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Influence of Deep Frying on the Unsaponifiable Fraction of Vegetable Edible Oils Enriched with Natural Antioxidants

Mara I. Orozco,[†,‡] Feliciano Priego-Capote,[*,†,‡] and María D. Luque de Castro[†,‡]

[†]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071 Córdoba, Spain

[‡]Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071 Córdoba, Spain

Supporting Information

ABSTRACT: The influence of deep frying, mimicked by 20 heating cycles at 180 °C (each cycle from ambient temperature to 180 °C maintained for 5 min), on the unsaponifiable fraction of vegetable edible oils represented by three characteristic families of compounds (namely, phytosterols, aliphatic alcohols, and triterpenic compounds) has been studied. The target oils were extra virgin olive oil (with intrinsic content of phenolic antioxidants), refined sunflower oil enriched with antioxidant phenolic compounds isolated from olive pomace, refined sunflower oil enriched with an autoxidation inhibitor (dimethylpolysiloxane), and refined sunflower oil without enrichment. Monitoring of the target analytes as a function of both heating cycle and the presence of natural antioxidants was also evaluated by comparison of the profiles after each heating cycle. Identification and quantitation of the target compounds were performed by gas cromatography−mass spectrometry in single ion monitoring mode. Analysis of the heated oils revealed that the addition of natural antioxidants could be an excellent strategy to decrease degradation of lipidic components of the unsaponifiable fraction with the consequent improvement of stability.

KEYWORDS: olive oil, sunflower oil, fatty alcohols, sterols, triterpenic compounds, phenolic antioxidants, oil enrichment, deep frying, GC-MS

INTRODUCTION

Deep fat frying is one of the most common processes used worldwide for the preparation of cooked food. Both physical and chemical changes occurring in oil as a result of frying are mainly due to temperature but also to interaction between oil, light, air, water, oxygen free radicals, enzyme action, and food components.[1] During the frying process, the oil or fat is exposed to high tem peratures in the presence of air and moisture. Under these conditions, a cascade of chemical reactions is activated, resulting in the loss of quality of the frying oil, and of the fried food, evidenced by the variation of the sensory and nutritional characteristics.

Although general modifications of the main fat constituents are well-known, it is not easy to foresee the rate of fat degradation due to the number of variables involved in the process. Among these variables, Dobarganes et al. emphasized variables linked to the process itself, such as temperature, heating time, continuous or discontinuous heating, and turnover rate,[2] and those associated with the oil or fat used (e.g., unsaturation degree, initial quality, and additives). The main chemical modifications affecting the quality of frying oil are caused by temperature, oxygen, and moisture. It is worth noting that oxygen plays an important role in the deterioration of the oil during frying, but rarely is it a limiting factor. Under ambient conditions, autoxidation occurs by a free radical mechanism in which hydroperoxides play a primary role.[2] With temperature increase, oxidation leads to the formation of hydroperoxides, which do not accumulate at the frying temperature due to their instability. These intermediates decompose spontaneously to form volatile organic compounds such as alcohols, ketones, aldehydes, epoxides, and hydrocarbons or generate dimers and trimers. At an advanced level, dimers and cyclic compounds can be polymerized. Oxidation plays a significant role in the development of rancid flavors, which negatively modify the organoleptic characteristics, and in the formation of oxidized products that may cause a health hazard.[3] Hydrolysis alteration is caused by moisture content, even at trace levels, resulting in free fatty acids, monoglycerides, diglycerides, and glycerol.[4] These reactions, which massively affect the major fraction of vegetable oils, composed of acylglycerols (95−98%), have been widely studied.[5,6] However, the effect on the minor fraction (2−5%), composed mainly by aliphatic alcohols, triterpenic compounds, sterols, hydrocarbons, volatile compounds, and antioxidants, has been less studied. Thus, Boskou evaluated the effect of frying on the stability of some of these families to retard lipid polymerization.[7] The minor fraction is of particular interest for the oil quality due not only to its contribution to organoleptic properties and product stability but also from a nutraceutical point of view thanks to health benefits linked to many compounds in this fraction. Three representative groups present in the minor fraction are phytosterols, aliphatic alcohols, and triterpenes.

Phytosterols are isoprenoid compounds with a sterol nucleus and an alkyl chain. They have nutritional interest because of their potential to lower both total serum cholesterol and LDL cholesterol in humans.[8,9] In the presence of oxygen and at frying temperature (150−190 °C), sterols can lead to the formation of

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Table 1. Name, Formula, m/z Value, Retention Time, and Segment for Each Compound and Ions for Quantification in SIM Mode

compound	formula	retention time	segment	ions for quantification
aliphatic alcohols				
1-eicosanol (IS)	$C_{20}H_{42}O$	26.247	1	355.3
1-docosanol	$C_{22}H_{46}O$	27.266	2	383.5
1-tetracosanol	$C_{24}H_{50}O$	29.471	3	411.3
1-hexacosanol	$C_{26}H_{54}O$	32.060	5	439.5
1-octasanol	$C_{28}H_{58}O$	34.987	6	370.2/467.2
sterols				
cholestanol (IS)	$C_{27}H_{48}O$	34.957	6	370.2/467.2
campesterol	$C_{28}H_{48}O$	36.613	7	382.3/394.4
stigmasterol	$C_{29}H_{48}O$	36.997	7	382.3/394.4
β-sitosterol	$C_{29}H_{50}O$	37.938	8	215.3/383.5/396.0
stigmastanol	$C_{29}H_{52}O$	38.128	8	215.3/383.5/396.0
triterpenes				
erythrodiol	$C_{30}H_{50}O_2$	40.365	9	216.1/496.5
uvaol	$C_{30}H_{50}O_2$	41.064	9	216.1/496.5
oleanolic acid	$C_{30}H_{48}O_3$	42.335	10	203.3/320.3
betulinic acid (IS)	$C_{30}H_{48}O_3$	41.794	10	203.3/320.3
ursolic acid	$C_{30}H_{48}O_3$	41.448	10	203.3/320.3
maslinic acid	$C_{30}H_{48}O_3$	44.069	11	203.1/320.1

oxysterols, which are absorbed in the small intestine and incorporated into chylomicrons with toxic effects. Some examples of these effects are alteration of the structure and function of the cellular membranes or changes in the activity and expression of enzymes involved in cholesterol biosynthesis.[10]

Aliphatic alcohols are derived from natural fats and oils not only originated in plants but also synthesized in animals and algae. Their historically overlooked significance in nutrition and health is at present recognized, as they are closely related to fatty acids, including the well-documented omega-3 fatty acids. Aliphatic alcohols are clinically interesting as they are endowed with anticancer, antiviral, antifungal, and anti-HIV properties and, thus, with potential in medicine and as health supplements.[10,11]

Seeds and drupes from which edible oils are extracted contain triterpenic compounds, which are partially extracted in the oil as a part of the unsaponifiable fraction. Several studies have shown that these compounds possess healthy properties,[12] and they have also been used as a purity parameter to detect olive oil frauds with pomace olive oil;[13] however, some papers have reported that high amounts of triterpenes deteriorate organoleptic oil quality.[14]

Antioxidants naturally present in or added to oils exert beneficial effects by avoiding oil chemical alteration during heating. Natural antioxidants such as phenolic compounds have demonstrated an antioxidant activity superior to that of synthetic antioxidants. Therefore, there is an increased trend to replace the latter with natural antioxidants.[15] Enrichment with phenols protects edible oils against oxidation; that means better oil quality and prevention of the formation of toxic oxidation products such as cholesterol oxides.[16] The aim of the present work was to evaluate the effect of the addition of natural antioxidants on the stability of vegetable oils subjected to 20 cycles of simulated deep frying at 180 °C (each cycle from ambient temperature to 180 °C, maintained for 5 min). Three different fractions present in the unsaponifiable fraction were monitored to detect changes during the heating process. The stability of the oil enriched with natural antioxidants was compared to that of the same oil spiked with a synthetic autoxidation inhibitor and with those of two pure oils (extra virgin olive oil and sunflower oil) as references

MATERIALS AND METHODS

Reagents. Fatty alcohols (docosanol, tetracosanol, hexacosanol, and octacosanol) and sterols (campesterol, stigmasterol, stigmastanol, and β-sitosterol) were purchased from Sigma-Aldrich (St. Louis, MO) and used as standards. The most representative and commercially available triterpenic compounds , that is, erythrodiol from Extrasynthese (Genay, France) and uvaol, oleanolic acid, and ursolic acid from Sigma-Aldrich, were also used. Maslinic acid (>97% purity) isolated by a previously reported protocol[17] was a gift from A. García-Granados (University of Granada, Spain).

Betulinic acid was used as internal standard (IS) for triterpene quantitation, whereas eicosanol and cholestanol (all from Sigma-Aldrich) were used as IS for alcohols and sterols, respectively. 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, four sterols, and five triterpenes, were prepared by appropriate dilution of the stock solutions. All solutions were stored at −20 °C in glass flasks until use.

n-Hexane LC gradient grade was provided by Panreac (Barcelona, Spain). Anhydrous sodium sulfate from Sigma-Aldrich was used as drying agent for the nonpolar phase in the derivatization step. A 2 M KOH methanolic solution (Panreac) was used for oil saponification.

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

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 laboratory-made stainless steel container with eight compartments to place test tubes. A Selecta Mixtasel centrifuge (Barcelona, Spain) was used to separate the immiscible phases after saponification. A mechanical electrical stirrer MS2 minishaker from IKA (Wilmington, NC) was used to assist the derivatization step.

A Varian CP-3900 gas chromatograph (Walnut Creek, CA) equipped with a programmable-temperature injector and coupled to a Saturn 2100 ion-trap mass spectrometer (Sunnyvalley, TX) was used for analysis of

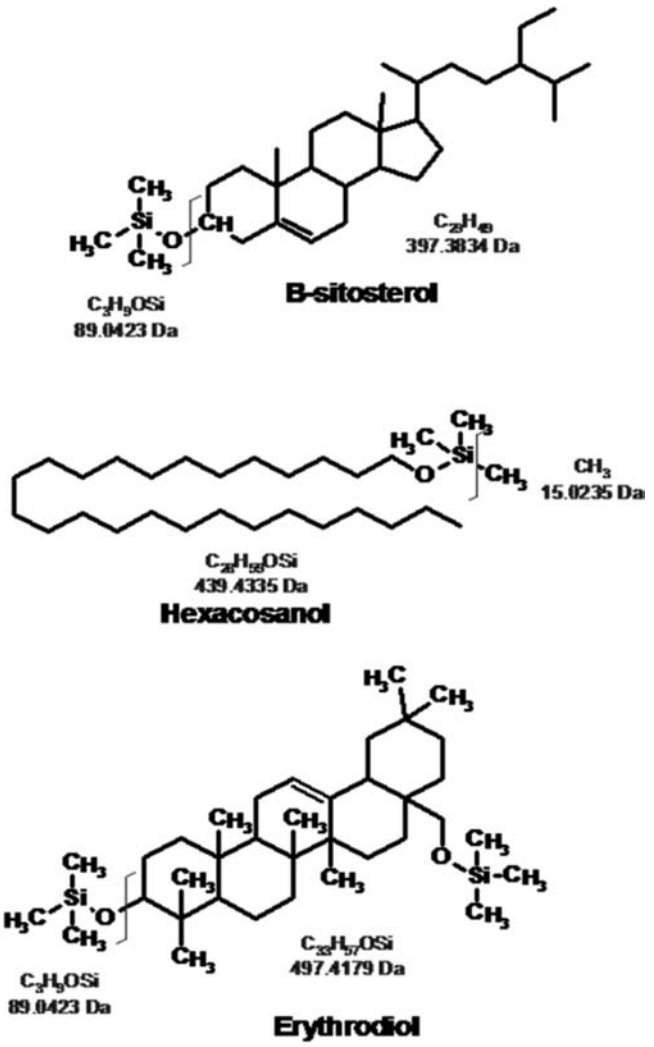

Figure 1. Fragmentation of the derivatization products of the most representative target analytes of each fraction (β-sitosterol, hexacosanol, and erythrodiol).

fatty alcohols, sterols, and triterpenic compounds. The chromatograph was equipped 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.

A stainless deep fryer (Fagor F-206, Barcelona, Spain) was used for oil heating (simulation of the frying process).

Preparation of Edible Oils. Edible oils with a known content in antioxidants [extra virgin olive oil as such (VOO), refined sunflower oil enriched with an extract of phenolic compounds from olive pomace (ASO), refined sunflower oil enriched with dimethylpolysiloxane as an artificial autoxidation inhibitor (DSO), and refined sunflower oil without enrichment (SO)] were used. Koipesol (SOS Cuétara S.A., Madrid) provided the oils for subsequent enrichment in the laboratory.

Deep Heating Oil Procedure. Two liters of the selected oil was placed in a stainless deep fryer. The oil was subjected to 20 heating cycles at 180 ± 5 °C for 5 min/cycle (each cycle from ambient temeperature to 180 °C, which was maintained for 5 min). Three milliliter aliquots from the target oil were removed after each heating period and stored at −20 °C until use for a unique treatment by in-triplicate analysis.

Sample Preparation. Two grams of the tested oil was placed in a test tube to which 2 mL of 2 M KOH and 10 μL of internal standard for sterols, fatty alcohols and triterpenic compounds (0.1% chloroform

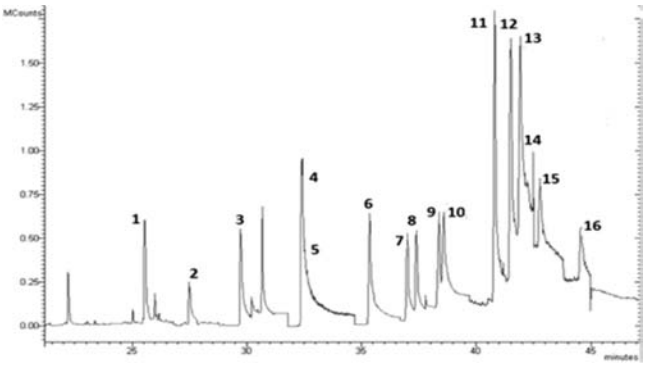

Figure 2. Total ion chromatogram of a 15 μg/L standard solution of sterols, aliphatic alcohols, and triterpenic compounds. Peaks: 1, eicosanol (internal standard); 2, docosanol; 3, tetracosanol; 4, hexacosanol; 5, cholestanol (internal standard); 6, octacosanol; 7, campesterol; 8, stigmasterol; 9, β-sitosterol; 10, stigmastanol; 11, erythrodiol; 12, uvaol; 13, oleanolic acid; 14, betulinic acid (internal standard); 15, ursolic acid; 16, maslinic acid.

solutions of cholestanol and 1-eicosanol and 0.1% ethanol solution of betulinic acid) were added. The tube was immersed in the water bath at 23 °C, where ultrasonic irradiation (duty cycle 70%, output amplitude 30% of the converter) was applied for 15 min. After cooling at room temperature, the unsaponifiable fraction was extracted with 4 mL of hexane, and the immiscible organic phase was separated by centrifugation for 10 min at 1500*g*. Finally, this phase was washed with distilled water until the aqueous phase resulted in neutral reaction. The unsaponifiable fraction was dried and the residue dissolved in 0.4 mL of hexane. The resulting solution was passed through an aminopropyl column, in which the sterols, fatty alcohols, and triterpenic dialcohols were bound to the sorbent, whereas the nonretained compounds were disposed of. The column was conditioned by two consecutive washing steps with 2 mL of hexane each. The analytical sample was then applied to the cartridge under vacuum suction. The interfering substances were removed by washing the cartridge with 4 mL of hexane. Then, the fraction containing the target compounds was eluted with 6 mL of 1:1 hexane/ethyl acetate.

Finally, conversion of sterols, fatty alcohols, and triterpenic dialcohols into their more volatile derivatives is a necessary step prior to GC individual separation. With this aim, 200 μL of clean extract was subjected to dryness by a nitrogen stream and the residue reconstituted with 100 μL of *N*-pyridine and homogeneized 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 chloride trimethylsilane was added, and the mixture was shaken vigorously in the vial for an additional 2 min and then subjected to ultrasound (output amplitude 40% of the converter, duty cycle 50%) for 10 min to accelerate the derivatization reaction.[18,19] Finally, the resulting solution was analyzed in triplicate by GC-MS.

Determination of Sterols, Fatty Alcohols, and Triterpenes by GC-MS. The individual separation of sterols, fatty alcohols, and triterpenes and determination by GC-MS were similar to those previously proposed by the authors.[20] Briefly, 1 μL of 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 at a 100:1 split ratio for 10 min. The oven temperature program was as follows: initial temperature of 70 °C (held for 1.20 min), increased at 25 °C/min to 120 °C, followed by a second gradient at 2 °C/min to 243 °C, and, finally, increased at 40 °C/min to 270 °C (held for 50 min). The total analysis time was 70 min, and 10 min of extra time was necessary for re-establishing and equilibrating the initial conditions.

Table 2. Calibration Curves, Limits of Detection (LODs), and Limits of Quantitation (LOQs) of Sterols, Aliphatic Alcohols, and Triterpenes

compound	calibration curve	R^2	linear range	LOQ (μg/L)	LOD (μg/L)
docosanol	$Y = 3.0 \times 10^{-4}X + 0.4559$	0.998	LOQ−0.50	0.06	0.19
tetracosanol	$Y = 3.0 \times 10^{-4}X + 0.3302$	0.990	LOQ−0.50	0.04	0.09
hexacosanol	$Y = 1.5 \times 10^{-3}X + 0.8455$	0.992	LOQ−0.50	0.02	0.06
octacosanol	$Y = 1.5 \times 10^{-3}X + 0.3643$	0.999	LOQ−0.50	0.11	0.36
stigmasterol	$Y = 8.0 \times 10^{-5}X + 0.0448$	0.996	LOQ−0.50	0.43	1.43
β-sitosterol	$Y = 1.0 \times 10^{-4}X + 0.1097$	0.997	LOQ−0.50	0.95	3.15
stigmastanol	$Y = 3.0 \times 10^{-4}X + 0.1126$	0.999	LOQ−0.50	0.98	3.26
campesterol	$Y = 5.0 \times 10^{-5}X + 0.0493$	0.991	LOQ−0.50	1.09	3.64
erythrodiol	$Y = 1.0 \times 10^{-3}X + 1.630$	0.994	LOQ−0.50	0.29	0.97
uvaol	$Y = 6.0 \times 10^{-4}X + 0.9327$	0.989	LOQ−0.50	0.55	1.86
oleanolic acid	$Y = 3.0 \times 10^{-4}X + 0.177$	0.999	LOQ−0.50	0.98	3.27
ursolic acid	$Y = 4.0 \times 10^{-4}X + 0.1004$	0.997	LOQ−0.50	0.37	1.23
maslinic acid	$Y = 9.0 \times 10^{-5}X + 0.0359$	0.999	LOQ−0.50	0.88	2.02

The ion-trap mass spectrometer was operated in single ion monitoring (SIM) mode after electron impact positive ionization (EI). The manifold, trap, and transfer line temperatures were set at 220, 200, and 50 °C, respectively. The analyses were performed with a filament−multiplier delay of 20 min, and data acquisition was performed in the range m/z 50−650 in full scan mode, with a background mass of m/z 45. The emission current of the ionization filament was set at 80 μA. The scan time for data acquisition was set at 1.0 s in SIM mode, whereas 3 microscans/s was selected in full scan mode. Table 1 includes time segments and ions monitored for each compound. The fragmentation schemes for representative compounds of each family (sterols, fatty alcohols, and triterpenes) are shown in Figure 1.

Identification of analytes was ensured with standard solutions and spiked samples by comparison of mass spectra and retention times. Figure 2 shows a chromatogram obtained from a multistandard solution. As can be seen, complete separation was achieved within 52 min. The retention times of sterols, fatty alcohols, and triterpenic compounds depend on the length of their chains, double bond number, position, geometry, and branches. Also, the elution temperature program affects the elution order of sterols and triterpenic compounds with different numbers of double bonds. In preliminary tests, the absence in the samples of the selected internal standards was checked by targeted analysis.

Statistical Analysis. The variables potentially influencing the saponification procedure were studied by a multivariate approach. Statgraphics Centurion XV, Statpoint Technologies, Inc. (Warranton, VA), was used as statistical software for this purpose.

RESULTS AND DISCUSSION

Preparation of Vegetable Oils. Four different oils were selected to study the influence of antioxidants on the stability of the three target groups of compounds present in the unsaponifiable fraction during simulated deep frying. Olive and sunflower oils were used as reference to assess the antioxidants' effect, as the former oil is characterized by the natural presence of phenolic antioxidants, whereas sunflower oil loses them in the refining process. To obtain a VOO with a total phenol concentration of 400 μg/mL, expressed as caffeic acid according to the Folin−Ciocalteu test, two different olive oils were mixed at the suitable proportion. On the other hand, pure refined sunflower was used as such and also enriched with natural antioxidants to compare their effect with that of a synthetic autoxidation inhibitor. The enrichment was as follows: (i) With an ethanolic extract of phenol compounds from an olive pomace residue using the protocol described by Giron et al.[19] Enrichment was carried out up to a total phenols concentration of 400 μg/mL, expressed as caffeic acid. The composition of the olive pomace extract is included in Supplementary Table 1 of the Supporting Information. (ii) With 400 μg/mL of a synthetic autoxidation inhibitor (dimethylsiloxane). This additive (E900) was originally added to frying oils to prevent foaming, but it also possesses an oxidation inhibition activity by an uncertain mechanism.[21] The speed and efficiency of the heating process depend on the temperature and quality of the oil. The heating temperature is usually between 150 and 190 °C, set for this experiment at 180 °C.

Characterization of the Individual Separation−Detection Method. Calibration plots were run for the 13 analytes with commercial standards. Eicosanol, cholestanol, and betunilic acid were used as IS for the quantitation of sterols, fatty alcohols, and triterpenes, respectively.

Calibration equations were set by using the ratio between the peak area of each compound and that of the IS of the given fraction as a function of concentration of each compound (see Table 2). The regression coefficients ranged between 0.9842 and 0.9988 for the linear dynamic range tested for each analyte, which was within its limit of quantification (LOQ) and 50 μg/mL. Characterization of the method was completed with the limits of detection (LODs) and those of quantification, which were calculated from the chromatograms obtained with standard solutions. 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 minimal accepted values of the signal-to-noise (S/N) ratio of 3 and 10, respectively. The LODs for each analyte ranged from 0.21 to 1.09 μg/L for sterols, from 0.02 to 0.11 μg/L for fatty alcohols, and from 0.29 to 0.98 μg/L for triterpenic compounds. The LOQs ranged from 0.70 to 3.64, from 0.06 to 0.36, and from 0.97 to 3.27 μg/L for sterols, fatty alcohols, and triterpenic compounds, respectively.

Isolation of Sterols, Fatty Alcohols, and Triterpenic Dialcohols from Oils. Saponification in a methanol alkaline medium was completed in 15 min using ultrasonic energy, which has proven high efficiency in forming emulsions and accelerating chemical reactions,[22] thus avoiding heating of the reactant medium and long processing times, on the order of 4 or 6 h.[3] In a

Table 3. Optimization of the Solid-Phase Extraction Step

variable	tested range	optimum value
SPE sorbent	aminopropyl	aminopropyl[a]
sample volume (μL)	100−500	400
volume of washing solvent (mL of hexane)	2−12	4
percentage of organic solvent in the elution phase (mL of hexane)	0−50	50
volume of eluant (mL of 1:1 hexane/ethyl acetate)	5−20	6

[a] Optimum in the method described by Orozco et al.

Table 4. Intraday Variability (s_r) and Interday Variability (s_{WR}), Expressed as Relative Standard Deviation, of the Proposed Method

	variability	
compound	s_r	s_{WR}
docosanol	3.0	7.5
tetracosanol	3.1	7.5
hexacosanol	2.7	8.6
octacosanol	3.8	7.9
stigmasterol	5.1	8.0
β-sitosterol	2.8	7.6
stigamastanol	3.7	7.9
campesterol	3.9	7.4
erythrodiol	4.0	12.5
uvaol	3.2	7.2
oleanolic acid	4.3	7.7
ursolic acid	4.2	7.8
maslinic acid	2.9	9.4

previous research from the authors (*20*), oil saponification was completed in 15 minutes using ultrasonic energy, which has proved a high efficiency to form emulsions and accelerate chemical reactions (*22*). The capability of this protocol, optimized for analysis of sterols and fatty alcohols in olive oil, was tested additionally for analysis of triterpenic compounds. The operational variables were duty cycle 70% and output amplitude 30% of the converter (135 W) at ambient temperature (23 °C). The development of the process at ambient temperature is a significant benefit because degradation of labile compounds is thus avoided. After saponification and liquid−liquid extraction to a nonpolar hexane phase, a cleanup step based on SPE was optimized, the results of which are shown in Table 3. The recovery of the SPE cleanup step was evaluated by spiking two olive oil samples with the target compounds at two concentrations (5 and 15 μg/L). The recoveries, calculated by a calibration curve for each compound in triplicate analysis, were within 89−91%, which demonstrated both an optimum efficiency of the SPE step and the accuracy of the method. Evaluation of within-day and between-days variability was performed in a single experimental setup with replicates using natural samples (tested oils) subjected to the proposed method. Two analyses of the samples per day were carried out for 7 days.

Equation 1 was used to determine the variance between days

$$s_{between}^2 = (MS_{between} - MS_{within})/n_j \quad (1)$$

where MS is the mean square (residual sum of squares rated by the freedom degrees) and n_j is the number of replicates per day.

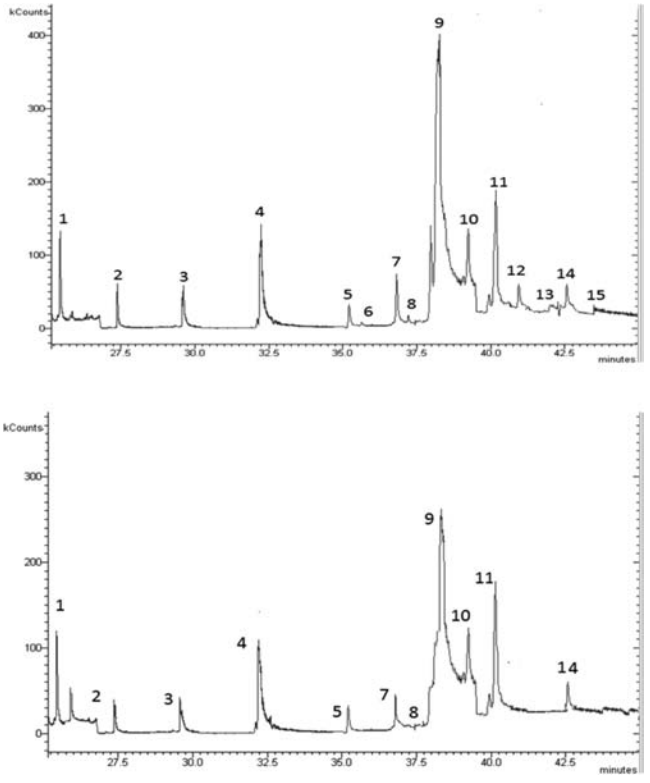

Figure 3. Chromatograms from virgin olive oil before heating (top) and after 20 heating cycles (bottom). Peaks: 1, eicosanol (internal standard); 2, docosanol; 3, tetracosanol; 4, hexacosanol; 5, cholestanol (internal standard); 6, octacosanol; 7, campesterol; 8, stigmasterol; 9, β-sitosterol; 10, stigmastanol; 11, erythrodiol; 12, uvaol; 13; oleanolic acid; 14, betulinic acid (internal standard); 15, ursolic acid.

The within-laboratory variability, s^2_{WR}, was calculated by eq 2

$$s_{WR}^2 = s_r^2 + s_{between}^2 \quad (2)$$

where s_r^2 is the variance due to the intraday effect and $s_{between}^2$ is the variance due to the interday effect. The results obtained are listed in Table 4.

Characterization and Quantification of Sterols, Fatty Alcohols, and Triterpenic Compounds in the Oils before Heating. The unsaponifiable fraction of the four oils selected for this study was chromatographically characterized. Figure 3 shows the chromatographic profile of the unsaponifiable fraction of VOO, identified according to Table 1.

Table 5 summarizes the results of monitoring alcohols, sterols, and triterpenic compounds in the four edible oils under study (note that the concentration of the target compounds is expressed as μg/mL) from heating cycle 0 to cycle 20. These results

Table 5. Monitoring of Aliphatic Alcohols, Sterols, and Triterpenic Compound Contents in the Target Vegetable Oils during the Simulated Frying Process[a]

		aliphatic alcohols[b]				sterols[b]				triterpenes[b]				
oil	cycle	DOC	TET	HEX	OCT	CAM	STE	STA	SIT	ERY	UVA	OLE	URS	MAS
VOO	0	4.75	11.93	13.07	<LOQ[c]	42.02	43.31	8.23	506.19	18.67	14.40	5.05	5.26	28.10
	4	5.71	11.86	15.25	<LOQ	30.34	38.36	8.31	447.95	18.98	12.40	5.93	4.11	26.17
	8	5.20	2.93	1.22	<LOQ	24.75	35.40	6.75	222.42	14.14	6.57	<LOQ	<LOQ	<LOQ
	12	4.73	3.30	2.20	<LOQ	23.30	38.43	6.91	237.77	20.17	6.44	<LOQ	<LOQ	<LOQ
	16	3.53	3.23	1.55	<LOQ	20.07	36.07	7.84	269.72	18.52	5.32	<LOQ	<LOQ	<LOQ
	20	3.57	3.83	1.12	<LOQ	11.40	30.16	10.97	244.20	17.70	4.24	<LOQ	<LOQ	<LOQ
SO	0	0.40	0.01	0.22	<LOQ	133.63	101.53	18.12	308.09	7.01	5.77	<LOQ	<LOQ	<LOQ
	4	1.71	1.78	1.44	<LOQ	160.79	145.31	21.16	257.48	6.20	8.36	<LOQ	<LOQ	<LOQ
	8	1.54	3.13	1.64	<LOQ	153.65	139.82	25.61	246.69	7.21	11.45	<LOQ	<LOQ	<LOQ
	12	2.05	1.55	0.56	0.14	151.32	133.71	8.89	275.11	6.66	9.55	<LOQ	<LOQ	<LOQ
	16	1.61	2.01	0.82	0.68	123.18	125.25	8.91	268.47	6.46	8.22	<LOQ	<LOQ	<LOQ
	20	0.34	0.65	0.70	0.80	72.02	22.43	12.84	155.32	7.32	11.12	<LOQ	<LOQ	<LOQ
ASO	0	3.64	14.00	2.09	0.08	65.56	28.44	16.25	173.26	9.05	9.06	8.77	6.21	16.81
	4	5.92	7.24	4.13	0.28	202.73	168.19	30.54	258.72	7.48	7.61	7.41	6.61	19.45
	8	5.82	13.71	5.06	1.53	148.16	139.06	23.09	284.65	9.38	7.54	5.84	6.22	17.22
	12	5.77	18.37	6.87	2.11	141.70	90.80	22.88	241.71	9.88	11.08	4.18	<LOQ	<LOQ
	16	3.84	16.54	5.04	2.19	74.47	119.08	21.97	270.13	8.52	4.96	<LOQ	<LOQ	<LOQ
	20	<LOQ	4.21	0.11	0.40	51.49	44.05	6.12	121.76	0.14	0.75	<LOQ	<LOQ	<LOQ
DSO	0	1.13	4.78	2.82	<LOQ	230.46	64.05	2.24	286.52	6.37	2.08	2.27	2.09	6.62
	4	0.22	1.40	4.19	<LOQ	235.50	37.60	6.34	119.60	5.78	1.98	2.10	2.55	5.28
	8	1.14	1.01	5.75	<LOQ	185.85	49.71	17.64	55.21	5.44	1.57	2.30	2.73	5.36
	12	5.25	7.70	5.59	0.46	202.84	95.78	22.78	23.52	7.90	2.11	1.22	2.60	5.69
	16	5.75	7.19	4.17	0.59	169.27	85.30	25.72	28.58	8.34	1.60	1.28	2.77	5.80
	20	<LOQ	2.71	2.53	<LOQ	62.92	57.66	13.76	20.36	0.98	1.02	1.13	2.77	5.35

[a] All results, expressed as μg/mL, were calculated as the average of in-triplicate analysis. [b] DOC, docosanol; TET, tetracosanol; HEX, hexacosanol; OCT, octacosanol; CAM, campesterol; STE, stigmasterol; SIT, β-sitosterol; STA, stigmastanol; ERY, erythrodiol;, UVA, uvaol; OLE, oleanolic acid; URS, ursolic acid; MAS, maslinic acid. [c] LOQ, limit of quantitation.

were obtained from three analytical replicates. Because standard deviations were below the precision values obtained with biological replicates, they were omitted. As shown in the table, SO and DSO provided levels of aliphatic alcohols significantly lower than VOO. These results could be ascribed to the refining process or, simply, to the low content of these compounds in the raw seeds used for oil extraction. By contrast, ASO analysis reported levels of aliphatic alcohols similar to VOO, except for hexacosanol, which was found at 2.1 μg/mL in ASO versus the 13 μg/mL present in VOO. This similarity between VOO and ASO in aliphatic alcohols can be ascribed to the enrichment process with the ethanolic extract from olive pomace.

A different trend was found for phytosterols. High levels of campesterol and stigmasterol were found in SO and DSO with concentrations of 133 and 230 μg/mL for campesterol and 101 and 64 μg/mL for stigmasterol, respectively. The concentrations of these sterols in VOO and ASO were below 65 μg/mL for campesterol and 43 μg/mL for stigmasterol. Both stigmastanol and β-sitosterol were found at higher concentrations in SO and VOO (18 and 308 μg/mL, respectively, for SO, and 8 and 506 μg/mL, respectively, for VOO as compared with their content in ASO and DSO). The concentration of sitosterol in sunflower oils was lower than in VOO.

As suspected, the highest concentrations of triterpenic compounds were found in VOO. The enrichment process caused an irregular increase in the concentration of triterpenic compounds in refined sunflower oil. The most concentrated triterpenes in VOO and ASO were maslinic acid and the two triterpenic dialcohols erythrodiol and uvaol.

Characterization and Quantification of Sterols, Fatty Alcohols, and Triterpenic Compounds in Fried Oils. The four target oils (VOO, ASO, DSO, and SO, the characteristics of which have been described above) were analyzed according to the proposed method to determine their concentrations of the target analytes after each heating cycle. Table 5 shows the concentrations of individual sterols, fatty alcohols, and triterpenic dialcohols found in the oils after 4, 8, 12, 16, and 20 heating cycles. As an example, Figure 3 compares the chromatograms obtained from VOO before and after 20 heating cycles, whereas the results for each monitored compound are plotted in Figure 4 and discussed below for each target fraction.

Triterpenic Compounds. Pentacyclic triterpenic acids such as oleanolic, ursolic, and maslinic acids were not detected in SO and DSO, whereas their presence in ASO was associated with the enrichment of the sunflower oil with extract from the solid residue

generated in olive oil production. The main trend observed in VOO for triterpenic acids was a dramatic decrease of their concentration after 6 heating cycles (30 min of heating), which led to their nondetection after eight frying cycles. As exposed above, similar levels of these compounds were transferred to ASO in the enrichment; however, ursolic and maslinic acids were practically undetected after 10 heating cycles, and the same happened to oleanolic acid after 14 heating cycles. Therefore, it is clear that degradation of triterpenic acids was delayed for ASO as compared to VOO. Also, clear differences were observed for maslinic acid levels in VOO and ASO during the first 7 cycles. Thus, the concentration of maslinic acid increased in ASO during heating, whereas the opposite trend was found in VOO. This behavior could be ascribed to the presence in the extract from olive pomace of a conjugated form of maslinic acid, which was transferred to the oil and deconjugated during the first heating cycles.

Triterpenic dialcohols were not detected in SO and DSO either. In VOO, the concentration of erythrodiol was practically not affected by heating. A different trend was observed for uvaol, the concentration of which decreased from 15 to 5 μg/mL, approximately, during the overall heating process. These two compounds were also detected in ASO because of the enrichment process. Uvaol showed a similar behavior in ASO and VOO: this triterpene was not detected in the aliquot sampled after 20 heating cycles. On the other hand, erythrodiol in ASO was decreased in concentration after 17 heating cycles practically below the detectable level.

Phytosterol Fraction. The concentrations of sterols in the oils under study decreased differently during the simulated frying process depending on the edible oil. With regard to campesterol, VOO contained the lowest level (42 μg/mL) and DSO contained the highest (230 μg/mL), both at cycle 0. These levels

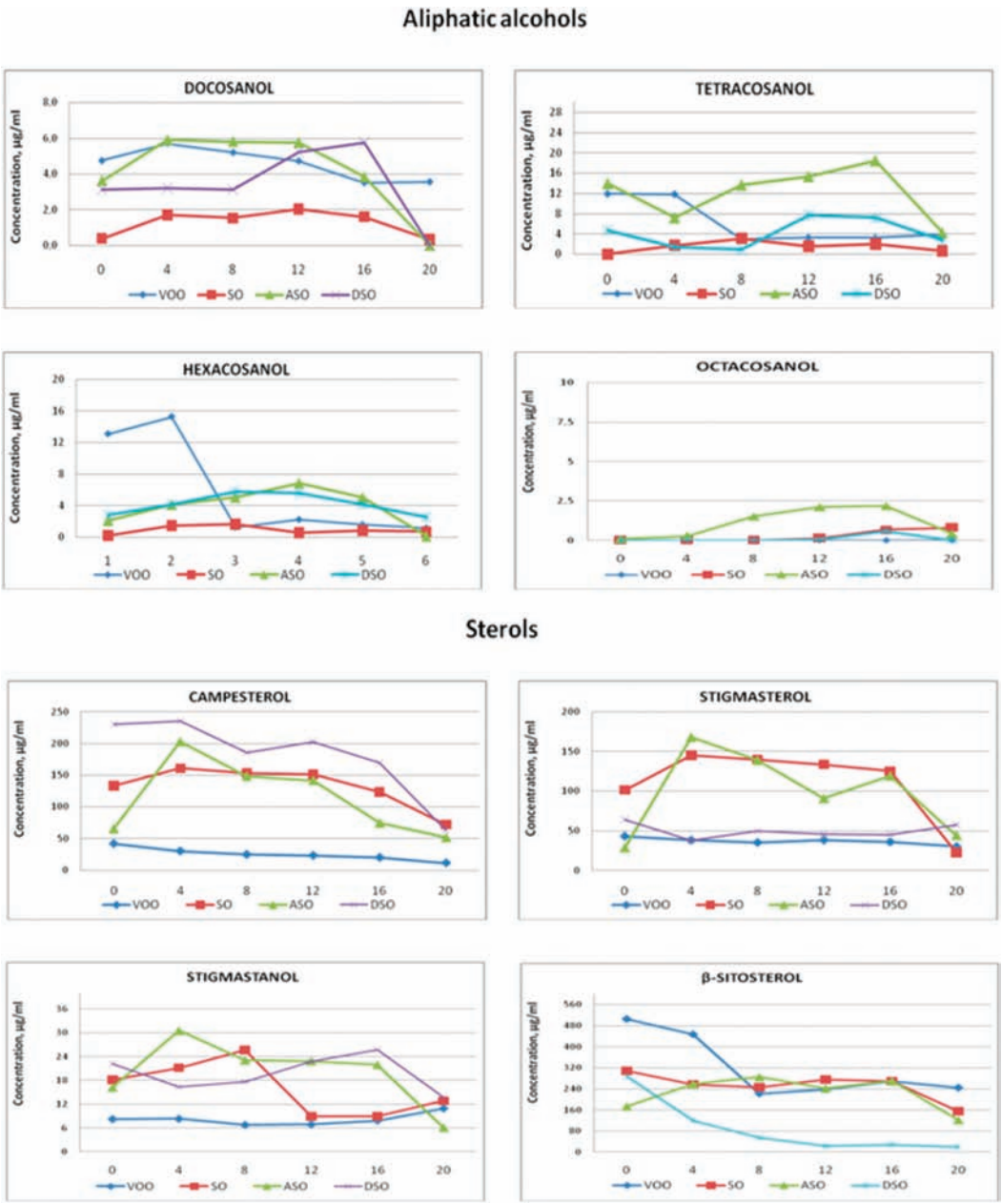

Figure 4. Continued

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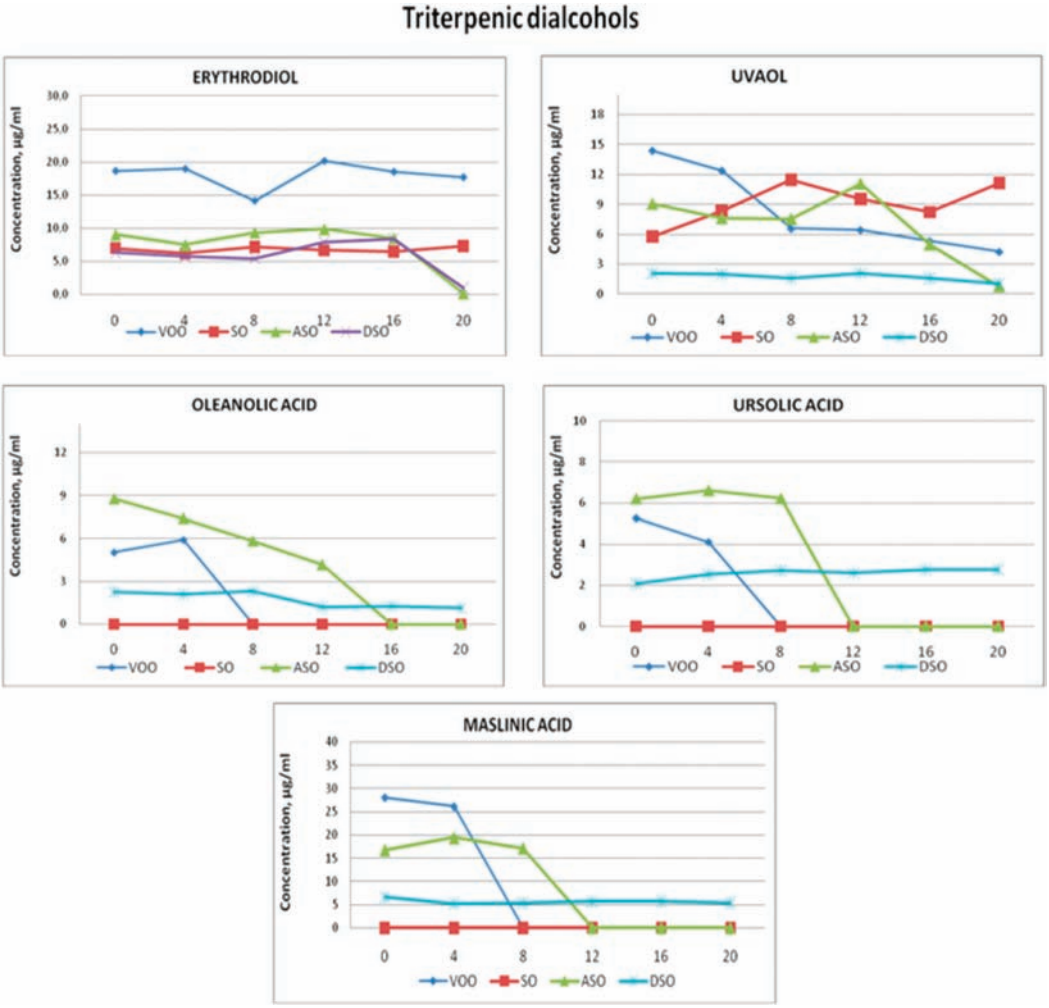

Figure 4. Monitoring of sterol, aliphatic alcohol, and triterpenic compound levels in the four tested oils (VOO, ASO, DSO, and SO) during the 20 heating cycles.

decreased to 11 and 62 μg/mL, respectively, after 20 heating cycles. Intermediate concentrations of campesterol were found in SO and ASO, which behave similarly: an increase of campesterol concentration during cycles 0−5/6 and a decrease of this sterol for subsequent heating cycles. This trend was also found for stigmasterol in SO and ASO. These results do not agree with a previous study on the stability of phytosterols, in which decreases of campesterol, stigmasterol, and β-sitosterol in vegetable oils were found after 1 h of heating at a temperature higher than 150 °C.[23]

β-Sitosterol was the most concentrated phytosterol in all tested oils. The concentration of this phytosterol in VOO decreased during the first 8 cycles and then remained at a stable value for the rest of the heating process. On the contrary, this sterol experienced a deep degradation in DSO after 12 heating cycles. SO and ASO underwent a slight nonsignificant decrease of β-sitosterol concentration with increasing number of heating cycles until the last part of the process.

Oxidation mechanisms of phytosterols are believed to follow the same pathways as cholesterol oxidation to form oxysterols. The evolution of the concentration of phytosterols during heating showed that autoxidation of β-sitosterol, which is the most concentrated phytosterol, is favored in VOO, with degradation from 506 to 228 μg/mL during the first 6 cycles and with slower degradation than the rest of phytosterols. The degradation process of phytosterols is more uniform in SO, with a significant decrease in the concentration of campesterol (from 133 to 72 μg/mL), stigmasterol (from 101 to 22 μg/mL), stigmastanol (from 16 to 9 μg/mL), and β-sitosterol (from 308 to 155 μg/mL) during the 20 cycles. The heating process of DSO affected principally campesterol and β-sitosterol, which decreased in concentration from 230 to 62 μg/mL for campesterol and from 286 to 20 μg/mL in the case of β-sitosterol. This dramatic decrease of phytosterols on heating has been attributed to the chemical reactivity of the hydroxyl function.[23]

With regard to the behavior of phytosterols in DSO and ASO, a characteristic trend was observed in DSO for stigmasterol and stigmastanol, the concentrations of which increased during the initial heating cycles, probably due to hydrolysis of conjugated forms. The concentration of target phytosterols also increased in ASO during the initial heating cycles. However, campesterol and stigmasterol decreased drastically their levels after 4 and 5 cycles, whereas stigmastanol and β-sitosterol concentrations remained constant up to cycle 16,; after that, they were also degraded. These results complement previous studies in which the effect of phytosterol structure on the thermal polymerization of a heated soybean oil was determined.[24] In

particular, the two evaluated aspects were the degree of unsaturation and the presence of an ethylidene group in the side chain: the former was found to be more important for its antipolymerization activity than the presence of an ethylidene group.[24]

Aliphatic Alcohol Fraction. The fraction of aliphatic alcohols was detected in all edible oils under study. In VOO, the concentrations of docosanol, tetracosanol, and hexacosanol clearly decreased with increasing number of heating cycles, whereas octacosanol was not detected at any time. Docosanol in sunflower oil experienced a behavior similar to that of VOO. In fact, this aliphatic alcohol was not detected in the last heating cycle, and the concentration of the rest of alcohols increased after a given number of heating cycles. Thus, the concentration of tetracosanol increased after 10 cycles (although this was lowered after 13 cycles) and that of octacosanol after 6 cycles (with a decrease after 18 cycles); hexacosanol experienced a slight increase during the first 12 cycles with a subsequent fall at higher numbers of cycles. Aliphatic alcohols are frequently found forming conjugated structures in edible oils such as waxes, glycosides, fatty acids, or sterols, which could justify this irregular behavior. The same trend of the different aliphatic alcohols was observed in DSO, except for octacosanol, which was not detected during the heating test. The presence of natural or artificial antioxidants influences the chemical behavior of oils during heating depending on the given compound. In any case, the use of natural antioxidants could be considered an excellent strategy to decrease the degradation of the lipidic fraction during heating, thus improving the stability of oils used for frying without affecting their quality.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone/fax: +34-957-218615. E-mail: q72prcaf@uco.es.

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