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Comparative study of phytosterol derivatives in monovarietal olive oils[#]

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Abstract: Plant sterols and their derivatives are minor compounds that have been extensively studied in vegetable oils, mainly in olive oil, where they are closely related with its identity. The objective of this work is to determine the content of free and esterified sterol glucosides and their profiles in olive oil in relation to different geographical situation of olive orchards, cultivar, farming modality, and sampling time. The orchards under study were located in the outer ring of the sub-metropolitan area of Madrid (Spain), where olives from Cornicabra, Manzanilla Cacereña, Manzanilla Castellana, and Picual varieties were grown under traditional and organic modes, and harvested in four different samplings. Conclusions state that cultivar, farming mode, and light-exposure, do not have outstanding effects, whereas pedoclimate might affect the sterol glucoside presence in a substantial way. Further studies are being carried out presently in order to confirm such statement. Also glucoside derivative profiles are discussed, and reasons for differences with results in previous studies pointed out.

Keywords: olive oil, Solid Phase Extraction (SPE), Sterol Glucoside (SG), Esterified Sterol Glucoside (ESG), pedoclimate

INTRODUCTION

Phytosterols, as well as their derivatives, are compounds of the unsaponifiable matter of vegetable oils, whose amount and profile are important parameters of oil identity. In case of sterol derivatives, SG consist of a glucose moiety bound at the C3 position of the sterol residue via an acetyl bond. If additionally a fatty acid is esterified at the C6 position of the sugar, ESG are formed.

The presence of free and esterified sterol glucosides in edible matrices has been widely studied, although their synthesis pathway and influencing factors are not clear yet.¹ The health promoting effects of sterol glucosides have been unknown for a long time. Their use for the treatment of pathologies related to elevated cholesterol in blood was never compared to those of FS solutions² or functional foods,³ and it was not until 2009 that studies on this field demonstrated the potential health benefits of glucosylated phytosterols in both free and esterified (acylated) forms.^{4,5} Actually, SG could get reductions of around 37% in cholesterol absorption in the gut of human subjects, a value similar to that 30% obtained with SE.⁴ As a consequence, research on possible sterol glucoside sources became more relevant and groups started publishing detailed data on the content and composition of glucosylated sterols in edible plant matrices to facilitate comparisons of the potential contribution of different foods as sources of sterol glucosides when included in the diet.⁶ Those studies did not usually included olive oil, which justifies our studies on the presence of both SG and ESG in this matrix.

One would expect the SG and ESG composition to be closely related to the free sterol pool, and therefore the contribution of both SG and ESG to be bound to the oil identity. The procedure for isolating sterol glucosides from olive oil had been developed earlier. Initially the method was applied to a small number of oil samples of

different categories and origins,⁷ and then to a wider number also from different cultivars and treated with different refining processes,⁸ reaching the conclusion that the only SG that might be present was β -sitosteryl glucoside (BSSG), whose concentration in olive oil was never higher than 3 mg kg⁻¹. Regarding ESG, mass spectrometry results confirmed that in the majority of the samples the only ESG present -at concentration sometimes above 8 mg kg⁻¹- was esterified β -sitosteryl glucoside (EBSSG), possibly eluting just before small quantities of esterified Δ 5-avenasteryl glucoside (EAvSG).

In this case we have determined the composition and content of free and esterified steryl glucosides in a more systematic way, paying special attention to the contribution of each steryl glucoside to the total glucoside concentration. We have focused on monovarietal olive oils from different geographical situation, farming modality, and sampling time. Our intention has been not only to start determining the factors affecting the steryl glucoside presence, but also to contribute in a direct way to nutrient databases.

MATERIALS AND METHODS

Reagents. The internal standard ChSG, commercial mixtures of ESG and SG, and the analytical-grade chemical reagents used in the course of this research were the same as those utilized previously.⁸

Samples and standard solutions. Virgin olive oils were obtained from Cornicabra, Manzanilla Cacereña, Manzanilla Castellana, and Picual olive varieties. They were directly extracted in the laboratory of Doctor Pérez Jiménez group (IMIDRA) using an Abencor® system identical to that described earlier.⁸ Our blank-sample (chemically refined olive-pomace oil) was obtained directly from the producers.

Stock solutions ($100 \mu\text{g mL}^{-1}$) and samples were treated as described formerly,^{7,8} in short: solutions of ESG standard were prepared by dissolving the standard mixture in chloroform. Stocks solutions of SG and of ChSG were made with a chloroform:methanol (2:1, by volume) blend. Samples consisted of 2 g oil dissolved in 4 mL chloroform, spiked with 100 μL of ChSG stock solution. The hydroxyl groups were derivatised (1h at 60 °C) with 200 μL of a mixture of Py:HMDS:TMCS (9:3:1, by volume) before carrying out the GC analysis.

Olive orchards and olive oil extraction. The orchards were situated in Madrid, the centre part of Spain, near the Manzanares River (Mediterranean climate), where they could be kept under optimal cultivation conditions. Specifically they were located in eight municipalities in the southern outer ring of the sub-metropolitan area (Table 1).

In these regions winters are cold due to the altitude (average of 667 m above sea level), being January the coldest month with temperatures between 3 and 10 °C, and 148 sunshine h. These are dry climate areas, actually the common annual precipitations are between 400 and 500 mm per year in municipalities A-E, and below 400 mm per year in municipalities F-H, that concentrate from October to May, being October and December are the wettest months (56 mm).

Fruits from four different cultivars -Cornicabra, Manzanilla Cacereña, Manzanilla Castellana, and Picual cv.- were obtained from irrigated traditional orchards (drip irrigation). Additionally, olives from Manzanilla Castellana and Cornicabra cultivars were also obtain from organic orchards (Table 1).

The olive plantations were in classical frame with around 300 trees per hectare. Fruits were handpicked according to the sampling time shown in Table 1.

To assure maximum oil quality, olives were kept in darkness at 4 °C, and oil was extracted within 24 h after harvesting using an Abencor® system and a small-quantity

mill simulating commercial oil-extraction procedures (MC2 Ingeniería de Sistemas, Sevilla, Spain): the olives were crushed with an Abencor® hammer mill equipped with a 4-mm sieve; 700 g paste were processed using the system's malaxer and centrifuge during 1 min at 3500 rpm. Conditions: malaxation temperature, 27 °C; malaxation time, 20 min, plus 10 min after water addition; amount of distilled water added to the paste, 300 mL; amount of talc added, 20 g. The mixtures were left to decant during 24 h before proceeding to oil separation and filtration. To facilitate the work comprehension the oil samples were coded according to the Table 1.

Instrumentation. GC analyses of the sterol glucosides were carried out with an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an Agilent 7683B Automatic Liquid Sampler and FID. Acquisition of data was done with the Agilent ChemStation for GC System program. The conditions for the GC assays were: TRB-5HT column (5% diphenyl-95% dimethylpolysiloxane; 30 m x 0.32 mm ID x 0.10 µm film; Teknokroma, Sant Cugat del Vallés, Barcelona, Spain), 1.0 µL injection volume, hydrogen carrier gas at 3 mL min⁻¹ and ECP cool on-column injection. The oven temperature program was: 80 °C (1 min), 50 °C min⁻¹ to 320 °C (12 min), and 40 °C min⁻¹ to 360 °C (19 min). The detector temperature was 360 °C.

Steryl glucoside analysis. The SPE procedure developed previously⁸ was utilized. The method consist of the conditioning of the cartridges (ExtraBond Si-1 g; Scharlab S.L., Barcelona, Spain) with 5 mL *tert*-butyl methyl ether, 5 mL hexane, 5 mL chloroform, and 10 mL of a newly prepared blend consisting of chloroform:methanol 4:1, by volume. Samples are then quantitatively transferred using 1 mL chloroform to wash the walls of the containers. The loaded cartridges are washed with 10 mL hexane, 10 mL *tert*-butyl methyl ether, and 10 mL chloroform, applied one after the other. The steryl glucosides are eluted with 5 mL of the chloroform:methanol 4:1, by

volume, solution. The procedure was carried out under negative pressure (1 mm.Hg) in a vacuum manifold. The eluted fractions are evaporated to dryness under nitrogen flux, derivatised and chromatographed using cool on-column injection.

The quantitative evaluation of the SG was carried out using ChSG as internal standard, whereas we quantified the ESG using three-point calibration curves (one specific curve for each ESG) built by spiking the in-house blank sample with the commercial standard. This evaluation together with the purity, qualitative and quantitative composition of the commercial standards is comprehensively described in our former work.⁸

All statistical analyses were run using the software GraphPad Prism[®] version 5.01 for Windows. The samples were compared by an ANOVA, with statistical significance of $p < 0.05$. After that they were differed by a TUKEY average comparison test.

RESULTS AND DISCUSSION

In the present study we have analysed 44 oil samples, each of them at least in triplicate, in a relatively short time (six determinations/h) and with fairly little solvent volumes, in comparison with former techniques, e.g. column chromatography.⁹ This has proved the suitability of the system to be applied on a routine basis.

The quantitative results of the present study are shown in Tables 2a-3b. For the SG, the total amount is within the range 0.59 ± 0.01 - 2.62 ± 0.04 mg kg⁻¹, being the lowest and the highest amounts those of Manzanilla Castellana samples from Valdelaguna, MCLL_T_VG_3 and MCLL_O_VG_4, respectively. If we compare them with those obtained previously⁸ it is clear that the SG concentration in virgin olive oils, devoid of defects, keeps constant within certain ranges. Consequently, we could assume that the SG concentration in virgin olive oils will be within the 0.6-2.7 mg kg⁻¹ range.

In case of the SG profile, BSSG is always the main SG present, contributing at least with $70.52 \pm 1.18\%$ to the total. Actually, MCLL_T_VG_3 and MCLL_T_VS_3 only show BSSG. On the other hand, CSG and AvSG have great importance in almost all samples. MCAC_T_CR_4 has the highest CSG concentration ($13.38 \pm 0.03\%$) whereas MCLL_T_AC_4 exhibits the highest amounts of AvSG ($14.96 \pm 0.28\%$). SSG was present only in two Manzanilla Cacereña samples: MCAC_T_VS_3 ($11.91 \pm 1.45\%$) and MCAC_T_CR_4 ($3.29 \pm 0.82\%$). If we compare these results with the FS profile from olive oil, it is clear that there is no relationship between them beyond the fact that β -sitosterol derivatives are always present.

ESG contents are within a range from 0.18 ± 0.01 to $3.07 \pm 0.07 \text{ mg kg}^{-1}$. Previous studies⁸ show varieties with very high ESG concentrations (e. g. Uovo di Pichone, $11.29 \pm 2.75 \text{ mg kg}^{-1}$, and Blanqueta, $9.63 \pm 0.71 \text{ mg kg}^{-1}$), which are not discussed here. If we compare the samples that were common to both studies (Cornicabra and Picual), ESG concentrations are nearly the same. Compiling global results from both studies it is possible to establish a concentration interval from 0.2 to 14.0 mg kg^{-1} for ESG presence in olive oil. As far as the profiles are concern, ECSG was never detected, while ESSG was seen only in two samples, which were those where SSG could be determined. EAvSG was detected in 70% of the samples (3.23 - 74.82% range). Except in the case of MCLL_T_VS_3, where EBSSG was $25.18 \pm 2.34\%$ of total ESG, in all the others samples, EBSSG was the main ESG, showing concentrations above 69%. Although it has already been demonstrated that there is no correspondence between the SG profile and that of their esterified counterparts,^{6,8} this lack of any equivalence is still striking. Once again, it becomes clear that there is no connection between both SG and ESG concentrations, and that the SG:ESG rate does not have to be lower than 1 (Tables 3a and 3b). Actually it is above 1 in more than

77% of the instances. Furthermore there is not a correlation between global steryl glucoside quantities and their corresponding profiles. The fact of having high SG and/or elevated ESG concentration(s) does not imply richer profile(s), and vice versa. Actually, all four SG have been found in sample MCAC_T_CR_4 (Figure 1), together with three other ESG, and the respective concentrations of both kinds of sterol glucoside derivatives are not significantly higher than with 'poorer' sets.

The cultivars tested in the present study cannot be considered different as far as the (E)SG content is concern. However, we may observe that the Cornicabra variety shows the highest SG:ESG relationship, being in some cases surprisingly elevated (samples COR_O_VG_4, COR_T_CH_4, and COR_O_AZ_3, with values of 8.6, 6.1, and 4.7, respectively). On the other hand the values are the lowest (around 1) in Manzanilla Cacereña cv. Finally, Picual cv. presents a more evenly distribution of the SG:ESG correlation.

Although correlation and cluster analysis, and other statistical analysis (PCA and HCA) did not let get a clear distribution pattern to correlate sample characteristics with their (E)SG composition and content, calculation of three times the standard deviation allowed us to group results provisionally. Table 4 shows the SG and ESG results grouped according to the origin of the olive fruits. The fact of having somehow acceptable relative SD values (between 1 and 30%) when observing the SG concentration as a function of the location of the olive tree, and not on dependence of the cultivar, drive us to think that it might actually be the pedoclimate (soil water, nutrients and aeration) what has any real influence on such values and not the fruit variety, as one might think *a priori*. Wider studies on this respect are presently being carried out. On the other hand, the ESG behaviour remains quite random, supporting our previous statement that both finds of phytosterols (SG and ESG) behave

independently from each other. The same trend is observed when classifying the results according to the sampling time (Table 5). Whereas results on the two different farming modes (traditional and organic) cannot be conclusive, mainly due to the lack of enough sample material.

To summarize we will conclude as follows: The performance of more than hundred analyses in a relatively short time and with no waste of solvent has demonstrated the suitability of the procedure to be applied on a routine basis.

The presence of steryl glucosides in olive oil is not just limited to BSSG and EBSSG as thought previously, but also AvSG, EAvSG, and CSG have been found in an extensive number of samples. This might also be symptomatic either of different sample treatment, or of differences during the oil extraction process. Actually it seems that industrial samples⁸, which have been obtained using a two or three phases decanter (higher water addition) have poorer (E)SG profiles than those samples obtained with the Abencor® system, where water addition has been scarce. This reasoning seems to be supported by the fact of finding certain molecules (e.g. CSG) in Abencor® samples, and not finding them in industrial oils. In any case a wider number of determinations under controlled circumstances must be carried out before drawing definite conclusions.

Olive cultivar, sampling time and farming mode have no influence on the parameters under study. Pedoclimate (soil aeration, micronutrient presence, and water content) seems to have some influence on the glucoside presence. In this way further studies are being carried out to confirm such relationship.

ABBREVIATIONS USED

ChSG cholesterol β -D-glucoside
(E)AvSG (esterified) Δ^5 -avenasteryl glucoside
(E)BSSG (esterified) β -sitosteryl glucoside
(E)CSG (esterified) campesteryl glucoside
ESG esterified sterol glucosides
(E)SSG (esterified) stigmasteryl glucoside
EVOO extra virgin olive oil
FID flame ionization detector
FS free sterols
GC gas chromatography
HMDS hexamethyldisilazane
LOO lampante olive oil
Py pyridine
SD standard deviation
SE sterol esters
SG free sterol glucosides
SPE solid phase extraction
TMCS trimethylchlorosilane
VOO virgin olive oil

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FIGURE CAPTION

Figure 1. GC-FID chromatograms for steryl glucosides in virgin olive oil extracted from olive fruits of the Manzanilla cacereña cultivar (sample MCAC_T_CR_4). This chromatogram has been obtained after analysing the sample according to the proposed method and it shows: 1. the internal standard cholesterol glucoside, 2. campesteryl glucoside, 3. stigmasteryl glucoside, 4. β -sitosteryl glucoside, 5. (presumptive) Δ^5 -avenasteryl glucoside, 6. esterified stigmasteryl glucoside, 7. esterified β -sitosteryl glucoside, 8. (presumptive) esterified Δ^5 -avenasteryl glucoside.

Table 1. Identification and Coding System of Studied Samples.

Variable	Coding	Assignment
Cultivar	MCAC	Manzanilla Cacereña
	COR	Cornicabra
	MCLL	Manzanilla Castellana
	PIC	Picual
Farming Modality	T	Traditional
	O	Organic
Municipality	AC	Anchuelo
	AG	Arganda
	AZ	Aranjuez
	CR	Campo Real
	CH	Chinchón
	TI	Tielmes
	VG	Valdelaguna
	VS	Vilarejo de Salvanés
Sampling Time	1	Nov, 14 th
	2	Nov, 28 th
	3	Dec, 12 th
	4	Dec, 26 th

Table 2a. Total Steryl Glucosides Contents (mg kg^{-1}) of Olive Oil from Different Cultivars, and Relative Composition Regarding Campesteryl Glucoside (CSG), Stigmasteryl Glucoside (SSG), β -Sitosteryl Glucoside (BSSG), and $\Delta 5$ -Avenasteryl Glucoside (AvSG). A Minimum of Two Independent Measures Was Made on Each Case. The Standard Deviation is Also Given; ND (Non Detected)

Samples	Steryl Glucosides														
	CGS			SSG			BSSG			AvSG			Total		
	%	SD	[†]	%	SD	[†]	%	SD	[†]	%	SD	[†]	mg kg ⁻¹	SD	[†]
MCAC_T_CR_1	2.46	2.40	^{a,b}	ND	-		86.89	3.14	^{c,d}	10.65	0.73	^{c,d}	2.08	0.05	^d
MCAC_T_VS_2	ND	-		ND	-		90.30	1.00	^d	9.70	1.00	^c	1.83	0.14	^{c,d}
MCAC_T_CR_3	5.25	0.37	^b	ND	-		79.94	0.51	^b	14.81	0.88	^{c,d}	2.22	0.01	^d
MCAC_T_VS_3	ND	-		11.91	1.45	^b	81.21	1.13	^{b,c}	6.88	0.33	^{b,c}	2.33	0.05	^{d,e}
MCAC_T_AZ_4	3.18	1.17	^{a,b}	ND	-		90.72	1.84	^d	6.10	0.66	^{b,c}	2.15	0.24	^d
MCAC_T_CR_4	13.38	0.03	^c	3.30	0.82	^a	70.52	1.18	^a	12.81	0.39	^{c,d}	2.28	0.02	^{d,e}
COR_T_AZ_1	2.41	2.51	^{a,b}	ND	-		91.38	0.13	^{d,e}	6.21	2.63	^{b,c}	1.74	0.07	^{c,d}
COR_T_CH_1	ND	-		ND	-		96.59	3.50	^e	3.41	3.50	^{a,b}	1.53	0.04	^{b,c}
COR_T_CH_2	2.06	2.04	^{a,b}	ND	-		94.17	1.77	^{d,e}	3.78	3.81	^{a,b}	1.36	0.01	^{b,c}
COR_T_AZ_2	4.67	0.70	^b	ND	-		93.12	1.48	^{d,e}	2.20	2.18	^{a,b}	2.16	0.02	^d
COR_T_CH_3	6.42	0.02	^b	ND	-		90.41	3.17	^d	3.17	3.15	^{a,b}	1.68	0.01	^{b,c}
COR_T_VS_3	5.29	0.83	^b	ND	-		86.12	0.98	^c	8.59	0.16	^{b,c}	1.68	0.02	^{b,c}
COR_T_AZ_3	4.59	0.57	^b	ND	-		89.53	0.39	^{c,d}	5.88	0.18	^{b,c}	2.08	0.05	^d
COR_T_CH_4	5.84	0.05	^b	ND	-		91.92	2.16	^{d,e}	2.24	2.21	^{a,b}	2.38	0.03	^{d,e}
COR_T_AZ_4	ND	-		ND	-		94.33	0.93	^{d,e}	5.67	0.93	^{b,c}	1.96	0.20	^{c,d}
COR_T_VS_4	3.16	0.51	^{a,b}	ND	-		89.29	0.81	^{c,d}	7.55	0.30	^{b,c}	2.14	0.11	^d
COR_E_AZ_1	1.77	1.78	^{a,b}	ND	-		92.29	1.23	^{d,e}	5.94	0.55	^{b,c}	1.63	0.01	^{b,c}
COR_E_VG_1	3.69	3.53	^{a,b}	ND	-		96.31	3.53	^e	ND	-		1.61	0.07	^{b,c}
COR_E_AZ_2	4.32	0.06	^b	ND	-		90.45	0.11	^d	5.22	0.05	^{b,c}	1.93	0.12	^{c,d}
COR_E_VS_2	1.77	1.78	^{a,b}	ND	-		92.29	1.23	^{d,e}	5.94	0.55	^{b,c}	1.63	0.01	^{b,c}
COR_E_CH_2	ND	-		ND	-		96.32	3.60	^e	3.68	3.60	^{a,b}	2.04	0.05	^{c,d}
COR_E_AZ_3	4.21	0.18	^{a,b}	ND	-		90.38	0.12	^d	5.40	0.30	^{b,c}	2.17	0.05	^d

^f The use of the same superscripts (^{a-e}) indicates the absence of statistic difference between samples (ANOVA/TUKEY test)

Table 2b. Total Steryl Glucosides Contents (mg kg^{-1}) of Olive Oil from Different Cultivars, and Relative Composition Regarding Campesteryl Glucoside (CSG), Stigmasteryl Glucoside (SSG), β -Sitosteryl Glucoside (BSSG), and $\Delta 5$ -Avenasteryl Glucoside (AvSG). A Minimum of Two Independent Measures Was Made on Each Case. The Standard Deviation is Also Given; ND (Non Detected)

Samples	Steryl Glucosides														
	CGS			SSG			BSSG			AvSG			Total		
	%	SD	f	%	SD		%	SD	f	%	SD	f	mg kg^{-1}	SD	f
COR_E_VG_4	ND	-		ND	-		96.77	3.22	e	3.23	3.22	a,b	2.60	0.01	e
MCLL_T_TI_1	ND	-		ND	-		96.80	3.20	e	3.20	3.20	a,b	1.95	0.00	c,d
MCLL_T_AC_1	ND	-		ND	-		91.06	0.58	d,e	8.94	0.58	b,c	1.34	0.04	b,c
MCLL_T_AC_2	1.62	1.57	a,b	ND	-		88.89	1.92	c,d	9.49	0.34	b,c	1.54	0.04	b,c
MCLL_T_TI_2	ND	-		ND	-		95.13	0.02	d,e	4.87	0.02	a,b,c	2.00	0.02	c,d
MCLL_T_VS_2	7.61	1.72	b	ND	-		83.49	1.54	b,c	8.90	0.17	b,c	1.70	0.02	c
MCLL_T_VG_3	ND	-		ND	-		100.00	-		ND	-		0.59	0.01	a
MCLL_T_VS_3	ND	-		ND	-		100.00	-		ND	-		1.33	0.09	b
MCLL_T_VS_4	0.82	0.80	a	ND	-		89.53	0.60	c,d	9.66	1.40	b,c	1.57	0.03	b,c
MCLL_T_TI_4	ND	-		ND	-		94.98	0.20	d,e	5.02	0.20	a,b,c	2.02	0.01	c,d
MCLL_T_AC_4	3.77	3.60	a,b	ND	-		81.28	3.32	b,c	14.96	0.28	d	1.71	0.08	c
MCLL_E_VG_2	ND	-		ND	-		91.06	0.52	d,e	8.94	0.52	b,c	1.98	0.04	c,d
MCLL_E_VG_4	ND	-		ND	-		94.77	0.04	d,e	5.23	0.04	b,c	2.62	0.04	e
PIC_T_AZ_1	6.57	0.39	b	ND	-		85.69	0.27	c	7.73	0.66	b,c	1.90	0.04	c,d
PIC_T_AG_1	ND	-		ND	-		92.16	0.42	d,e	7.84	0.42	b,c	1.85	0.01	c,d
PIC_T_AG_2	ND	-		ND	-		94.26	0.42	d,e	5.74	0.42	b,c	1.89	0.02	c,d
PIC_T_CR_2	3.55	3.61	a,b	ND	-		87.85	0.25	c,d	8.59	3.36	b,c	2.41	0.04	d,e
PIC_T_AZ_3	5.25	0.93	b	ND	-		89.66	1.35	c,d	5.09	0.42	a,b,c	1.75	0.04	c,d
PIC_T_CR_3	ND	-		ND	-		94.56	0.00	d,e	5.44	0.00	b,c	2.61	0.14	e
PIC_T_CR_4	ND	-		ND	-		94.96	0.09	d,e	5.04	0.09	a,b,c	1.69	0.01	b,c
PIC_T_AG_4	ND	-		ND	-		94.49	0.79	d,e	5.51	0.79	b,c	1.99	0.03	c,d
PIC_T_AZ_4	ND	-		ND	-		94.25	0.69	d,e	5.75	0.69	b,c	2.13	0.06	d

^f The use of the same superscripts (^{a-e}) indicates the absence of statistic difference between samples according to ANOVA and TUKEY test.

Table 3a. Total Steryl Glucosides Contents (mg kg^{-1}) of Olive Oil from Different Cultivars, and Relative Composition Regarding Esterified Stigmasteryl Glucoside (ESSG), Esterified β -Sitosteryl Glucoside (EBSSG), and Esterified $\Delta 5$ -Avenasteryl Glucoside (EAvSG). A Minimum of Two Independent Measures Was Made on Each Case. The Standard Deviation is Also Given; ND (Non Detected)

Samples	Esterified Steryl Glucosides											Ratio		
	ESSG			EBSSG			EAvSG			Total		SG:ESG		
	%	SD	^f	%	SD	^f	%	SD	^f	mg kg ⁻¹	SD	^f	%	^f
MCAC_T_CR_1	ND	-		84.50	1.52	^{c,d}	14.03	1.52	^{b,c}	3.07	0.11	^h	0.68	^a
MCAC_T_VS_2	ND	-		84.14	3.88	^{c,d}	15.86	3.88	^c	2.01	0.08	^{e,f}	0.92	^{a,b}
MCAC_T_CR_3	ND	-		73.28	1.84	^{b,c}	26.72	1.84	^d	1.55	0.12	^{d,e}	1.44	^{a,b}
MCAC_T_VS_3	24.20	0.30	^b	69.74	0.12	^b	6.06	0.21	^{a,b}	2.24	0.05	^{f,g}	1.04	^{a,b}
MCAC_T_AZ_4	ND	-		87.80	1.49	^d	12.20	1.49	^{b,c}	1.78	0.18	^{e,f}	1.20	^{a,b}
MCAC_T_CR_4	10.96	0.70	^a	74.15	0.68	^{b,c}	14.88	0.04	^{b,c}	2.74	0.05	^{g,h}	0.83	^{a,b}
COR_T_AZ_1	ND	-		100.00	-		ND	-		0.64	0.06	^{b,c}	2.75	^b
COR_T_CH_1	ND	-		95.98	4.34	^e	4.02	4.34	^{a,b}	1.69	0.12	^e	0.91	^{a,b}
COR_T_CH_2	ND	-		89.93	0.69	^{d,e}	10.07	0.69	^{b,c}	0.85	0.06	^{c,d}	1.60	^{a,b}
COR_T_AZ_2	ND	-		100.00	-		ND	-		0.89	0.03	^{c,d}	2.43	^b
COR_T_CH_3	ND	-		89.05	0.78	^{d,e}	10.95	0.78	^{b,c}	1.82	0.02	^{e,f}	0.92	^{a,b}
COR_T_VS_3	ND	-		76.42	4.62	^{b,c}	23.58	4.62	^d	1.48	0.07	^{d,e}	1.14	^{a,b}
COR_T_AZ_3	ND	-		81.92	6.60	^{c,d}	18.08	6.60	^{c,d}	1.04	0.02	^{c,d}	2.00	^{a,b}
COR_T_CH_4	ND	-		79.27	1.00	^c	20.73	1.00	^{c,d}	0.39	0.03	^b	6.20	^c
COR_T_AZ_4	ND	-		96.77	3.45	^e	3.23	3.45	^{a,b}	1.10	0.07	^{c,d}	1.77	^{a,b}
COR_T_VS_4	ND	-		84.85	3.11	^{c,d}	15.15	3.11	^c	0.83	0.03	^c	2.58	^b
COR_E_AZ_1	ND	-		91.11	1.92	^{d,e}	8.89	1.92	^{b,c}	2.34	0.08	^{f,g}	0.70	^a
COR_E_VG_1	ND	-		100.00	-		ND	-		1.04	0.04	^{c,d}	1.54	^{a,b}
COR_E_AZ_2	ND	-		100.00	-		ND	-		0.96	0.06	^{c,d}	2.01	^{a,b}
COR_E_VS_2	ND	-		86.21	3.84	^{c,d}	13.79	3.84	^{b,c}	1.71	0.06	^{e,f}	0.96	^{a,b}
COR_E_CH_2	ND	-		100.00	-		ND	-		1.20	0.06	^d	1.71	^{a,b}
COR_E_AZ_3	ND	-		100.00	-		ND	-		0.46	0.01	^b	4.73	^c

^f The use of the same superscripts (^{a-e}) indicates the absence of statistic difference between samples according to ANOVA and TUKEY test.

Table 3b. Total Steryl Glucosides Contents (mg kg^{-1}) of Olive Oil from Different Cultivars, and Relative Composition Regarding Esterified Stigmasteryl Glucoside (ESSG), Esterified β -Sitosteryl Glucoside (EBSSG), and Esterified $\Delta 5$ -Avenasteryl Glucoside (EAvSG). A Minimum of Two Independent Measures Was Made on Each Case. The Standard Deviation is Also Given; ND (Non Detected)

Samples												Ratio	
	ESSG		EBSSG			EAvSG			Total			SG:ESSG	
	%	SD	%	SD	f	%	SD	f	mg Kg ⁻¹	SD	f	%	f
COR_E_VG_4	ND	-	100.00	-		ND	-		0.30	0.05	a,b	8.94	d
MCLL_T_TI_1	ND	-	88.31	1.52	d,e	11.69	1.52	b,c	1.30	0.07	d	1.51	a,b
MCLL_T_AC_1	ND	-	91.87	0.74	d,e	8.13	0.74	b,c	2.36	0.16	f,g	0.57	a
MCLL_T_AC_2	ND	-	80.73	1.53	c,d	19.27	1.53	c,d	2.33	0.07	f,g	0.66	a
MCLL_T_TI_2	ND	-	100.00	-		ND	-		1.36	0.10	d,e	1.47	a,b
MCLL_T_VS_2	ND	-	88.61	0.27	d,e	11.39	0.27	b,c	0.72	0.13	b,c	2.47	b
MCLL_T_VG_3	ND	-	100.00	-		ND	-		0.18	0.03	a,b	3.45	b,c
MCLL_T_VS_3	ND	-	25.18	2.34	a	74.82	2.34	e	0.45	0.05	b	3.01	b
MCLL_T_VS_4	ND	-	81.72	1.38	c,d	18.28	1.38	c,d	1.10	0.06	c,d	1.44	a,b
MCLL_T_TI_4	ND	-	89.53	4.28	d,e	10.47	4.28	b,c	1.01	0.06	c,d	2.00	a,b
MCLL_T_AC_4	ND	-	73.78	0.32	b,c	26.22	0.32	d	2.58	0.07	g	0.67	a
MCLL_E_VG_2	ND	-	83.61	4.05	c,d	16.39	4.05	c,d	1.36	0.01	d,e	1.46	a,b
MCLL_E_VG_4	ND	-	89.88	2.99	d,e	10.12	2.99	b,c	2.24	0.01	f,g	1.17	a,b
PIC_T_AZ_1	ND	-	90.72	2.04	d,e	9.28	2.04	b,c	2.05	0.18	f	0.93	a,b
PIC_T_AG_1	ND	-	100.00	-		ND	-		1.41	0.06	d,e	1.32	a,b
PIC_T_AG_2	ND	-	100.00	-		ND	-		0.86	0.04	c,d	2.21	b
PIC_T_CR_2	ND	-	94.58	1.38	d,e	5.42	1.38	a,b	2.04	0.30	e,f	1.21	a,b
PIC_T_AZ_3	ND	-	92.48	2.39	d,e	7.52	2.39	b	0.66	0.10	b,c	2.70	b
PIC_T_CR_3	ND	-	100.00	-		ND	-		0.90	0.01	c,d	2.89	b
PIC_T_CR_4	ND	-	100.00	-		ND	-		1.27	0.12	d	1.34	a,b
PIC_T_AG_4	ND	-	100.00	-		ND	-		1.17	0.06	c,d	1.70	a,b
PIC_T_AZ_4	ND	-	91.41	0.58	d,e	8.59	0.58	b,c	1.46	0.04	d,e	1.46	a,b

^f The use of the same superscripts (^{a-e}) indicates the absence of statistic difference between samples according to ANOVA and TUKEY test.

Table 4. SG and ESG Contents of the Olive Oil Samples Under Study Classified According the Municipality of Origin (A-H). Three Times the Standard Deviation is Also Given.

Municipality	SG $\pm$ 3SD	ESG $\pm$ 3SD
AC	1.52 $\pm$ 0.10	2.42 $\pm$ 0.05
AG	1.89 $\pm$ 0.01	1.15 $\pm$ 0.23
CR	2.19 $\pm$ 0.29	2.00 $\pm$ 2.58
CH	1.78 $\pm$ 0.50	1.19 $\pm$ 1.06
VG	2.28 $\pm$ 0.69	1.09 $\pm$ 1.77
AZ	1.88 $\pm$ 0.16	1.22 $\pm$ 0.98
TI	1.97 $\pm$ 0.01	1.22 $\pm$ 0.11
VS	1.80 $\pm$ 0.26	1.47 $\pm$ 1.41

Table 5. SG and ESG Contents of the Olive Oil Samples Under Study Classified According Sampling. Three Times the Standard Deviation is Also Given.

Sampling	SG $\pm$ 3SD	ESG $\pm$ 3SD
1	1.72 $\pm$ 0.13	1.63 $\pm$ 1.81
2	1.99 $\pm$ 0.34	1.44 $\pm$ 0.90
3	2.03 $\pm$ 0.58	1.40 $\pm$ 1.56
4	1.94 $\pm$ 0.35	1.38 $\pm$ 1.75

Figure 1

Table of Contents (TOC) Graphic