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Ultrasound-assisted extraction and derivatization of sterols and fatty alcohols from olive leaves and drupes prior to determination by gas chromatography–tandem mass spectrometry

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ABSTRACT

A method for simultaneous determination of sterols and fatty alcohols in olive leaves and drupes based on ultrasound-assisted extraction and derivatization prior to individual identification–quantitation by chromatographic separation and mass spectrometry detection (single ion monitoring mode) is reported here. The sample preparation procedure involves the following steps: (i) leaching of the raw material accelerated by ultrasound; (ii) saponification of the leachate, also accelerated by ultrasound, and separation of the unsaponifiable matter; (iii) cleaning of the extract by solid-phase extraction; (iv) silylation of the target analytes—also assisted by ultrasound; (v) injection into the gas chromatograph for identification–simultaneous quantitation of the two families of compounds. Individual separation–determination of the fatty alcohols and sterols provide limits of detection (LOD) in the range 9.8×10^{-2} to 2 μg/l and 5.0–6.0 μg/l, respectively. The LOQs range from 0.3 to 0.9 μg/l and 17.0 to 21.0 μg/l, and the linear dynamic ranges are between LOQ and 25.0 μg/ml. The between-day precision, expressed as relative standard deviation (RSD), ranges between 3.6 and 6.1% and the within-laboratory reproducibility, also expressed as RSD, between 6.4 and 9.2%. Within the study of the metabolomic profile of the unsaponifiable fraction in olive tree, the method has been applied to the determination of the target analytes in different varieties of olive trees cultivated in the same zone, so that differences in this unsaponifiable fraction can be attributed to characteristics of the target varieties. As compared with its European Union counterpart, the method is endowed with similar analytical characteristics and drastic shortening of the operational time.

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1. Introduction

Information on the unsaponifiable matter of olive oil and its components is essential for oil classification into categories [1]. These compounds have been widely studied because of their healthy properties, mainly phytosterols, with recognized biological effects. Clinical research has shown that phytosterols reduce biliary cholesterol absorption in the intestine [2], thereby increasing faecal excretion of cholesterol [3]. Decrease in plasma cholesterol levels is important for prevention of cardiovascular diseases, which are the main causes of death in Europe [4]. Fatty alcohols in olive oil were found to be minor components of the unsaponifiable matter as compared to phytosterols. 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—first, cold press).

Sterols and alcohols profiles are used to characterize virgin olive oils and to detect adulteration of olive oil with hazelnut oil [5] or virgin olive oil with olive-pomace oil [6]. Chemometric studies have been used to characterize varietal olive oils based on their sterols and other fatty components (fatty acids, tocopherols, diacylglycerols and/or triacylglycerols) [7] to classify Portuguese olive oils according to the Protected Denomination of Origin [8] and discriminate between virgin olive oils from different olive-tree varieties [9].

Fatty aliphatic alcohols and phytosterols in vegetable oils have been usually analyzed by GC with flame ionization detection (FID) [10–20] and with MS detection [10,12,18–21]. HPLC coupled to mass detectors has been scarcely used to analyze the alcoholic fraction of oils [22] due to low ionization efficiency [23,24]. Atmospheric-pressure chemical-ionization mass spectrometry has gained interest for sterol characterization in the last few years [25], despite its price.

Analytical applications of ultrasound, particularly sample preparation, have experienced a significant increase in the last decade [26]. In addition to assistance to leaching [27], a number

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of chemical reactions including both enzymatic [28] and non-enzymatic steps [29], and physical processes such as emulsification [30], have evidenced the usefulness of this energy to improve and/or shorten analytical methods.

In this work, a GC–MS method to characterize the unsaponifiable fraction of drupes and olive leaves from different varieties, with individual identification–quantitation of sterols and fatty alcohols has been developed. Prior to CG separation, a fast method for sample preparation, in which the different steps have been efficiently assisted by ultrasound, has also been developed. The potential influential variables on these steps were studied by a multivariate approach, and the contents of these alcohols and sterols in different samples were established.

2. Experimental

2.1. Samples

Samples used in this research—leaves and drupes from 3 varieties of olive trees (*i.e.* manzanilla, picual and gordal)—were collected in the region of Guadalquivir valley, Encinarejo (Córdoba). All samples (5 olive leaf samples and 5 drupe samples) were taken from the same geographical area to avoid variations caused by soil characteristics and environmental factors. The samples were homogenized and kept at −20 °C until use. Leaves previously dehydrated were milled, sized (60 μm) and kept at −20 °C until use.

2.2. Reagents

Alcohols—docosanol, tetracosanol, hexacosanol and octacosanol—and sterols—campesterol, stigamasterol and stigmastanol—obtained from Sigma–Aldrich (St. Louis, MO, USA), with purity ≥98%, were used as standards. Eicosanol and cholesterol also from Sigma–Aldrich were used as internal standards (IS) in the determination step. Solutions at concentrations 1000 μg/ml were prepared in chloroform. All solutions were stored at −20 °C in glass vials and kept in the dark at room temperature until use. Aminopropyl-phase cartridges (500 mg) from Waters (Millipore, Milford, MA, USA) and silica cartridges from Supelco (Bellefonte, PA, USA) were used for solid-phase extraction. Bis-(trimethylsilyl)-fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma–Aldrich were used as silylation reagents for derivatization. Pyridine from Merck (Darmstadt, Germany) was used as derivatization solvent.

2.3. Apparatus and instruments

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 lab-made stainless-steel container with eight compartments to place test tubes [31]. A Varian CP 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable-temperature injector and coupled to a Saturn 2200 ion-trap mass spectrometer (Sunnyvalley, TX, USA) was used for the determination of sterols and fatty alcohols profiles in the target unnsaponifiable 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.

2.4. Conventional extraction method

Two grams of olive drupes or leaves and 10-ml of a 2:1 dichloromethane–hexane mixture were placed in a flask and agitated vigorously for 24 h; then, the extract was centrifuged for 10 min at 3000 rpm, the solid particles were separated from the liquid phase and the extract was subjected to the saponification step. All the experiments were carried out in duplicate [32].

2.5. Proposed method

The proposed method can be divided into five steps: (1) leaching of fatty alcohols and sterols from the target sample; (2) saponification step; (3) preconcentration and cleanup of the solution from the saponification step by solid-phase extraction; (4) silylation of the target analytes to a more volatile products; (5) individual separation of the silylated sterols and fatty alcohols and determination by GC–MS.

2.5.1. Leaching step

Two grams of olive 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 placed in the stainless-steel container, which was immersed into the water-bath at 20 °C, where ultrasonic irradiation under the optimal working conditions—duty cycle 10% (*viz.* ultrasound application 0.1 s/s), output amplitude 10% of the converter, applied power 50 W, position of the ultrasonic probe-tip 1 cm from the bath bottom, and irradiation time 10 min—was applied. The extract (oleaginous fraction) was centrifuged for 10 min at 3000 rpm to separate solid particles both from the liquid phase and extract.

2.5.2. Saponification step

The clean extract from the previous step was mixed with 2-ml 2 M KOH, and 10-μl internal standards for sterols and fatty alcohols (0.1% chloroform solutions of cholesterol and 1-eicosanol, respectively) were added. The mixture was subjected to ultrasound (output amplitude 45% of the converter, applied power 200 W, duty cycle 50%) for 10 min, the unsaponifiable was extracted with 2-ml hexane, and this phase separated by centrifugation for 10 min at 3000 rpm. A gentle N_2 stream was used to dry the unsaponifiable fraction.

2.5.3. Solid-phase extraction

200 μg of the residue from the previous step was reconstituted into 0.5-ml 4:1 hexane–chloroform and the resulting solution circulated through an aminopropyl column. The sterols and fatty alcohols were bounded to the functional groups of the sorbent and the compounds not retained by the column were disposed off. The column was rinsed with 10-ml 1:1 hexane–ethyl ether to remove matrix remainings, dried with a nitrogen stream and the analytes eluted with 8-ml hexane and then with 6-ml 5:1 hexane–chloroform.

2.5.4. Derivatization procedure

Conversion of sterols and fatty alcohols into their more volatile derivatives is a necessary step prior to gas chromatography individual separation. 200 μl of the clean extract was subjected to dryness by a nitrogen stream and the residue reconstituted with 100-μl *N*-pyridine and homogenized in a vial for 1 min; then, 98-μl BSTFA was added and the mixture shaken vigorously in the vial for 1 min. Finally, 2-μl chloride trimethylsilyl 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 favour derivatization.

2.5.5. Individual sterols and fatty alcohols separation and determination by GC–MS

After derivatization, 1 μl of the analytical sample was injected into the chromatograph. 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

Table 1
Ions monitored in SIM mode for identification-quantitation of fatty alcohols and sterols.

Alcohol/Sterol	Retention time (min)	*m/z*[a]
Eicosanol (IS)	24.5	80, **435**
Docosanol	24.7	41, 75, 207, 281, **355** (89), 384
Tricosanol	25.9	353, 280, 103(45), 73
Tetracosanol	30.2	80, 226, **412** (91), 491
Pentacosanol	33.0	80(90), 95, 226, 374, 464
Hexacosanol	33.3	80, **440** (75), 519
Heptacosanol	32.6	69, 8 (92), 147
Octacosanol	38.2	52, 80, 147, 226, **468** (90), 547
Cholesterol (IS)	37.9	44, 75, 147, 207, 330, 368(83)
Brassicasterol	38.3	73, 121, 265, **380** (42)
24-Methylene cholesterol	38.5	57, **147** (75), 192, 355
Campesterol	38.6	281, 344, **382** (83), 473, 503
Campestanol	38.6	185, 218, 343, **503** (89)
Stigmasterol	42.2	83, 129, 256, **395** (89), 485
Clerosterol	44.8	73, 170, **221** (42), 341
β-Sitosterol	45.1	73, 229, 255, 382, **396** (93)
Sitostanol	45.3	79, 147, 216, **384** (91), 474
Δ^5-Avenasterol	48.2	69, **80** (95), 161, 393, 408
Δ^7-Avenasterol	53.9	**89** (92), 203, 380, 379

[a] In brackets, third column, are given the percentages of the most abundant fragments

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 were kept at 220, 200 and 50 °C, respectively. The recording window was set between 40 *m*/*z* and 650 *m*/*z* and the data were acquired using total ion current (TIC) scan mode. Digital selected ion monitoring (SIM) was applied as data treatment in order to remove the chromatographic background.

2.6. Statistical analysis

The variables potentially influencing the leaching procedure were studied by a multivariate approach. Statgraphics Centurion XV, Statpoint technologies, Inc. (Warrenton, VA, USA) was used as statistical software with this purpose.

3. Results and discussion

Both raw materials under study, olive-tree leaves and drupes, were used in the optimization study, the sequence of which consisted of four steps. First, individual separation of the silylated analytes by gas chromatography and mass-detection variables were optimized for proper monitoring of sample preparation. In this experiments silylation was performed without ultrasound assistance, as described in the literature [33]. Then, the optimal working conditions for ultrasound-assisted extraction of the target compounds were established; those to accelerate saponification by ultrasound as well as those for solid-phase extraction were also optimized. The two last steps were optimized both with standards and extracts in order to detect potential interferents in the extracts. Finally, silylation was optimized.

3.1. Optimization of the determination step

The experimental chromatographic variables were optimized resulting in the operating conditions described in the experimental section. Splitless and split injections were tested to check the influence of the split ratio on the GC–MS analysis. A 1:50 split ratio was found optimum. Optimization was carried out both with standard solutions and extracts derivatized by the conventional procedure [33]. Cholesterol and 1-eicosanol were used as internal standards, for sterols and alcohols, respectively, because of their physical and chemical behaviours were similar to those of the target analytes and after checking their absence in the samples.

Concerning detection, the emission current of the filament was optimized with two purposes: (1) selection of the most characteristic ions to identify each analyte with the highest selectivity, and (2) selection of the ion with the highest sensitivity for quantitation of each alcohol and sterol in SIM mode. The optimum filament current was 80 μA for all analytes.

Identification of target analytes with commercially available standards (*viz.* 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 (*viz.* tricosanol, pentacosanol and some sterols as cholestanol, brassicasterol, 24-methylene cholesterol, campestanol, chlerosterol, Δ^5-avenasterol and Δ^7-avenasterol) were identified by comparing their retention times and mass spectra with those in the literature. These values and the principal ions for quantitation are listed in Table 1. Complete separation of the target compounds was achieved within 70 min, as shows Fig. 1.

3.2. Optimization of ultrasound-assisted leaching

This study was focused on the eight more influential variables on the leaching step in order to obtain the best values of them to remove the target analytes with the highest efficiency and without degradation. The eight variables with potential interrelation in the leaching of the unsaponifiable fraction from the two raw materials—and therefore studied by a multivariate design—a Plackett-Burman design $2^8 \times 3/64$ type III resolution allowing six degrees of freedom and involving 12 randomized runs plus three centre points—were ultrasound radiation amplitude, percent of ultrasound exposure (duty cycle), probe position (*viz.* distance between the bottom of the water-bath and the tip probe), irradiation time, water-bath temperature, extractant volume, extractant composition and number of leaching cycles. The upper and lower values given to each variable were selected from the experience gathered in preliminary experiments and are shown in Table 2A.

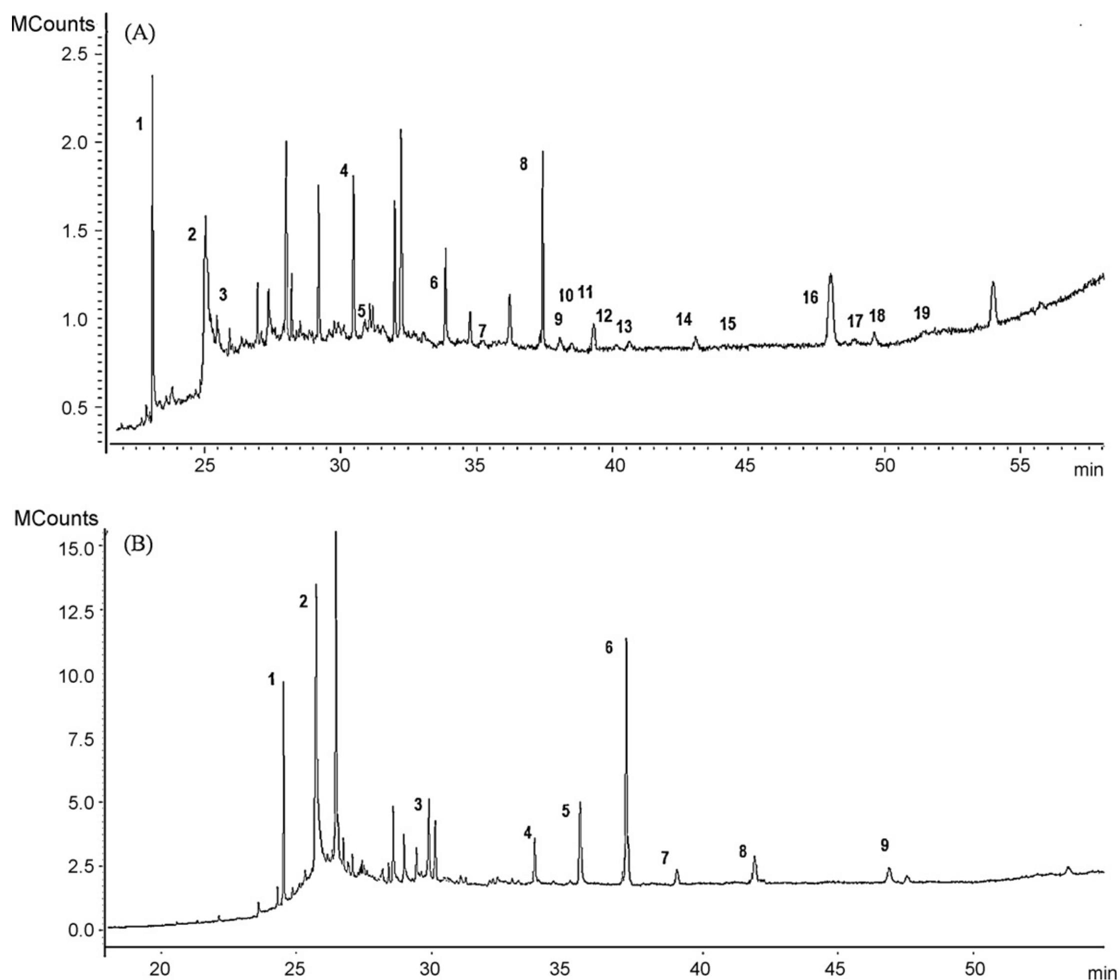

Fig. 1. (A) Chromatogram from an alcohol + sterol extract of olive drupes. Peak identification: 1, eicosanol (internal standard); 2, docosanol; 3, tricosanol; 4, tetracosanol; 5, pentacosanol; 6, hexacosanol; 7, heptacosanol; 8, cholesterol (internal standard); 9, octacosanol; 10, brassicasterol; 11, 24-methylene cholesterol; 12, campesterol; 13, campestanol; 14, stigmasterol; 15, clerosterol; 16, β-sitosterol; 17, sitostanol; 18, Δ^5-avenasterol and Δ^7-avenasterol. (B) Chromatogram from a 25 μg/ml standard solution of sterol and alcohol compounds. Peak identification: 1, eicosanol (internal standard); 2, docosanol; 3, tetracosanol; 4, hexacosanol; 5, cholesterol (internal standard); 6, octacosanol; 7, campesterol; 8, stigmasterol; 9, sitostanol.

The response variable was the extraction efficiency expressed as the ratio between the peak areas of each compound and that of the IS.

The conclusions of this screening study were that the irradiation amplitude, duty cycle, extract volume, probe position and water-bath temperature were not statistically influential factors within the ranges under study. However, the results showed higher extraction efficiencies with the lowest values tested for the irradiation amplitude (10%), duty cycle (10%), probe position (1 cm from the bath bottom and water-bath temperature (20 °C); and the highest value tested for the extractant volume (10 ml). The extractant composition, the number of extraction cycles and irradiation time were influential factors within the range under study. The first and second factors had positive influence on the process and the influence of the third was negative.

A second experimental design involving higher values for the extractant composition and the number of extraction cycles, and lower values for the irradiation time was carried out using the optimum conditions for the rest of the variables. The tested and optimum values obtained for each variable are shown in Table 2A. The conclusions for this screening were that the extractant composition was not a statistical influential factor in the range under study. However, the best results were obtained with a 66:33 dichloromethane–hexane mixture. The number of extraction cycles

Table 2A
Optimization of the ultrasound-assisted leaching of the target compounds from olive drupes and leaves.

Variable	Tested range (1st screening)	Tested range (2nd screening)	Optimum value
Radiation amplitude (%)	10–50		10
Duty cycle (%)	10–50		10
Irradiation time (min)	10–20	5–10	10
Extractant composition (dichloromethane–hexane)	33.3–66	66–100	66
Extractant volume	5–10		10
Cycles number	1–3	3–5	5[a]
Water-bath temperature (°C)	15–25		20
Probe position (cm)	1–3		1

[a] From univariate optimization

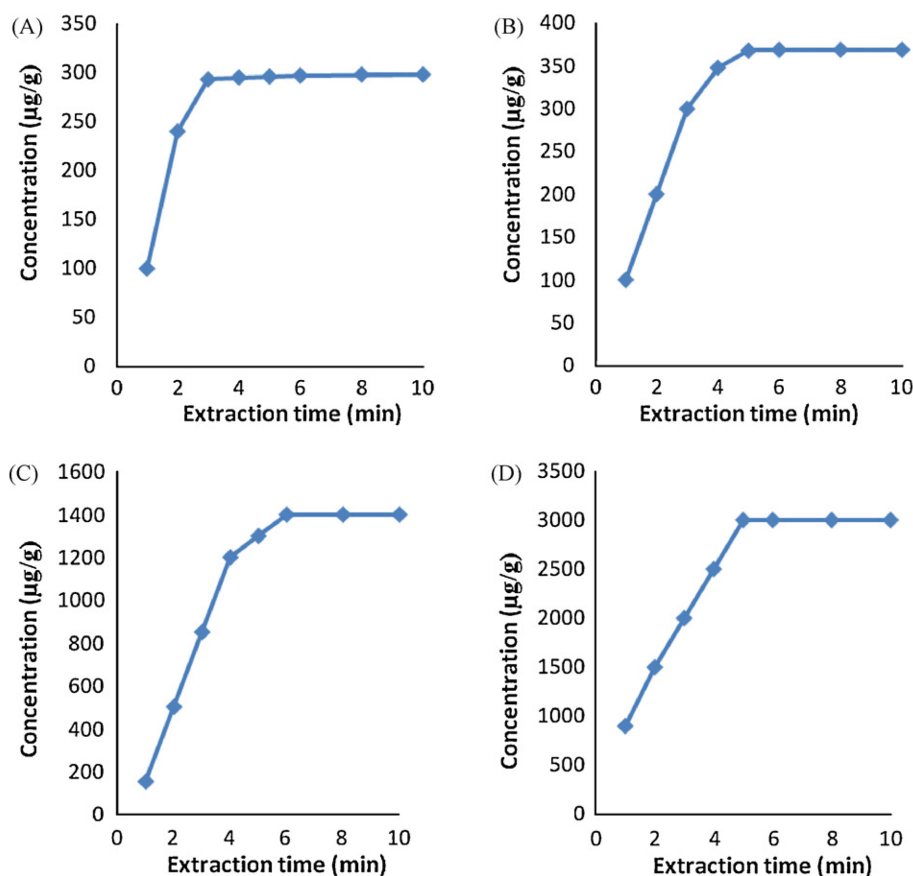

Fig. 2. Leaching kinetics of olive materials. (A) Fatty alcohols from leaves; (B) sterols from leaves; (C) fatty alcohols from drupes; and (D) sterols from drupes.

and irradiation time were influential factors, positive influence, within the range under study. Taking into account the fact that the irradiation time had also a significant but negative influence on the first design, 10 min was selected for further experiments. Finally, the number of cycles was studied to know obtain the number of extraction cycles necessary for exhaustive extraction of the target compounds. Fig. 2 shows that 5 cycles were necessary for extraction of the two target families in the case of olive drupes; and 3 and 5 cycles for alcohols and sterols, respectively, in case of leaves.

3.3. Saponification step

Saponification developed in homogeneous media which proceeded via radical or radical–ion intermediates, so it is sensitive to ultrasound energy [26]. For this reason, ultrasound energy was also used to accelerate saponification of the target analytes. In this case, the ultrasonic variables (duty cycle, irradiation amplitude and irradiation time) were optimized giving to the chemical variables (concentration and volume of the KOH solution) the optimum values proposed by Cunha et al. [34]. The temperature was fixed at 30 °C, as temperatures close to (or higher than) 40 °C are not recommended. This step was optimized with extracts from the two raw materials.

Table 2B
Optimization of the ultrasound-assisted saponification step.

Variable	Tested range	Optimum value
Duty cycle (%)	10–50	50
Irradiation time (min)	10–50	10
Irradiation amplitude (%)	10–50	45

The ultrasound variables were optimized by a response surface using a central composite design 2^3 + star, involving 16 plus 3 centre points. The tested ranges and the optimum values obtained from the design are shown in Table 2B. Comparison of the results obtained with the proposed method—assisted by ultrasound—and those provided by the conventional method showed that the reaction yield was nine times higher for the proposed method for a reaction time shortened from 50 to 10 min.

3.4. Solid-phase extraction

The isolation and cleanup of fatty alcohols and sterols using normal-phase sorbents—silica or aminopropyl—and appropriate

Table 2C
Optimization of the solid-phase extraction step.

Variable	Tested range	Optimum value
SPE sorbent	Aminopropyl, silica	Aminopropyl
Weight of analytical sample (μg)	100–300	200
Volume of cleaning solvent (ml 1:1 hexane–ethyl ether)	5–15	10
Volume of eluent for sterols (ml 5:1 hexane–chloroform)	3–12	6
Volume of eluent (ml hexane)	5–20	8

Table 3
Calibration curves, limits of detection (LODs), limits of quantitation (LOQs) of sterols and fatty alcohols found (intercept and slope errors in brackets).

Compound	Calibration curve	Linear range	LOD (mg/kg)	LOQ (mg/kg)
Docosanol	$Y = 11.8\ (0.1)X + 0.9\ (0.1)$	LOQ–50	1.3	4.5
Tricosanol	As docosanol			
Tetracosanol	$Y = 44.2\ (1.3)X + 6.4\ (0.9)$	LOQ–0.5	0.1	0.3
	$Y = 5.2\ (0.1)X + 65.1\ (2.3)$	0.5–25		
Pentacosanol	As tetracosanol			
Hexacosanol	$Y = 181.7\ (4.2)X + 1.3\ (0.1)$	LOQ–0.25	2.8	9.5
	$Y = 4.5\ (0.1)X + 38.4\ (0.6)$	0.25–25		
Heptacosanol	As hexacosanol			
Octacosanol	$Y = 338.9\ (2.8)X + 1.5\ (0.1)$	LOQ–0.25	0.2	0.7
	$Y = 3.5\ (0.1)X + 47.8\ (1.2)$	0.25–25		
Brassicasterol	As campesterol			
24-Methylene cholesterol	As campesterol			
Campesterol	$Y = 7.9\ (0.1)X + 1.3\ (0.1)$	LOQ–25	5.3	17.9
Campestanol	As campesterol			
Stigmasterol	$Y = 10.0\ (0.2)X + 2.9\ (0.2)$	LOQ–25	6.3	21.0
Clerosterol	As stigmasterol			
β-Sitosterol	As stigmasterol			
Sitostanol	$Y = 68.0\ (3.2)X + 0.1\ (0.1)$	LOQ–0.5	6.1	20.5
	$Y = 9.1\ (0.2)X + 41.9\ (3.2)$	0.5–25		
Δ^5-Avenasterol	As sitostanol			
Δ^7-Avenasterol	As sitostanol			

eluting solutions—hexane or hexane–chloroform mixtures—have been reported [35].

Five variables—sorbent type, and volumes of: (a) analytical sample as obtained after applying the previous step; (b) solvent circulated through the column after sample application to eliminate no retained species; (c) fatty alcohols eluent; (d) sterols eluent—were tested by a univariate design to obtain the optimum conditions for cleanup of the target analytes. The results obtained are shown in Table 2C.

3.5. Derivatization step

Ultrasound energy was also used to accelerate silylation of fatty alcohols and sterols. Ultrasound variables irradiation amplitude and duty cycle were studied in a multivariate mode using 5 min as irradiation time using a 5-μg/ml standard solution and leachates from olive leaves and drupes. The best conditions were 40% irradiation amplitude (180 W, applied power) and 50% of duty cycle (that is, 0.5 s/s), which were used to check the reaction time from 1 to 15 min. Only 10 min was required for maximum silylation of the target analytes; drastic decrease as compared with 120 min proposed by Janicsak et al. [33].

3.6. Characterization of the individual separation–detection method

Calibration plots were run for the seven analytes with commercial standards (peak area *versus* standard concentration—see Table 3). Compounds with no commercial standards were quantified by the calibration curve of the most similar alcohol or sterol and their typical characterization parameters also appear in Table 3. Eicosanol and cholesterol were used as IS for quantitation of fatty alcohols and sterols, respectively.

Calibration equations were established by using the ratio between the peak area of each compound and that of its family as a function of concentration of each compound. The regression coefficients ranged between 0.9991 and 0.9998 for the linear dynamic range tested for each analyte, which was LOQ–25 μg/ml. The characterization of the method was completed with the limits of detection (LODs) and quantification (LOQs), which were calculated from the GC–MS chromatograms obtained with natural samples. The peak height to averaged background noise ratio was calculated, for which the background noise was estimated by the peak to peak baseline near the analyte peak. LODs and LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise (S/N) ratio of 3 and 10, respectively.

The LODs for each analyte ranged between 9.8×10^{-2} and 2.0 μg/l for fatty alcohols and between 5.0 and 6.0 μg/l for sterols. The LOQs ranged from 0.3 to 0.9 μg/l and from 17.0 to 2.0 μg/l for fatty alcohols and sterols, respectively. LODs and LOQs were estimated for standard solutions and are shown in Table 3.

3.7. Assessment of precision

Within-laboratory repeatability was evaluated in a single experimental setup with duplicates with natural samples (leaves and

Table 4
Repeatability (s_r) and within-laboratory reproducibility (s_{WR}), expressed as relative standard deviation, of the proposed method.

Compound	Leaves		Drupes	
	s_r	s_{WR}	s_r	s_{WR}
Docosanol	4.0	6.8	4.4	7.5
Tricosanol	3.6	6.4	4.3	7.2
Tetracosanol	3.7	6.6	4.2	7.8
Pentacosanol	3.8	6.9	4.5	8.1
Hexacosanol	4.0	7.0	5.1	8.5
Heptacosanol	4.2	7.2	5.2	8.3
Octacosanol	5.1	8.0	6.1	9.2
Brassicasterol	3.8	6.6	4.0	6.9
24-Methylene	3.7	6.9	4.2	7.3
Campesterol	3.9	7.1	4.7	7.2
Campestanol	4.0	6.6	4.9	7.4
Stigmasterol	4.2	7.2	5.1	7.9
Clerosterol	4.1	7.7	5.0	8.2
β-Sitosterol	4.4	7.8	4.0	8.0
Sitostanol	3.9	6.4	4.8	8.1
Δ^5-Avenasterol	3.8	6.5	4.7	7.3
Δ^7-Avenasterol	4.2	7.8	5.1	8.4

Table 5
Analysis of sterols and fatty alcohols in olive leaves and drupes using the proposed method (all the results are expressed as μg/g—errors, in brackets, expressed as %, $n = 3$ replicates).

Compound	DRUPES[a]																			
	1				2				3				4				5			
	CONV		US		CONV		US		CONV		US		CONV		US		CONV		US	
Docosanol	3.0	(1.9)	7.8	(1.8)	11.5	(1.9)	83.7	(2.0)	4.1	(2.1)	7.3	(2.3)	14.2	(2.1)	22.5	(2.0)	6.7	(2.0)	2.4	(2.0)
Tricosanol	<LOD		22.1	(2.1)	<LOD		21.7	(2.0)	0.3	(1.8)	5.5	(1.9)	1.3	(1.9)	7.0	(1.6)	0.1	(2.1)	14.4	(2.2)
Tetracosanol	22.3	(1.6)	218.9	(1.8)	34.5	(1.8)	339.9	(2.3)	20.4	(2.9)	46.3	(1.8)	25.5	(1.7)	40.5	(1.6)	50.8	(1.7)	48.2	(1.7)
Pentacosanol	5.6	(2.0)	372.8	(1.9)	4.8	(2.9)	370.2	(2.1)	4.9	(2.8)	10.3	(2.4)	3.2	(2.7)	9.1	(2.5)	<LOD		38.7	(1.9)
Hexacosanol	52.0	(1.9)	403.9	(1.4)	35.6	(2.6)	386.7	(2.7)	55.0	(1.7)	99.6	(1.6)	19.1	(1.9)	56.8	(1.8)	95.2	(1.5)	36.4	(2.3)
Heptacosanol	0.9	(2.3)	104.2	(2.5)	0.5	(2.3)	102.1	(2.6)	0.1	(2.9)	1.0	(2.9)	0.1	(2.8)	0.5	(2.9)	0.0	(3.2)	2.5	(2.5)
Octacosanol	20.3	(3.3)	144.1	(3.2)	11.8	(3.5)	130.6	(3.3)	17.6	(3.4)	33.8	(3.6)	1.8	(3.2)	17.9	(3.1)	18.5	(3.3)	7.3	(3.4)
Brassicasterol	0.2	(1.8)	4.0	(2.3)	0.1	(2.5)	3.7	(2.2)	0.3	(2.8)	2.1	(2.2)	0.2	(2.4)	1.8	(2.8)	0.2	(2.8)	0.2	(2.9)
24-Methylene	<LOD		0.2	(2.8)	<LOD		<LOD		<LOD		0.9	(2.7)	<LOD		0.9	(2.5)	<LOD		<LOD	
Campesterol	3.9	(1.6)	6.5	(2.1)	21.0	(2.5)	258.2	(1.6)	7.5	(1.9)	16.1	(1.8)	28.6	(1.7)	38.4	(1.5)	5.0	(1.9)	2.9	(2.6)
Campestanol	0.1	(1.9)	0.8	(2.0)	0.6	(2.3)	7.8	(1.9)	0.2	(2.7)	0.6	(3.0)	0.8	(2.1)	1.4	(1.9)	0.1	(2.3)	8.1	(1.8)
Stigmasterol	8.2	(2.0)	19.8	(2.3)	8.2	(2.4)	79.3	(2.7)	14.6	(2.9)	24.8	(2.3)	7.1	(2.2)	19.2	(1.8)	1.3	(2.3)	1.1	(2.8)
Clerosterol	0.1	(2.2)	1.9	(2.4)	0.1	(2.9)	6.7	(2.6)	0.8	(2.6)	2.5	(2.1)	0.4	(2.1)	2.2	(2.1)	2.0	(1.9)	3.1	(2.6)
β-Sitosterol	229.7	(1.5)	2399.7	(1.8)	137.1	(1.8)	2594.6	(1.8)	475.1	(2.4)	1407.0	(1.5)	141.5	(1.7)	1205.4	(1.5)	272.3	(1.8)	1138.8	(1.5)
Sitostanol	0.7	(2.3)	71.7	(2.7)	0.6	(2.6)	71.7	(2.9)	0.4	(2.7)	2.1	(2.4)	0.4	(2.9)	2.1	(2.3)	1.3	(2.5)	18.2	(1.8)
Δ^5-Avenasterol	2.0	(2.2)	8.5	(2.5)	2.5	(2.8)	19.4	(2.6)	9.0	(2.0)	15.5	(2.5)	10.3	(2.4)	29.9	(1.9)	1.0	(2.4)	6.3	(1.9)
Δ^7-Avenasterol	2.3	(2.3)	10.4	(2.1)	4.2	(2.9)	21.9	(2.8)	2.2	(3.0)	3.8	(2.3)	2.8	(2.2)	6.8	(2.0)	1.2	(2.1)	8.4	(1.8)
Alcohols	104.0		1273.9		98.7		1434.9		102.4		203.9		65.2		154.3		171.2		149.8	
Sterols	247.1		2523.5		174.5		3063.3		510.0		1475.4		192.1		1308.1		284.1		1187.1	
Compound	LEAVES[a]																			
	1				2				3				4				5			
	CONV		US		CONV		US		CONV		US		CONV		US		CONV		US	
Docosanol	1.4	(1.7)	2.4	(1.9)	1.7	(1.5)	3.0	(1.8)	0.2	(1.9)	0.7	(2.1)	0.3	(2.2)	2.8	(2.3)	0.7	(2.1)	15.3	(2.0)
Tricosanol	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		0.1	(3.2)	1.0	(2.3)
Tetracosanol	2.8	(1.5)	7.9	(1.8)	1.4	(1.9)	2.8	(1.6)	0.2	(2.0)	3.2	(1.9)	0.2	(1.8)	5.3	(1.6)	2.9	(1.8)	26.6	(1.5)
Pentacosanol	<LOD		<LOD		<LOD		<LOD		0.1	(2.2)	1.2	(1.9)	0.1	(2.8)	1.6	(1.9)	0.7	(1.9)	12.6	(1.6)
Hexacosanol	13.9	(1.6)	27.3	(1.4)	10.5	(1.8)	16.0	(1.7)	2.4	(1.8)	13.7	(1.6)	2.1	(1.9)	8.6	(1.7)	9.1	(1.6)	169.0	(1.6)
Heptacosanol	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		0.1	(1.7)
Octacosanol	3.4	(3.3)	6.4	(3.1)	2.7	(3.0)	4.2	(3.4)	1.1	(3.0)	4.8	(3.1)	1.0	(3.1)	2.8	(3.6)	3.5	(3.4)	73.9	(3.3)
Brassicasterol	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LDO		<LOD		0.1	(2.4)	0.5	(2.0)
24-Methylene	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Campesterol	1.4	(3.0)	2.5	(1.7)	1.7	(1.7)	2.9	(1.7)	0.3	(2.0)	1.0		0.4	(1.7)	3.2	(1.5)	1.4	(1.9)	32.7	(2.0)
Campestanol	<LOD		<LOD		<LOD		0.1	(2.6)	<LOD		<LOD		<LOD		0.1	(1.8)	<LOD		0.8	(2.4)
Stigmasterol	0.4	(1.8)	0.7	(1.7)	0.4	(2.4)	0.6	(2.2)	0.7	(1.8)	2.2	(1.8)	0.7	(2.1)	1.1	(1.5)	2.7	(1.8)	66.5	(1.9)
Clerosterol	0.6	(2.8)	1.1	(2.8)	0.6	(2.8)	0.9	(3.0)	<LOD		<LOD		<LOD		<LOD		0.1	(3.0)	3.8	(1.9)
β-Sitosterol	75.7	(1.7)	129.9	(1.6)	67.1	(1.6)	101.0	(1.5)	17.8	(1.5)	77.3	(1.6)	16.7	(1.6)	32.6	(1.4)	85.7	(1.6)	204.6	(1.5)
Sitostanol	0.3	(2.7)	0.5	(2.4)	0.2	(3.0)	0.4	(2.9)	<LOD		0.2	(2.8)	<LOD		0.2	(2.0)	<LOD		<LOD	
Δ^5-Avenasterol	0.4	(2.9)	0.2	(1.9)	0.5	(2.0)	0.2	(1.8)	0.2	(1.8)	0.6	(2.2)	0.2	(2.1)	0.9	(1.7)	1.8	(2.6)	49.0	(1.8)
Δ^7-Avenasterol	0.5	(2.9)	0.9	(2.0)	0.6	(2.0)	0.9	(1.7)	0.3	(1.9)	0.7	(2.1)	0.3	(2.0)	1.1	(1.7)	0.4	(1.9)	11.4	(1.7)
Alcohols	21.4		44.0		16.3		26.0		4.0		23.6		3.6		21.2		16.9		298.5	
Sterols	79.3		135.9		71.1		107.0		19.3		82.0		18.3		39.2		92.0		369.1	

[a] CONV and US refer to conventional and ultrasound-assisted method, respectively.

drupes) subjected to the proposed method. Two analyses of these samples per day were carried out for 7 days.

Eq. (1) was used to determine the variance due to inter-day repeatability:

$$s^2_{\text{between}} = \frac{\text{MS}_{\text{between}} - \text{MS}_{\text{within}}}{nj} \quad (1)$$

where MS is the mean square (residual sum of squares rated by the freedom degrees) and nj is the number of replicates per day. The within-laboratory reproducibility, s^2_{WR}, was calculated by Eq. (2).

$$s^2_{\text{WR}} = s^2_{\text{r}} + s^2_{\text{between}} \quad (2)$$

where s^2_{r} is the residual mean squares within-days, and s^2_{between} is the variance due to the inter-day effect.

The results obtained are listed in Table 4. Between-day precision, expressed as relative standard deviation (RSD), ranges between 3.6 and 6.1% and the within-laboratory reproducibility, also expressed as RSD, between 6.4 and 9.2%.

3.8. Characterization of alcohol and sterol fractions

Ten samples (drupes and leaves) obtained from different varieties were used to assess the proposed method. The chromatogram from an alcohol + sterol extract of olive drupes is shown in Fig. 2A, and the results for each analyte and sample are summarized in Table 5.

3.8.1. Fatty alcohols fraction

In general terms, the concentration of the alcohols in the drupes ranged between 154.3 and 1434.9 μg/g, which is higher than in leaves, where the content of these compounds ranged between 21.2 and 298.5 μg/g. The concentration of each compound in a target raw material depends on the variety.

The study of the composition of the alcoholic fraction shows that all the studied alcohols are present in the samples. The concentration of each analyte depends on the sample, but in general, the alcohols present at higher concentrations are hexacosanol followed by tetracosanol; the alcohols at lower concentrations are tricosanol and docosanol. The concentration of octacosanol, one of the most important bioactive alcohols according to the literature, ranges between 7.3 and 144.1 μg/g.

Concerning leaves, they do not contain alcohols with an impair number of carbon atoms, hexacosanol is the alcohol at the highest concentration in all the varieties, meanwhile the concentration of octacosanol ranges between 2.8 and 73.9 g/g.

3.8.2. Sterols fraction

The concentration of sterols in drupes and leaves is higher than that of alcohols. Furthermore, their concentration in drupes (between 1187.1 and 3064.3 μg/g) is higher than that in leaves (between 39.2 and 369.1 μg/g).

The results obtained are in agreement with those found in the literature on the composition of sterols fraction: the predominant sterol is β-sitosterol, and minor components are campesterol, stigmasterol, Δ^5-avenasterol, Δ^7-avenasterol and brassicasterol.

All sterols under study were quantifiable in drupe samples. In the case of leaves, 24-methylene cholesterol was under the LOD in all cases and brassicasterol was detected only in one sample (sample number 5) and at low concentration.

3.9. Comparison of the efficiency of the proposed method versus that of the conventional method

The efficiency of the conventional method based on maceration extraction applied for 24 h was calculated taking as 100% the efficiency of the proposed method. In this way, 22% and 36% were the efficiencies provided by the former method for the fatty alcohol and sterol fractions, respectively. In addition to the high efficiency of the proposed ultrasound-assisted extraction, no degradation of target compounds was detected, as demonstrated by the plateaux in Fig. 2 when the number of extraction cycles increased and thus, the subjection of the extracted compounds to longer times of ultrasonic irradiation.

4. Conclusions

The method reported here is a clear demonstration of the enormous capability of ultrasound to favour and/or accelerate a number of steps of the analytical process [34]. Solid–liquid extraction and free-radical involved reactions, such as saponification or silylation, are drastically shortened and also their yields improved by ultrasonic assistance.

In short, the contributions of the method here proposed are as follows:

(1) It is the first time that a method allows the simultaneous determination of sterols and fatty alcohols from olive drupes and leaves in a single chromatogram, thus saving time and resources.
(2) The leaching step of these compounds accelerated by ultrasonic irradiation is also reported for the first time (the time required for this step was shortened from 24 h to 10 min).
(3) The use of aminopropyl sorbent for solid-phase extraction provides a clean extract.
(4) Saponification of the target analytes, also assisted by ultrasound, shortens the time for this step from 2 h to 10 min with no degradation of analytes.
(5) The extraction efficiency of the proposed method is 5 and 3 times higher than that provided by the conventional maceration method in the case of alcohols and sterol fractions, respectively.

The results obtained for the composition of the alcohol and sterol fractions are in agreement with those provided by the literature.

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