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Characterization of Fatty Alcohol and Sterol Fractions in Olive Tree

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The determination of sterols and fatty alcohols is a part of the study of the metabolomic profile of the unsaponifiable fraction in olive tree. Leaves and drupes from three varieties of olive tree (Arbequina, Picual, and Manzanilla) were used. The content of the target compounds was studied in five ripeness stages and three harvesting periods for olive drupes and leaves, respectively. A method based on ultrasound-assisted extraction and derivatization for the individual identification and quantitation of sterols and fatty alcohols, involving chromatographic separation and mass spectrometry detection by selected ion monitoring, was used. The concentrations of alcohols and sterols in the drupes ranged between 0.1 and 1086.9 μg/g and between 0.1 and 5855.3 μg/g, respectively, which are higher than in leaves. Statistical studies were developed to show the relationship between the concentration of the target analytes and variety, ripeness stage, and harvesting period.

KEYWORDS: Olive tree; leaves; drupes; fatty alcohols; sterols; GC-MS; statistical analysis

INTRODUCTION

Olive drupes are one of the most extensively cultivated fruit crops in the world. The cultivation area of olive trees has tripled in the past 44 years, from 2.6 to 8.6 million hectares (*1*). Hundreds of olive tree varieties have been selected over centuries for their adaptation to different microclimates and soil types. Among them, some cultivars are characteristic of a given zone, whereas others can be found in several countries. With regard to the names of the different varieties, the same name is sometimes given to clearly different varieties and different names are used for identical varieties (*2*).

Olive trees produce a wide variety of metabolites comprising relatively simple primary compounds to very complex products of the secondary metabolism with great chemical diversity (*3*).

Sterols and aliphatic alcohols are secondary metabolites, the determination of which is of great interest as the content of these compounds influences the quality of vegetal oils, including olive oil. Clinical studies have demonstrated that plant sterols, also known as phytosterols, ingested in the normal diet or as dietetic supplements, decrease cholesterol levels in blood, inhibiting their absorption in the thin intestine (*4*). Also, it is recognized that phytosterols are biologically active substances in cancer prevention, although it has still not been demonstrated by epidemiological studies (*5*). In addition, sterols and alcohol profiles are used to characterize virgin olive oil and, especially, to detect adulteration of olive oil by hazelnut oil (*6*). The content of these compounds, present in the unsaponifiable fraction of olive oils and other vegetal oils, has been regulated by Regulation 2568/91/EEC and later amendments (*7*).

Sterols are widely occurring natural substances in plant fats and make up the greatest proportion of the unsaponifiable fraction of lipids (*8*, *9*). Their composition depends on the plant species (*10*) and, in oils, the composition may vary according to agronomic and climatic conditions, the quality of the fruits or seeds, extraction and refining procedures, and storage conditions. The predominant phytosterol is β-sitosterol; minor components are campesterol, stigmasterol, Δ^5-avenasterol, Δ^7-avenasterol, and brassicasterol. Sterols, including precursors of cholesterol and their metabolites, can be biologically active, although many of these activities have to be clearly defined. However, the potential of sterols to bind to nuclear receptors and activate target gene transcription has encouraged much new research in this area (*11*). These compounds are membrane components and, as such, they regulate its fluidity and permeability. In plants, where they are always present in mixtures, sterols act as substrates for the production of a wide variety of secondary metabolites, such as cardenalids, glycoalkaloids, pregnane derivatives, and saponins.

With regard to fatty alcohols, the smallest molecules are used in cosmetics and food and as industrial solvents. Some of the largest molecules are simply seen as biofuels, but little research had been done until 2006 with regard to many of them, even though they had shown to be endowed with anticancer, antiviral, antifungal, and anti-HIV properties and, thus, with potential in medicine and as health supplements (*12*). Due to their amphipathic nature, fatty alcohols behave as nonionic surfactants. They find use as emulsifiers, emollients, and thickeners in the cosmetics and food industries and are common components of waxes, mostly as esters of fatty acids but also as free alcohols. Very long-chain fatty alcohols (VLCFA) obtained from plant waxes and beeswax have been reported to lower plasma cholesterol in humans (*13*). They can be found in unrefined cereal grains, beeswax, and many plant-derived foods. Reports suggest that 5−20 mg per day of mixed C24−C34 alcohols, including octacosanol and triacontanol, lowers low-density lipoprotein (LDL) cholesterol by 21−29% and

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raises high-density lipoprotein (HDL) cholesterol by 8−15%. Wax esters are hydrolyzed by a bile salt-dependent pancreatic carboxyl esterase, releasing long-chain alcohols and fatty acids that are absorbed in the gastrointestinal tract. Studies of fatty alcohol metabolism in fibroblasts suggest that very long-chain fatty alcohols, fatty aldehydes, and fatty acids are reversibly interconverted by a fatty alcohol cycle (*14*). The main alcohols present in olive oil are hexacosanol, octacosanol, tetracosanol, and docosanol (always at higher concentration in the oil from second press than in extra virgin olive oil) (*15*).

In the present research, the study of the profile of sterols and fatty alcohols was planned to be extended to three olive tree varieties, drupes collected during five maturation stages and leaves collected during three different seasons, grown in the same agricultural zone. Therefore, the differences found in the unsaponifiable fraction can be attributed to the characteristics of the referred varieties, the sampling period, or the maturation stage, taking into account that climate, type of soil, and other environmental conditions are common to all three.

MATERIALS AND METHODS

Samples, Chemicals and Instruments. The samples (leaves and drupes) used in this research were collected in the region of the Guadalquivir valley, Encinarejo (Córdoba), Spain. Three genetic varieties of olive trees, *Olea europaea* (Manzanilla, Picual, and Gordal) were used in this study. The olive drupes were harvested from September 2008 to January 2009 in each of the five maturity stages of the fruit (1, green; 2, green-yellow; 3, yellow-purple; 4, purple; and 5, black); meanwhile, leaves were harvested in three seasons of the year, autumn (October 2008), winter (January 2009), and spring (April 2009). All samples were taken from the same agricultural zone to avoid variations caused by soil characteristics and environmental factors. For representativeness, the samples were taken from four different trees of each variety. The drupes were milled, homogeneized by agitation in a vortex, and kept at −20 °C until use. The leaves, previously dehydrated, were milled, sized (60 μm), and kept at −20 °C until use.

Chemicals were docosanol, tetracosanol, hexacosanol, and octacosanol as alcohols and the sterols campesterol, stigmasterol, and stigmastanol, obtained from Sigma-Aldrich (St. Louis, MO). These were used as standards. Eiocosanol and cholesterol, also from Sigma-Aldrich, were used as internal standards (IS) in the determination step. The stock standard solutions of alcohols and sterols were prepared at 1000 μg/mL in chloroform, whereas campesterol was prepared at 100 μg/mL also in chloroform. The standard solutions, which contained four alcohols and three sterols, were prepared by dilution of the appropriate volume of the stock solution. All solutions were stored at −20 °C in glass vials and kept in the dark at room temperature before use.

Aminopropyl-phase cartridges (500 mg) from Waters (Millipore, Milford, MA) and silica-phase cartridges from Supelco (Bellefonte, PA) were used in the solid-phase extraction step. Bis(trimethylsilyl)fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma-Aldrich were used as silylation reagents in the derivatization step. Pyridine from Merck (Darmstadt, Germany) was used as derivatization solvent.

Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium alloy probe (12.70 mm diameter), which was immersed into a laboratory-made stainless steel container with eight compartments in which to place test tubes (*16*). A Selecta Mixtasel (Barcelona, Spain) centrifuge was used to separate solid particles from the extract. A Büchi R-200 (Postfach, Switzerland) rotary evaporator furnished with a B-490 heating bath was used to concentrate the sterol and fatty alcohol extracts after ultrasound-assisted extraction (UAE). A mechanical electrical stirrer MS2 minishaker from Ika (Staufen, Germany) was used to assist the derivatization step. A dry nitrogen stream was used to remove dichloromethane from the unsaponifiable fraction.

A Varian CP-3800 gas chromatograph (Walnut Creek, CA) equipped with a programmable temperature injector and coupled to a Saturn 2200 ion-trap mass spectrometer (Sunnyvalley, TX) was used for the determination of sterol and fatty alcohol profiles in the target unsaponifiable fraction. The chromatograph was furnished with a Varian CP 8400 autosampler and a Factor Four VF-5 ms fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) provided by Varian.

Sample Preparation. The sample preparation step was similar to that proposed by Orozco-Solano et al. (*17*). Briefly, 2 g of sample, either drupes or leaves, was placed in test tubes to which 10 mL portions of a 2:1 dichloromethane/hexane mixture was added. The tube was immersed in the water bath at 20 °C, where ultrasonic irradiation (duty cycle, 10%; output amplitude, 10% of the converter; applied power, 50 W; position of the ultrasonic probe tip, 2 cm from the bath bottom; and irradiation time, 10 min) was applied.

After extraction, the extract was centrifuged for 10 min at 3000 rpm for separation of the solid particles from the liquid phase. The clean extract was mixed with 2 mL of 2 M KOH and 10 μL of internal standards for sterols and fatty alcohols (0.1% chloroform solutions of cholesterol and 1-eicosanol, respectively). The mixture was subjected to ultrasound (output amplitude, 45% of the converter; applied power, 200 W; duty cycle, 50%) for 10 min, and, finally, the unsaponifiable fraction was extracted with 2 mL of hexane; the immiscible organic phase was separated by centrifugation for 10 min at 3000 rpm. A gentle N_2 stream was used to dry the unsaponifiable fraction.

Two hundred micrograms of the residue from the previous step was dissolved in 0.5 mL of 4:1 hexane/chloroform. The resulting solution was passed through an aminopropyl column, the sterols and fatty alcohols were bound to the functional groups of the sorbent, and the compounds not retained by the column were disposed of. The column was rinsed with 10 mL of 1:1 hexane/ethyl ether to remove matrix and dried with a nitrogen stream; the analytes were eluted from it with 8 mL of hexane and then with 6 mL of 5:1 hexane/chloroform.

Finally, conversion of sterols and fatty alcohols into their more volatile derivatives is a necessary step prior to GC individual separation. Two hundred microliters of the clean extract was subjected to drying with a nitrogen stream and the residue reconstituted with 100 μL of *N*-pyridine and homogenized in a vial for 1 min; then, 98 μL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide was added and the mixture shaken vigorously in the vial for 1 min. Finally, 2 μL of TMCS was added, the mixture was shaken vigorously in the vial for 2 min more and then subjected to ultrasound (output amplitude, 40% of the converter; applied power, 180 W; duty cycle, 50%) for 10 min to favor reaction.

Individual Separation and Determination of Sterols and Fatty Alcohols by GC-MS. The individual sterols and fatty alcohols separation and determination by GC-MS was similar to that previously proposed in ref *17*. Briefly, 1 μL of analytical sample was injected into the chromatograph for GC-MS analysis. The injector temperature was fixed at 250 °C, and the injection was in the split−splitless mode. The splitter was opened (50:1) for 0.5 min, closed for 3.5 min, and then opened at 100:1 split ratio for 10 min. The oven temperature program was as follows: initial temperature, 50 °C (held for 2 min), increased at 8 °C/min to 250 °C, followed by a second gradient at 3 °C/min to 260 °C (held for 20 min), and, finally, increased at 3 °C/min to 300 °C (held for 10 min). The total analysis time was 70 min, and 5 min extra time was necessary for re-establishing and equilibrating the initial conditions.

The ion-trap mass spectrometer was operated in the electron impact ionization (EI) positive mode, for which the instrumental parameters were set at the following values: filament emission current, 80 μA; transfer line, ion trap, and manifold temperatures, 220, 200, and 50 °C, respectively. The recording window was set between m/z 40 and 650, and the data were acquired using total ion current (TIC) scan mode. Digital selected ion monitoring (SIM) was applied as data treatment to remove the chromatographic background.

Identification of target analytes with commercially available standards (docosanol, tetracosanol, hexacosanol, octacosanol, campesterol, stigmasterol, and stigmastanol) was based on comparison of the retention times and mass spectra. Those compounds with no commercial standards, low purity, or expensive standards (tricosanol, pentacosanol, and some sterols such as cholestanol, brassicasterol, 24-methylenecholesterol, campestanol, chlerosterol, Δ^5-avenasterol, and Δ^7-avenasterol) were identified by comparing their retention times and mass spectra with those in the literature. Calibration plots were run for the seven analytes with commercial standards (peak area versus standard concentration). Compounds with

Table 1. Analysis of Sterols and Fatty Alcohols in Olive Leaves and Drupes[a]

	drupes																									
	Gordal										Manzanilla										Picual					
compound	1		2		3		4		5		1		2		3		4		5		1		2		3		4		5	
docosanol	3.4	(1.9)	14.3	(2.0)	3.7	(2.3)	180.1	(2.1)	0.1	(2.0)	9.9	(2.0)	33.9	(1.9)	7.1	(2.1)	210.7	(2.3)	6.5	(2.1)	10.1	(2.2)	55.5	(2.5)	9.6	(2.2)	29.1	(2.4)	2.2	(2.4)
tricosanol	0.1	(2.1)	0.2	(2.0)	0.1	(1.9)	<LOD		43.0	(2.2)	0.1	(3.0)	0.1	(2.2)	<LOD	(2.3)	20.8	(2.4)	44.2	(2.3)	1.0	(2.1)	5.6	2.7)	0.6	(2.4)	2.9	(1.9)	14.9	(1.8)
tetracosanol	28.3	(1.8)	17.4	(2.3)	2.7	(1.9)	466.5	(1.7)	113.4	(1.8)	74.6	(1.9)	37.4	(1.6)	7.6	(2.0)	407.6	(1.7)	574.7	(1.6)	91.7	(1.9)	74.3	(1.6)	7.5	(2.0)	56.3	(1.9)	39.9	(2.0)
pentacosanol	0.1	(2.0)	0.1	(2.1)	<LOD		2.7	(2.6)	116.0	(2.0)	23.5	(1.7)	11.7	(1.9)	8.2	(2.0)	12.1	(2.0)	1086.9	(1.7)	18.0	(1.9)	14.4	(1.9)	8.3	(2.0)	1.7	(2.3)	4.5	(2.0)
hexacosanol	50.5	(1.4)	8.1	(2.8)	35.2	(1.7)	212.6	(1.9)	23.4	(2.3)	189.2	(1.5)	24.8	(1.8)	90.1	(1.7)	203.2	(1.8)	932.2	(1.6)	162.8	(1.8)	34.3	(1.7)	99.9	(1.9)	28.1	(2.0)	36.2	(1.9)
heptacosanol	<LOD	(2.6)	<LOD	(2.7)	<LOD	(3.0)	0.2	(3.0)	7.5	(2.6)	4.6	(2.0)	<LOD		1.5	(2.5)	<LOD		306.4	(1.8)	1.7	(2.0)	<LOD		0.4	(3.0)	<LOD		1.0	(2.4)
octacosanol	9.9	(3.2)	0.8	(3.3)	9.8	(3.7)	78.8	(3.1)	2.3	(3.5)	69.8	(3.2)	5.3	(3.4)	46.0	(3.1)	69.9	(3.7)	316.6	(3.3)	53.9	(3.1)	6.2	(3.4)	47.6	(3.6)	9.7	(3.4)	<LOD	
brassiscasterol	0.1	(2.3)	<LOD		<LOD		5.8	(3.0)	0.4	(3.0)	0.9	(2.9)	0.2	(3.0)	0.1	(3.0)	<LOD		11.0	(2.0)	2.2	(2.0)	1.4	(2.2)	<LOD		4.6	(2.3)	4.0	(2.0)
24 methylene cholesterol	<LOD		<LOD		<LOD		<LOD		<LOD		0.2	(3.0)	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		7.1	(2.0)	2.7	(2.3)
campesterol	2.4	(2.1)	10.8	(1.7)	4.3	(1.9)	489.3	(1.6)	1.9	(2.7)	10.7	(1.8)	38.3	(1.8)	<LOD		733.8	(1.6)	2.4	(2.0)	13.9	(1.9)	80.6	(2.0)	21.6	(1.9)	101.4	(1.8)	12.9	(2.0)
campestanol	0.1	(2.1)	0.3	(2.0)	0.1	(3.0)	18.8	(2.0)	24.1	(1.9)	0.2	(3.0)	0.9	(2.7)	0.5	(2.9)	20.5	(1.9)	1.8	(2.4)	0.4	(3.0)	2.8	(2.3)	0.6	(2.9)	2.8	(2.0)	0.8	(3.0)
stigmasterol	0.6	(2.3)	0.2	(2.8)	1.4	(2.3)	165.4	(1.9)	1.4	(2.9)	21.3	(1.8)	4.4	(2.2)	28.4	(1.9)	223.8	(1.6)	9.7	(2.0)	25.1	(1.9)	8.4	(2.0)	44.6	(1.9)	30.9	(2.0)	4.7	(2.0)
clerosterol	1.0	(2.5)	0.3	(2.7)	2.1	(2.2)	4.1	(2.1)	6.3	(2.7)	0.2	(2.9)	0.1	(3.0)	0.5	(2.8)	15.2	(1.9)	4.9	(2.0)	1.4	(3.0)	0.5	(3.0)	2.8	(2.0)	2.1	(2.0)	3.3	(2.1)
β-sitosterol	134.5	(1.9)	34.3	(1.9)	224.9	(1.6)	1101.2	(1.6)	3057.0	(1.6)	634.1	(1.7)	131.9	(1.6)	709.6	(1.7)	1796.5	(1.5)	5855.3	(1.5)	910.7	(1.8)	305.8	(1.8)	1343.7	(1.7)	248.3	(1.8)	1966.8	(1.9)
sitostanol	0.6	(2.8)	0.2	(3.0)	0.8	(2.5)	4.7	(2.3)	53.2	(1.9)	2.2	(2.5)	0.7	(3.0)	1.8	(2.9)	3.4	(2.0)	211.0	(1.7)	0.7	(3.0)	0.7	(2.9)	0.6	(2.8)	0.5	(2.2)	4.9	(2.0)
Δ^5-avenasterol	0.3	(2.5)	1.3	(2.7)	1.3	(2.5)	12.1	(2.0)	17.3	(2.0)	3.2	(2.0)	10.8	(1.9)	8.7	(2.2)	33.8	(1.8)	13.6	(1.9)	8.4	(2.1)	51.6	(1.9)	33.7	(1.9)	4.7	(2.0)	4.5	(2.0)
Δ^7-avenasterol	0.4	(2.1)	1.6	(2.9)	1.7	(2.4)	57.9	(2.0)	23.0	(1.9)	3.7	(2.0)	12.9	(1.8)	10.3	(2.2)	35.8	(1.8)	17.1	(1.8)	2.2	(2.3)	11.1	(2.0)	7.5	(2.0)	5.0	(2.0)	1.7	(2.3)
alcohols	92.3		40.8		51.4		940.8		305.6		371.6		113.1		160.5		924.3		3267.4		339.1		190.3		173.8		127.7		98.7	
sterols	140.2		48.9		236.6		1859.3		3184.6		676.8		200.3		760.0		2862.9		6126.8		964.8		462.8		1455.1		407.3		2006.3	

	leaves																	
	Gordal						Manzanilla						Picual					
compound	winter		spring		autumn		winter		spring		autumn		winter		spring		autumn	
docosanol	1.6	(2.1)	2.8	(2.0)	3.2	(2.0)	0.7	(2.9)	5.0	(2.0)	0.7	(2.9)	4.0	(2.0)	6.3	(2.0)	26.6	(2.0)
tricosanol	0.1	(2.7	0.1	(3.0)	0.1	(2.6)	<LOD		<LOD		<LOD		0.4	(2.9)	0.6	(2.8)	1.5	(2.8)
tetracosanol	13.5	(1.9)	3.4	(2.0)	2.2	(2.0)	1.2	(2.1)	5.4	(2.0)	5.3	(2.1)	35.8	(1.7)	8.3	(1.9)	17.4	(1.9)
pentacosanol	<LOD		<LOD		<LOD		0.7	(2.4)	1.6	(2.3)	1.6	(2.2)	6.6	(2.0)	1.5	(2.6)	18.6	(1.9)
hexacosanol	24.2	(1.9)	1.6	(2.1)	30.4	(1.9)	13.8	(1.9)	3.6	(2.2)	13.6	(1.9)	64.1	(1.8)	3.8	(2.1)	274.0	(1.7)
heptacosanol	<LOD		<LOD		<LOD		0.1	(3.1)	<LOD	(3.1)	0.1	(2.8)	0.1	(3.0)	<LOD	(3.1)	0.1	(3.0)
octacosanol	4.6	(3.1)	0.1	(3.4)	8.3	(3.1)	4.6	(3.2)	0.7	(3.4)	5.0	(3.2)	20.9	(3.1)	0.5	(3.3)	126.9	(2.9)
brassiscasterol	0.1	(2.1)	<LOD		<LOD	(3.1)	0.1	(3.0)	0.1	(3.0)	0.1	(3.0)	0.8	(2.7)	0.1	(2.9)	0.1	(2.9)
24-methylene	<LOD		<LOD		<LOD		<LOD		<LOD		0.1	(3.0)	<LOD		<LOD		<LOD	
campesterol	1.2	(2.1)	2.1	(2.1)	3.7	(2.0)	1.3	(2.3)	5.6	(2.0)	0.8	(2.8)	5.5	(2.0)	9.2	(2.0)	59.9	(2.0)
campestanol	0.1	(2.2)	0.1	(2.9)	0.1	(2.2)	0.1	(2.6)	0.1	(2.8)	0.1	(3.0)	0.1	(3.0)	0.3	(2.8)	1.4	(2.3)
stigmasterol	0.3	(2.3)	0.1	(3.0)	1.2	(2.0)	2.9	(2.0)	0.7	(3.0)	1.5	(2.0)	9.9	(2.0)	1.0	(2.5)	123.1	(1.7)
clerosterol	0.5	(2.3)	0.1	(3.0)	1.8	(2.0)	0.1	(2.9)	<LOD	(3.0)	<LOD	(3.1)	0.5	(2.4)	0.1	(2.8)	7.1	(2.1)
β-sitosterol	64.6	(1.9)	6.7	(1.9)	195.3	(1.7)	108.8	(1.9)	19.4	(1.9)	45.8	(1.8)	360.7	(1.6)	35.0	(1.8)	3733.1	(1.6)
sitostanol	0.3	(3.0)	0.1	(2.9)	0.7	(2.0)	0.2	(2.8)	0.1	(2.9)	0.2	(2.8)	<LOD		<LOD		<LOD	
Δ^5-avenasterol	0.2	(3.2)	0.3	(2.9)	0.2	(2.8)	0.9	(2.7)	1.6	(2.1)	0.3	(2.6)	3.4	(2.0)	5.9	(2.0)	94.7	(1.8)
Δ^7-avenasterol	0.2	(3.3)	0.3	(2.9)	1.5	(2.3)	1.1	(2.3)	1.9	(2.3)	0.3	(2.2)	1.0	(2.1)	1.3	(2.4)	21.8	(1.9)
alcohols	44.0		7.9		44.1		21.0		16.2		26.1		131.8		21.0		465.1	
sterols	67.3		9.6		204.4		115.2		29.5		48.8		381.9		52.8		4041.0	

[a] All results are expressed as μg/g; errors, given in parentheses, are expressed as %, n= 3 replicates.

no commercial standards were quantified by the calibration curve of the most similar alcohol or sterol.

Statistical Analysis. Models based on principal component analysis (PCA) were developed to study the influence of the olive tree variety and harvest period on the concentration of sterols and fatty alcohols. Those results were confirmed by means of models based on hierarchical cluster analysis (HCA). The concentrations obtained by the proposed method for these compounds, expressed as micrograms per gram, were used as variables for development of the models. Statistical analysis was performed using the data obtained after normalization in the case of leaves, and both the raw data and those obtained after the normalization and differentiation processes (mean normalization $X(i,k) = X(I,k)/\text{Abs}(\text{mean}-(X(i,*)$ and Norris derivative (segment size for averaging equal to 3, and difference ($Xk - Xk - 1$) equal to 2) in the case of drupes.

Unscrambler 9.0 from CAMO (Oslo, Norway) was used as statistical software (*15*) in the case of the PCA models. Statgraphics Centurion XV, Statpoint technologies, Inc. (Warranton, VA) was used as statistical software for the development of the HCA models.

RESULTS AND DISCUSSION

Alcohol and sterol fractions of olive drupes and leaves have been characterized using the proposed method. Although both wet and dry material was used as samples for the extraction of the target analytes in the case of drupes and leaves, respectively, all of the results are expressed as dry material to make possible the comparison between different matrices. Humidity was calculated in the case of the drupe samples for correction of the results.

The properties of the target compounds, alcohol and sterols, in addition to the presence in the leaves of other added-value compounds, such as triterpene dialcohols and phenols, and the extensive amount of olive leaves (in Spain 110 tonnes approximately) that is annually generated from pruning and also after separation of olive fruit in mills prior to oil production (*18*), give an additional interest to the characterization of these compounds in leaf samples and their subsequent use in the industry as a source of biologically active compounds.

Five ripeness stages (1, green; 2, green-yellow; 3, yellow-purple; 4, purple; and 5, black) were studied in the case of the drupes and three harvesting periods (winter (drupes, ripeness stage 5), spring (no drupes or flowers in the tree), and autumn (drupes, ripeness stage 1)) were studied in the case of the leaves.

There is no information in the literature about the composition of alcohol and sterol fractions in olive leaves. Therefore, all discussion of the results is based on the results obtained for olive drupes.

Characterization of the Alcohol and Sterol Fractions. Table 1 shows the concentrations of individual fatty alcohols and sterols found in each variety, expressed as micrograms per gram in dry material, and the precision of the method, calculated by triplicate analysis.

Drupes. The total concentration of alcohols in the drupes ranges between 40.8 and 3267.4 μg/g. These values are in agreement with the results provided by Ranalli et al. (*19*) for oil obtained from whole olive fruit using an organic solvent as extractant and are 10 times higher than in extra virgin olive oil (*20*). This difference can be easily explained by taking into account the procedure used for the extraction of extra virgin olive oil.

A detailed study of the composition of this fraction reveals that the fatty alcohols present at higher concentration are hexacosanol, tetracosanol, and docosanol, for all of the varieties. This result is in agreement with the results obtained for extra virgin olive oil, but not with those provided by organic solvent extraction, in which docosanol is present at low concentrations. This can be explained by the ripeness stage of the drupes. Drupes in stage 4 are used for the extraction of extra virgin olive oil. As can be seen in **Table 1** the concentration of docosanol for these drupes

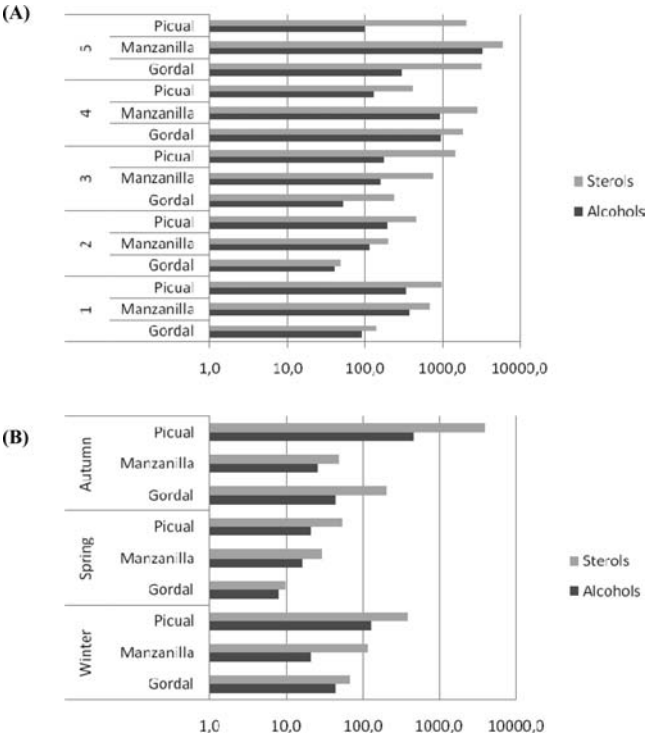

Figure 1. Concentration of fatty alcohols and sterols in the three varieties under study: (**A**) in drupes; (**B**) in leaves (all results are expressed as μg/g of dry material).

is high for this ripeness stage but not for the others, which could have been used in the case of the organic solvent extraction.

Two trends were observed in the case of drupes (**Figure 1A**) for the variation of the concentration of fatty alcohols as a function of the ripeness stage. In one of them, found in Gordal and Manzanilla varieties (used for production of table olives), the concentration of alcohols in drupes in stages 4 and 5 is higher than in those in stages 1−3. Furthermore, the concentration of fatty alcohols in the drupes of the ripeness stage 1 is higher than in stages 2 and 3. This behavior was also found by Ranalli et al. in 1998 (*21*) for other varieties. In that case, samples in stage 1 had the same concentration of total fatty alcohols as samples in stages 4 and 5.

The other trend was found in the Picual drupes, a variety used for olive oil production, in which alcohols concentration decreases at higher maturation stages. This can be explained by taking into account that the amount of these compounds present in olive oil is limited by legislation and fatty alcohol concentrations found for drupes of all the varieties in ripeness stages 4 and 5 are in agreement with those found by Ranalli et al. for Frantoio and Lechin varieties.

The concentration of total sterols in drupes (ranging between 48.9 and 6126.8 μg/g) is higher than that of fatty alcohols (**Table 1**). This is in agreement with the information found in the literature about the ratio between the alcohol and sterol fractions, which ranges between 1:3 and 1:10 (*19*).

The concentrations of the sterol fraction were higher than the results provided for oil obtained from whole olive fruit, but they were similar to the values provided by the oil obtained from the drupe seed (*19*). This behavior can be easily explained by taking into account that the whole drupe, seed included, was used in this study and the proposed extraction method was optimized for total extraction of these compounds (*17*).

A detailed study of the composition of this fraction reveals that sitosterol is by far the predominant sterol followed by campesterol

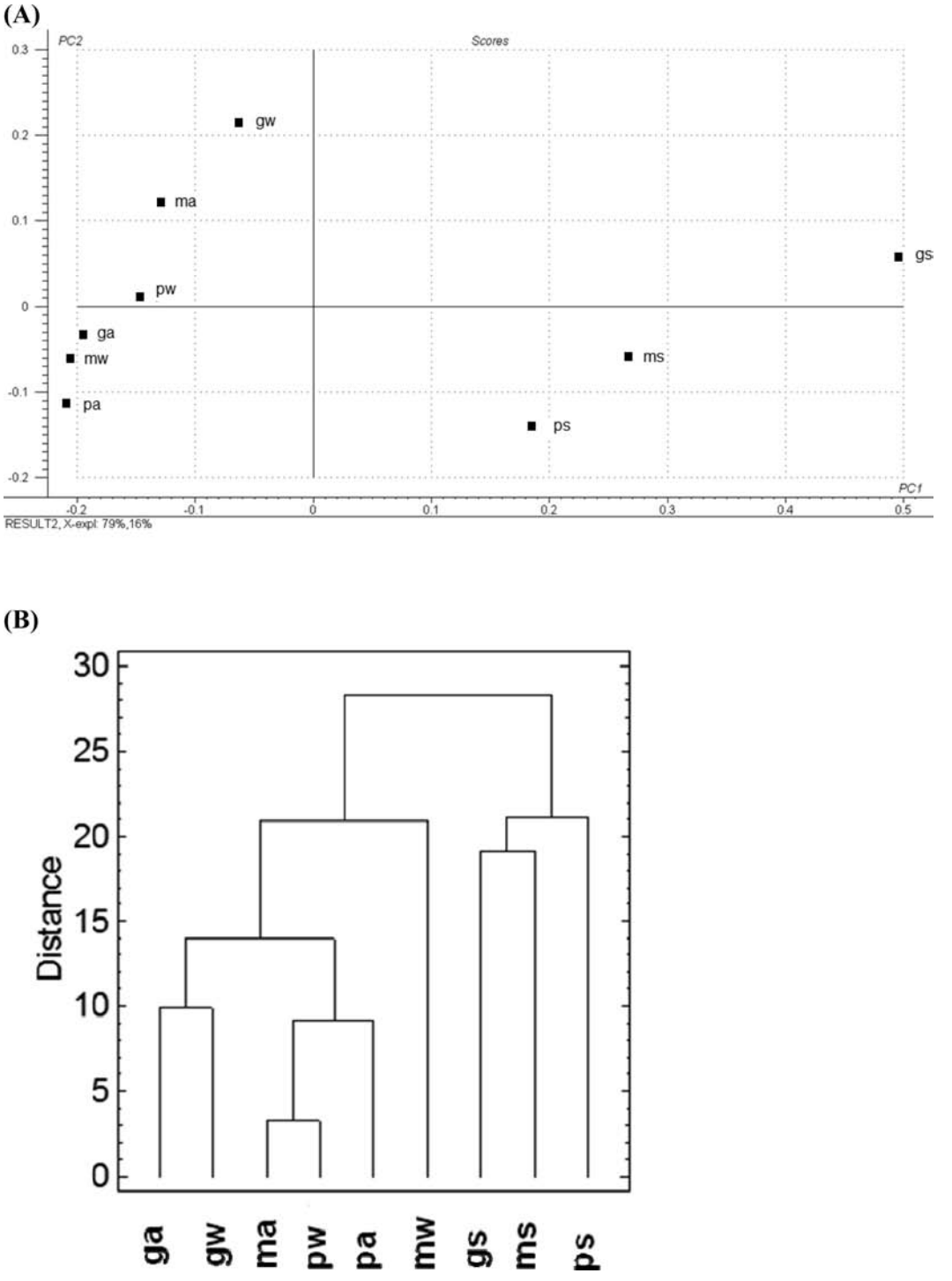

Figure 2. Statistical analysis of main sterols and fatty alcohols families: (**A**) PCA and (**B**) HCA of olive leaves (normalized data). Samples are identified using two letters. The first letter is associated with the variety (g, Gordal; m, Manzanilla; p, Picual) and the second one with the harvest period (a, autumn; s, spring; w, winter).

and stigmasterol. These results are in agreement with the information found in the literature for whole drupe (*22*). It should be emphasized that the concentration of Δ^5-avenasterol found in the samples under study is smaller than that found by other authors (*22*).

Two trends were also observed in drupes for the variation of the concentration of sterols as a function of the ripeness stage (**Figure 1A**). The first one was found for Gordal and Manzanilla drupes. The concentration of sterols increased with the ripeness stage, with the exception of stage 2, which showed the smallest concentration of these compounds. This result is similar to that proposed by Stiti et al. in 2007 (*22*). In the case of Picual drupes the behavior was similar to that in the other varieties, with the exception of stage 4, which showed a concentration similar to that of stage 2. This behavior was also found by Ranalli et al. (*21*).

Leaves. The concentration of fatty alcohols in leaves ranges between 7.9 and 465.1 μg/g. This concentration is 5 times smaller than the concentration present in drupes. A study of the individual composition of this fraction shows that hexacosanol is the alcohol present at the highest concentration. Furthermore, the concentrations of tetracosanol and docosanol are 10 times smaller than the concentration of hexacosanol. This is the main difference in the composition of drupes.

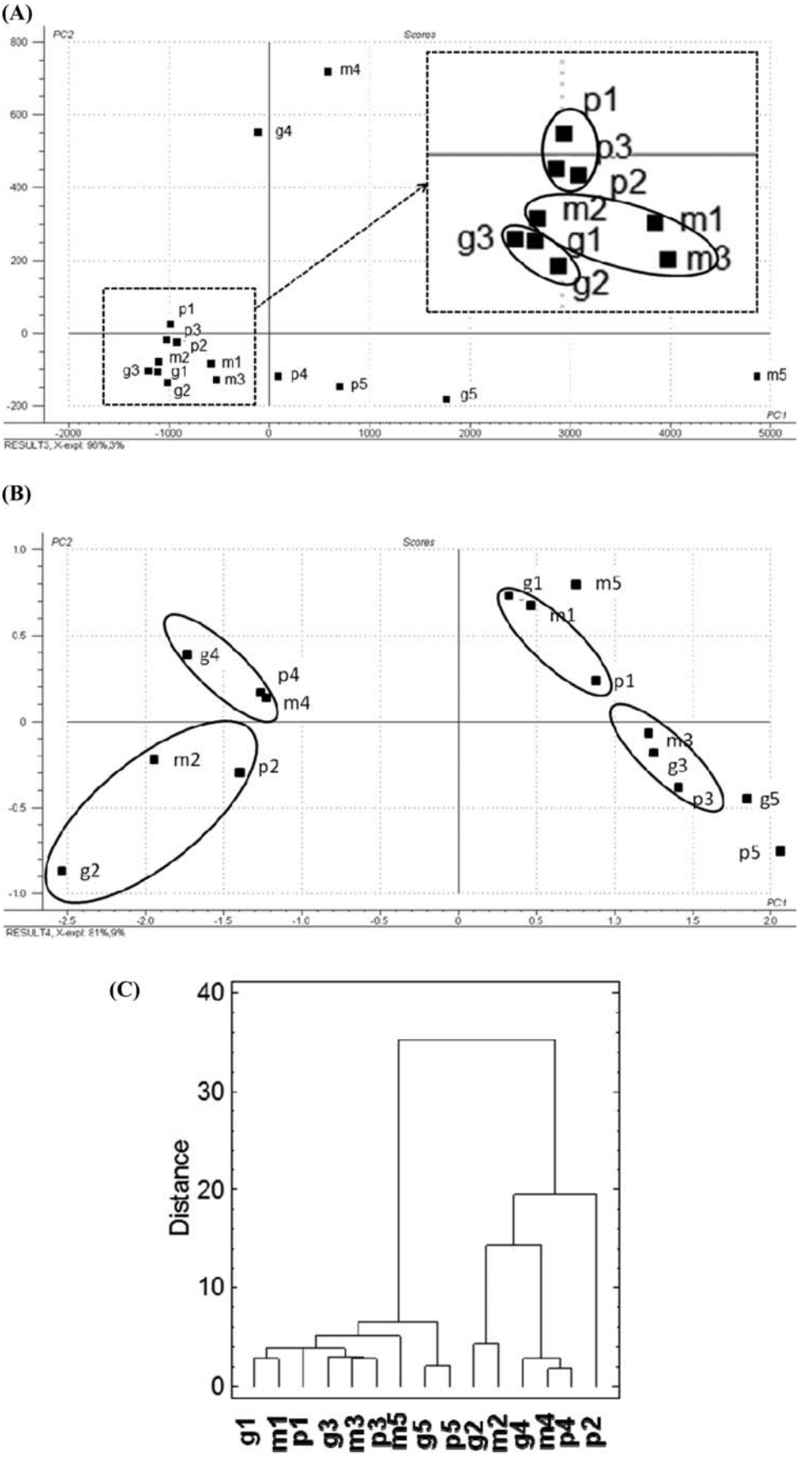

Figure 3. Statistical analysis of main sterols and fatty alcohols families: (**A**) PCA of olive drupes (raw data); (**B**) PCA of olive drupes (normalized and differentiated data); (**C**) HCA of olive drupes (normalized and differentiated data). Samples are identified using a letter and a number. The letter is associated with the variety (g, Gordal; m, Manzanilla; p, Picual) and the number from 1 to 5 with the ripeness stage.

In leaves, the harvesting period has a clear influence (**Figure 1B**). The concentration of alcohols in autumn and winter is higher than in spring for all of the varieties. It should be emphasized that the concentrations found for Picual variety leaves were higher than those found for the other varieties. This could be related with the behavior of Picual drupes, which present low fatty alcohol concentrations in ripeness stages 4 and 5.

The concentration of sterols in leaves ranges between 9.6 and 4041.0 μg/g. This concentration is 10 times smaller than that present in drupes in the case of Gordal and Manzanilla varieties, but the concentration found for Picual leaves is twice the concentration found in drupes. This fact can also be related with the presence of the smallest amount of sterols in Picual drupes. Although there is no information in the literature about the concentration of these compounds in olive leaves, there is information about the composition of the different fractions of the olive drupes, and the highest concentration of sterols is present in the seed, which could be compared with leaves.

Two trends have also been observed in the modification of the sterols concentration with the season. In one of them, involving Gordal and Picual varieties, the concentration of sterols decreased from autumn to spring. The other trend was found in Manzanilla leaves, in which sterols concentration increased from autumn to winter and decreased in spring.

Chemometric Analysis. Statistical studies based on nonsupervised pattern recognition techniques, such as PCA and HCA, were developed to study the influence of the olive tree variety and the harvest period on the concentration of sterols and fatty alcohols. The small number of samples used for development of the models hindered the use of supervised pattern recognition techniques, such as *K*-nearest neighbor (KNN) or soft independent modeling of class analogy (SIMCA). Therefore, these results should be considered as a first approach to be completed in future research and as a useful tool to visualize differences between samples.

In the case of olive leaves, normalization of the data was necessary. As can be seen in **Figure 2A**, the fatty alcohol and sterol compositions in leaves harvested in winter and autumn are, respectively, similar to and different from that provided by leaves harvested in spring. Furthermore, identification of variety as a function of composition of these two groups of compounds is impossible. Target analytes that exert a higher influence in the development of the model are hexacosanol, docosanol, campesterol, octacosanol, and Δ^5-avenasterol. Three principal components were necessary to explain 96% of the data variability. This information was confirmed by means of the dendrogram obtained on the HCA (**Figure 2B**). This dendrogram can divided into two main groups, which are separated by a Euclidean squared distance of 30. One group contains the samples harvested in spring and the other group contains the rest of the samples. Additional information can be obtained from this dendrogram for the samples harvested during autumn or winter. Gordal and Picual leaf samples can be easily differentiated (Euclidean squared distance of 14). The composition of the Manzanilla leaves harvested in autumn is similar to that provided by the Picual leaves harvested in winter. The distance between these samples is smaller than 5. Therefore, it could be difficult to find differences between these samples. Manzanilla leaves harvested during winter are totally different from the rest of the leaves harvested during autumn or winter but are associated with that group of samples. This behavior can be explained by taking into account the fact that Manzanilla olives are harvested in advance.

Principal component models were developed using the raw data obtained after the analysis of the sterol and fatty alcohol composition of drupes from 5five different ripeness stages. As can be seen in **Figure 3A**, drupes belonging to ripeness stage 5 for all varieties and those of stage 4 for Manzanilla and Gordal are different. Apart from this behavior is that of stages 1−3 for the three varieties. The joint use of these ripeness stages allows differentiation between varieties as a function of sterol and fatty alcohol composition (see inset in **Figure 3A**, in which ellipses have been used to join samples from the same variety). Three PCs were necessary to explain 99% of the variability. The analytes with higher influence in the model were β-sitosterol, campesterol, stigmasterol, docosanol, and pentacosanol.

After data normalization and differentiation, it becomes clear that the samples are grouped as a function of the ripeness stage (**Figure 3B**, in which ellipses have been used to join samples from the same variety). Manzanilla drupes in ripeness stage 5 have a different behavior from the rest of the varieties at the same ripeness stage. This behavior can be associated with the high concentration of fatty alcohols present in the sample. The explained variability for three PCs was 96%, and the most influential variables were campesterol, β-sitosterol, hexacosanol, tetracosanol, octacosanol, and docosanol.

HCA was also developed in this case. As can be seen in **Figure 3C**, the obtained dendrogram can be divided into two groups: the first contains the samples in the ripeness stages 2 and 4 and the second contains the rest of the ripeness stages. The Euclidean squared distance for the samples from the same ripeness stage is lower than 6 for ripeness stages 1, 3, and 4. Therefore, these samples can be easily differentiated. In the case of ripeness stages 2 and 5, one sample is not grouped correctly. Picual drupes in ripeness stage 2 appear totally isolated from the rest of the samples of the same ripeness stage. This can be explained by taking into account **Figure 3B**. It is possible to see in this figure that the distance between the Picual sample and the rest of the samples of ripeness stage 2 is the largest in the plot. Therefore, in HCA this sample cannot be grouped correctly. Something similar has been found for Manzanilla drupes in ripeness stage 5.

Therefore, it is possible to say that drupe sterol and fatty alcohol composition can be used for the development of models based on supervised pattern recognition techniques (KNN and SIMCA) for empirical identification of the ripeness stage of olive drupes. These models were not developed in this case owing to the small number of samples analyzed.

LITERATURE CITED

(1) Conde, C.; Delrot, S.; Gerós, H. Physiological, biochemical and molecular changes occurring during olive development and ripening. *J. Plant Physiol.* **2008**, *165*, 1545–1562.

(2) Japón, R.; Ruiz-Jiménez, J.; Luque de Castro, M. D. Discrimination and classification of olive tree varieties and cultivation zones by biophenol contents. *J. Agric. Food Chem.* **2006**, *54*, 9706–9712.

(3) Ercisle, S.; Barut, E.; Ipek, A. Molecular characterization of olive cultivars using amplified fragment length polymorphism markers. *Genet. Mol. Res.* **2009**, *8*, 414–419.

(4) Ostlund, R.; Lin, X. Regulation of cholesterol absorption by phytosterols. *Curr. Atheroscler. Rep.* **2006**, *8*, 487–491.

(5) Berger, A.; Jones, P.; Abumweis, S. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. *Lipid Health Dis.* **2004**, *3*, 1–19.

(6) Angerosa, F.; Camera, L.; Cumitini, S.; Gleixner, G.; Reniero, F. Carbon stable isotopes and olive oil adulteration with pomace oil. *J. Agric. Food Chem.* **1997**, *45*, 3044–3048.

(7) Reglamento n° 702/2007 de la Comisión, de 21 de junio de 2007, por el que se modifica el Reglamento n° 2568/91 relativo a las características de los aceites de oliva y de los aceites de orujo de oliva y sobre sus métodos de análisis. *Diario Of. Union Eur.* **2007**, *L161* (June 22).

(8) Segura, A.; Carrasco, A.; Cortacero, S.; Gori, A.; Cerretani, L.; Fernández, A. A simplified method for HPLC-MS analysis of sterols in vegetable oil. *Eur. J. Lipid Sci. Technol.* **2008**, *110*, 1142–1149.

(9) Lagarda, M. J.; García, G.; Farré, R. Analysis of phytosterols in foods. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1486–1496.
(10) Giacomelli, L.; Mattea, M.; Ceballos, C. Analysis and characterization of edible oils by chemometric methods. *J. Am. Oil Chem. Soc.* **2006**, *83*, 303–308.
(11) Salter, A. M.; Tarling, E. J. Regulation of gene transcription by fatty acids. *Animal* **2007**, *1*, 1314–1320.
(12) Hilmarsson, H.; Traustason, B.; Kristmundsdottir, T.; Thormar, H. Virucidal activities of medium- and long-chain fatty alcohols and lipids against respiratory syncytial virus and parainfluenza virus type 2: comparison at different pH levels. *Arch. Virol.* **2007**, *152*, 2225–2236.
(13) Volin, P. Analysis of steroidal lipids by gas and liquid chromatography. *J. Chromatogr., A* **2001**, *935*, 125–140.
(14) Kim, Y.; Dohnalek, Z.; Kay, B.; Rousseau, R. Competitive oxidation and reduction of aliphatic alcohols over $(WO3)_3$ clusters. *J. Phys. Chem.* **2009**, *113*, 9721–9730.
(15) Paganuzzi, V. Distribution of the alcoholic components of the unsaponifiable matter within the olive drupe. I. Sterols. *Riv. Ital. Sostanze Grasse* **1979**, *56*, 431–437.
(16) Mijangos, O.; Ruiz-Jiménez, J.; Lagunez-Rivera, I.; Luque de Castro, M. D. Fast ultrasound-assisted extraction of polar (phenols) and nonpolar (lipids) fractions in *Heterotheca inuloides*. *Phytochem. Anal.* **2010**, submitted for publication.
(17) Orozco-Solano, M.; Ruiz-Jiménez, J.; Luque de Castro, M. D. Ultrasound-assisted extraction and derivatization of sterols and fatty alcohols from olive leaves and drupes prior to determination by GC-tandem MS. *J. Chromatogr., A* **2010**, *1217*, 1227–1235.
(18) Sánchez, N.; Priego Capote, F.; Luque de Castro, M. D. Ultrasound-assisted extraction and silylation prior to gas chromatography−mass spectrometry for the characterization of the triterpenic fraction in olive leaves. *J. Chromatogr., A* **2007**, *1165*, 158–165.
(19) Ranalli, A.; Pollastri, L.; Contento, S.; Di Loreto, G.; Lannucci, E.; Lucera, L.; Russi, F. Sterol and alcohol components of seed, pulp and whole olive fruit oils. Their use to characterise olive fruit variety by multivariates. *J. Sci Food Agric.* **2002**, *82*, 854–859.
(20) Rivera del Álamo, R M.; Fregapane, G.; Aranda, F.; Gómez-Alonso, S.; Salvador, M. D. Sterol and alcohol composition of cornicabra virgin olive oil: the campesterol content exceeds the upper limit of 4% established by EU regulations. *Food Chem.* **2004**, *84*, 533–537.
(21) Ranalli, A.; Tombesi, A.; Ferrante, M.; De Mattia, G. Respiratory rate of olive drupes during their ripening cycle and quality of oil extracted. *J. Sci. Food Agric.* **1998**, *77*, 359–367.
(22) Stiti, N.; Triki, S.; Hartmann, M. Formation of triterpenoids throughout *Olea europaea* fruit ontogeny. *Lipids* **2007**, *42*, 55–67.

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