylene-cholesterol and Δ^5-avenasterol (r = 0.6789). In addition, there were other correlations between $\Delta^{5,24}$-stigmastadienol and Δ^5-avenasterol

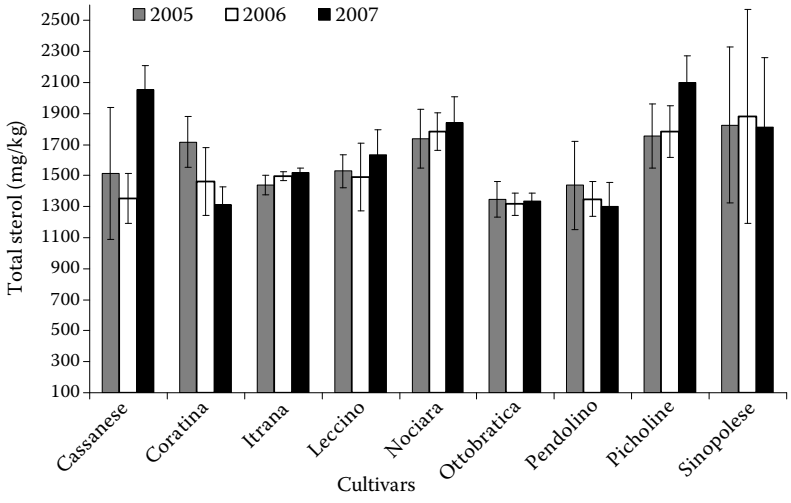

Figure 5. Variation in Δ^5-avenasterol content for the three harvest seasons 2005–2007 and for the different cultivars. The values represent the means of triplicates ± standard error

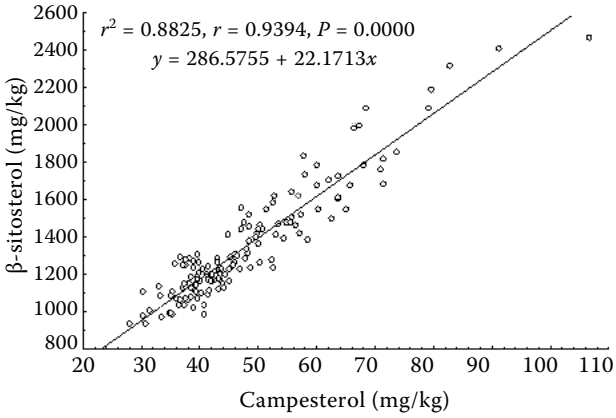

Figure 6. The variation in β-sitosterol content in the function of campesterol content

(r = 0.6209), and between the β-sitosterol and chlerosterol with a positive correlation (r = 0.6720).

CONCLUSIONS

It was shown in this study that the sterol composition in the olive oils extracted from the studied cultivars is in conformity with the two regulations used as references. No previous data exist on the influence of crop season and cultivar on the sterol composition of monovarietal olive oils in Reggio Calabria (Calabria, Southern Italy). These results also demonstrated statistically significant differences in the sterol composition of olive oil of different cultivars grown in the same geographical area. The crop season effect influences only stigmasterol, β-sitosterol, campesterol/stigmasterol and Δ^7-avenasterol. Variation in sterols is probably due to climatic and genetic factors and to the interaction between genes of each olive cultivar and environment. The results show a very good adaptation of the allochthonous cultivars to the local environment. The cluster analysis allowed us also to classify the cultivars in different classes linked with each other. Qualitative and quantitative analyses could be used to characterize extra virgin olive oil of the studied cultivars, using sterols as markers of the growing area.

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