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Presence of Phytosterol Oxides in Crude Vegetable Oils and Their Fate during Refining

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The content of phytosterol oxidation products was determined in samples of crude vegetable oils: peanut, sunflower, maize, palm nut, and lampante olive oils that were intended for refining and not for direct consumption. The 7α- and 7β-hydroxy derivatives of β-sitosterol, stigmasterol, and campesterol and the 7-keto-β-sitosterol were the principal phytosterol oxides found in almost all of the oils analyzed. In some oils, the epoxy and dihydroxy derivatives of β-sitosterol were also found at very low levels. The highest total concentrations of phytosterol oxides, ranging from 4.5 to 67.5 and from 4.1 to 60.1 ppm, were found in sunflower and maize oils, respectively. Lower concentrations were present in the peanut oils, 2.7−9.6 ppm, and in the palm nut oil, 5.5 ppm, whereas in the lampante olive oils, only three samples of the six analyzed contained a low concentration (1.5−2.5 ppm) of oxyphytosterols. No detectable levels of phytosterol oxides were found in the samples of palm and coconut oils. Bleaching experiments were carried out on a sample of sunflower oil at 80 °C for 1 h with 1 and 2% of both acidic and neutral earths. The bleaching caused a reduction of the hydroxyphytosterol with partial formation of steroidal hydrocarbons with three double bonds in the ring system at the 2-, 4-, and 6-positions (steratrienes). The same sunflower oil was deodorized at 180 °C under vacuum for 1 h, and no dehydration products were formed with a complete recovery of the hydroxyphytosterols. A bleaching test with acidic earths was carried out also with an extra virgin olive oil fortified with 7-keto-cholesterol, dihydroxycholesterol, and α-epoxy-cholesterol. There was no formation of steratrienes from these compounds, but dihydroxycholesterol underwent considerable decomposition and α-epoxycholesterol underwent ring opening with formation of the dihydroxy derivative, whereas 7-ketocholesterol was rather stable

KEYWORDS: Phytosterol oxides; cholesterol oxides; vegetable oils; steroidal hydrocarbons; steradienes; steratrienes; refined oils; bleaching; deodorization; gas chromatography−mass spectrometry

INTRODUCTION

The refining of vegetable oils is generally necessary to obtain a product acceptable to consumers. This process comprises various steps (degumming, neutralization, bleaching, deodorization, and dewaxing), the type and intensity of each step depending on the kind of oil being treated. In particular, bleaching with acidic earths and deodorization at high temperatures can cause major changes, which are related to the conditions used, to the minor components present in the unsaponifiable matter of oils. In the case of sterols, isomerization with a shift of the double bonds and dehydration with formation of steroidal hydrocarbons (steradienes) and disteryl ethers have been reported (*1*−*8*). The analysis of steradienes represents, in fact, a useful method for detecting the addition of refined oil to extra virgin olive oils and is one of the official methods in the European Union (*9*−*13*).

Besides the steradienes, other steroidal hydrocarbons have been detected in refined oils; their molecular weights were 2 mass units less than those of the steradienes derived from the dehydration of sterols, indicating the presence of another double bond in the molecule (*7*, *8*, *14*). Grob et al. (*7*) have tentatively identified the product formed from β-sitosterol as the 24-ethylcholest-2,4,6-triene, claiming that oxidation is involved in its formation. Smith (*15*) reported the formation of cholest-2,4,6-triene among the degradation products of 7-hydroperoxy and 7-hydroxy derivatives of cholesterol. The formation of steroidal hydrocarbons and ketosteroids from 7-hydroxy derivatives of β-sitosterol treated with bleaching earths was also long ago reported by Niewiadomski et al. (*4*) and Kaufman et al. (*5*, *6*). Bortolomeazzi at al. (*14*) demonstrated the correlation between the oxidation products of β-sitosterol, campesterol and stigmasterol and the formation of compounds with three double bonds in the ring system. The formation of these products can be rationalized by hypothesizing the loss of two molecules of water from the hydroxy derivative of a sterol. The same authors

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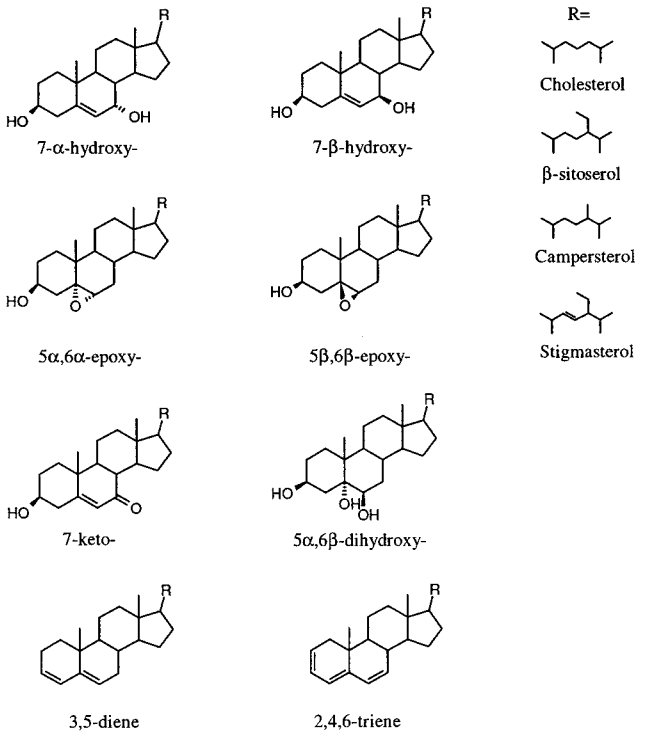

Figure 1. Structures of the principal sterol oxides and of the 3,5-diene and 2,4,6-triene derivatives.

(*16*) have identified these compounds (steratrienes) as 24-ethylcholest-2,4,6-triene (2,4,6-stigmastatriene), 24-methylcholest-2,4,6-triene (2,4,6-campestatriene), 24-ethylcholest-2,4,6,22-tetraene (2,4,6,22-stigmastatetraene), and 24-methylcholest-2,4,6,22-tetraene (ergostatetraene), confirming the hypothesis of the hydroxy sterols as precursors of these compounds. The structures of the principal sterol oxides and the 3,5-diene and 2,4,6-triene derivatives are shown in **Figure 1**.

The investigations of Bortolomeazzi et al. (*14*, *16*) were, however, based on model systems in which the oil was fortified with the hydroxyphytosterols before bleaching. Data on the actual presence of hydroxyphytosterols in crude vegetable oils are very scarce, the research being focused mainly on the determination of the phytosterol oxidation products in refined and in fried oils (*17*−*23*) due to the possible pathological effect of these compounds, as with the corresponding cholesterol oxidation products. The only previous data were reported by Nourooz-Zadeh and Appelqvist (*22*), who studied the effect of refining and storage and found no detectable levels of β-sitosterol oxides in a crude soybean oil at a detection limit of 0.2 ppm.

In the first part of this work we investigated the presence of phytosterol oxides in different crude vegetable oils. All of the oils analyzed were intended for refining before consumption. Sunflower, maize, and peanut oils were solvent extracted, whereas the lampante olive oils were virgin olive oils with an acidity of >3.3% and undesirable organoleptic characteristics and not suitable for direct consumption without refining. To verify the formation of steratrienes in a real sample, in the second part of the work, a sunflower oil rich in hydroxysterols was chosen for bleaching experiments with both acidic and neutral earths. The same oil was also tested under the conditions used in the deodorization process. The behavior of other phytosterol oxides, namely, the epoxy, 7-keto, and dihydroxy derivatives present at low concentration in the oils analyzed, was investigated. For this purpose, an extra virgin olive oil was fortified with 5α,6α-epoxycholesterol, 7-ketocholesterol, and dihydroxycholesterol and then subjected to the bleaching process.

MATERIALS AND METHODS

Samples. Samples of crude oils were obtained from an oil refining industry. Five samples of sunflower oil (SF1−SF5), five samples of peanut oil (PE1−PE5), six samples of corn oil (CO1−CO6), six samples of lampante virgin olive oil (LOO1−LOO6), two samples of palm oil (PA1−PA2), one sample of coconut oil (CN1), and one sample of palm nut oil (PN1) were analyzed.

Materials and Reagents. All solvents were of analytical grade. Cholesterol, 5α,6α-epoxycholesterol, 7-ketocholesterol, dihydroxycholesterol, and cholest-3,5-diene (cholestadiene) were purchased from Sigma (St. Louis, MO). β-Sitosterol, the silica gel 60 (70−230 mesh), and the thin-layer chromatography (TLC) silica gel plates, 0.25 mm thick, were purchased from Merck (Darmstadt, Germany). The GC and GC-MS analyses of the β-sitosterol standard as its trimethylsilyl ether (TMS) derivative revealed the following composition: β-sitosterol, 76%; campesterol, 8%; campestanol, 1.4%; and sitostanol, 14.6%. Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were from Fluka (Buchs, Switzerland).

7α-Hydroxycholesterol and 7α-hydroxy-β-sitosterol were synthesized as reported by Bortolomeazzi et al. (*24*). Due to the presence of ~8% campesterol in the β-sitosterol standard, its corresponding hydroxy derivative was formed during the synthesis.

Mega Bond Elut silica solid phase extraction (SPE) cartridges (1 g) were purchased from Superchrom (Milan, Italy), whereas NH_2 SPE cartridges (500 mg) were from Merck,

Two types of bleaching earths, Tonsil, acid (pH 2.95), and neutral (pH 7.50), were obtained from an oil refining industry.

Gas Chromatography (GC) and GC−Mass Spectrometry (GC-MS). Quantitative analysis of the steradienes and steratrienes was performed on a Fisons GC 8000 TOP gas chromatograph equipped with a split−splitless injector and a flame ionization detector. The column was a 30 m × 0.25 mm i.d., 0.25 μm, fused silica Carbowax (Supelco, Bellefonte, PA). The column temperature was 260 °C isothermal, and the detector and injector temperatures were 270 °C. The carrier gas (helium) flow rate was 1.2 mL/min and the split ratio 1:15 (v/v).

GC-MS analysis was performed for the qualitative and quantitative determination of the phytosterol oxides. Analyses were carried out on a Varian 3400 gas chromatograph coupled to a Varian Saturn ion trap detector. The column was a 30 m × 0.25 mm i.d., 0.25 μm, fused silica SPB5 (Supelco). The injection was in splitless mode (time = 0.6 min) with helium as carrier gas at a flow rate of 1 mL/min. The column temperature was set at 80 °C for 1 min, then programmed to 250 °C at 25 °C/min, and then to 300 °C at 3 °C/min. The injector, transfer line, and ion trap temperatures were 280, 300, and 170 °C, respectively. The electron impact (70 eV) spectra were recorded at 0.5 s/scan with a filament emission current of 10 μA. All of the samples were analyzed as TMS derivatives. The *m*/*z* ratios of the ions used for the quantitative analysis of the 7α- and 7β-OH-β-sitosterol TMS, 7α- and 7-β-OH-campesterol TMS, 7α- and 7-β-OH-stigmasterol TMS, and 7-keto-β-sitosterol TMS were 484 ($M^+ - 90$), 470 ($M^+ - 90$), 482 ($M^+ - 90$), and 500 (M^+), respectively. For the two internal standards 7α-OH-cholesterol TMS and 7-ketocholesterol TMS, the ions used were 456 ($M^+ - 90$) and 472 (M^+), respectively.

GC-MS analysis was also performed to confirm the identity of steradienes and steratrienes by using both the SPB5 column and the Carbowax column.

Cold Saponification of Oils. Cold saponification of the oils was carried out according to the method of Dutta and Appelqvist (*20*) with minor changes. Prior to saponification, 20 μL of a 7-ketocholesterol solution (0.98 mg/mL) in $CHCl_3$ and 50 μL of a 7α-hydroxycholesterol solution (0.20 mg/mL) in $CHCl_3$, as internal standards, were poured into a Teflon-lined screw-cap tube. After removal of the solvent under nitrogen, 0.5 g of oil and 5 mL of an ethanolic 2 M KOH solution were added. The mixture was vigorously mixed and left overnight at room temperature. After the addition of 10 mL of water, the unsaponi-

fiable material was extracted with 10 mL of diethyl ether. The ethereal phase was washed with water and dried over anhydrous sodium sulfate, and the solvent was removed under nitrogen.

Purification of Phytosterol Oxidation Products by SPE. The unsaponifiable matter was dissolved in 1 mL of *n*-hexane/diethyl ether (8:2, v/v) and loaded onto a silica SPE column previously conditioned with 5 mL of *n*-hexane. Nonpolar compounds and sterols were eluted with two 5 mL aliquots of *n*-hexane/diethyl ether (1:1, v/v). The phytosterol oxidation products were then eluted with 5 mL of diethyl ether/methanol (1:1, v/v) and, after removal of the solvent under nitrogen, were derivatized to TMS ethers with ~100 μL of a mixture of pyridine/HMDS/TMCS (5:2:1, v/v/v) for 1 h at room temperature. The solvent was removed under nitrogen and the residue dissolved in ~100 μL of *n*-heptane prior to GC-MS analysis. Two injections were made for each sample. This procedure was used for all of the oils analyzed.

Purification with the NH_2 SPE column was performed as reported by Rose-Sallin et al. (*25*) without modifications. The phytosterol oxidation products containing fractions were treated in a similar way as the corresponding fractions eluted from the silica gel columns before GC-MS analysis.

Bleaching with Earths. Two series of experiments were carried out: (a) bleaching of a sunflower oil that contained the hydroxy derivatives of β-sitosterol, campesterol, and stigmasterol and 7-keto-β-sitosterol; (b) bleaching of an extra virgin olive oil to which α-epoxycholesterol, 7-ketocholesterol, and dihydroxycholesterol had been added.

(a) To 10.00 g of oil in a 25 mL round-bottom flask was added 100 mg of acidic or neutral earths (1%, w/w). The flask was then connected to a water pump and the mixture warmed to 80 °C by a water bath. Decoloration was carried out for 1 h at this temperature, under vacuum and vigorous magnetic stirring. After cooling, the mixture was immediately filtered on a Büchner funnel to remove the earths. Bleaching was also carried out with 2% (w/w) of either acidic or neutral earths.

(b) Two milliliters of an α-epoxycholesterol solution (0.5 mg/mL) was placed in a 25 mL round-bottom flask. After evaporation of the solvent under reduced pressure at room temperature, 10.00 g of extra virgin olive oil was weighed into the same flask, and the mixture was vigorously stirred by a magnetic bar until complete dissolution of the compound in the oil. After the addition of 100 mg (1% w/w) of acidic earths, the flask was connected to a water pump and decoloration was carried out as in the case of the sunflower oil. Bleaching under the same conditions was also performed with extra virgin olive oil fortified with 7-ketocholesterol and dihydroxycholesterol.

Deodorization. Ten grams of the sunflower oil was weighed into a round-bottom flask. The flask was then connected to a water pump and the mixture warmed to 180 °C by an oil bath. Deodorization was carried out for 1 h at this temperature, under vacuum and vigorous magnetic stirring.

Isolation of the Steroidal Hydrocarbons from the Bleached Oils. The method used was essentially the European Union Official Method for the determination of stigmastadienes in vegetable oils (*11*), without the saponification step. To 1 g of bleached or deodorized oil was added 0.2 mL of a cholest-3,5-diene (internal standard, I.S.) solution (0.204 mg/mL) in hexane, and the mixture was then loaded onto a silica gel column (1.5 cm × 15 cm) with two 1 mL portions of *n*-hexane. The elution was carried out with *n*-hexane as mobile phase. The first fraction, of ~30 mL, containing alkanes, was discarded, and the second 40 mL fraction, containing the steroidal hydrocarbons, was reduced to dryness under reduced pressure. The residue, dissolved in *n*-heptane, was transferred to a conical vial and the volume adjusted to ~100 μL prior to GC and GC-MS analyses.

RESULTS AND DISCUSSION

Purification of the Phytosterol Oxidation Products. The first step in the purification procedure of the phytosterol oxidation products is the saponification of the oil. Cold saponification, as reported by Dutta and Appelqvist (*20*), was used to minimize the formation of artifacts and the decomposition of the keto and epoxy derivatives (*26*). The second step consisted of the isolation and enrichment of the phytosterol oxides from the unsaponifiable matter. This operation is generally carried out by SPE with silica gel or aminopropyl as stationary phases (*25*, *27*−*31*). In this work, 1 g silica columns were used, and the results were compared with those of a procedure that used NH_2 columns (*25*). Some authors (*25*, *32*) reported the possible formation of artifacts due to interactions of cholesterol with silicic acid, but in these works the purification of the cholesterol oxidation products was carried out on TLC silica plates, where the time of the exposure of the sample to air is much higher than in an SPE column.

Two other aspects must be considered in the use of SPE for the purification of the phytosterol oxidation products: their separation from the sterols and the complete recovery of the less and more retained phytosterol oxidation products. Sterols are likely to represent the class of compounds that can most interfere in the purification of phytosterol oxides, due to their relatively high amount present in the unsaponifiable matter as well as their polarity characteristics. The sterols are only a little less retained with respect to 25-hydroxy, 7-keto, and epoxy derivatives of sterols as shown in a silica gel TLC separation (*25*). These compounds are the less retained among the phytosterol oxidation products, whereas the dihydroxy derivatives are the more strongly retained. Generally, in the quantitative analyses of phytosterol oxidation products and cholesterol oxidation products, 19-hydroxycholesterol is used as internal standard. The chromatographic behavior of this compound on a silica TLC plate is similar (slightly more retained) to those of 7α-OH and 7β-OH derivatives, which have in turn an intermediate behavior between those of the 25-hydroxy, 7-keto, and epoxy derivatives and the dihydroxy derivatives. 19-Hydroxycholesterol could not then be a good internal standard for the less and the more retained phytosterol oxidation products for the SPE separation. The critical point regards mainly the less retained phytosterol oxides, which could be selectively lost with respect to the I.S., whereas a sufficiently strong solvent can elute quantitatively the I.S. and the dihydroxy derivatives.

In this work the corresponding cholesterol oxides were used as internal standards for the determinations of the phytosterol oxides. In particular, 7α-OH-cholesterol was used as I.S. for all of the hydroxy derivatives of phytosterols and 7-ketocholesterol as I.S. for the 7-keto derivatives of phytosterols. Moreover, the use of 7-ketocholesterol as I.S. can better simulate the behavior of the analytes toward possible decomposition during cold saponification. This approach, although more laborious, could improve the accuracy of the quantitative determination of phytosterol oxidation products in oils and eventually in other complex matrices. From this point of view, the oxidation products of cholesterol are very suitable. In principle, the procedure can be reversed and the oxidation products of a phytosterol could be used as internal standards in the analysis of the cholesterol oxidation products, although in this case the availability of the compounds would be a limiting factor. The only previous case in the literature regarding the use of 7α-hydroxycholesterol as I.S. for the analysis of phytosterol oxides in soybean oil was reported by Nourooz-Zadeh and Appelqvist (*22*), but without details about this aspect of the quantitation methodology.

GC-MS Analysis. GC-MS chromatograms of the phytosterol oxidation products containing fractions of some crude oils are shown in **Figure 2**. The phytosterol oxidation products were identified on the bases of the mass spectrometric data reported in the literature (*20*, *21*). The 7α-OH and 7β-OH derivatives of

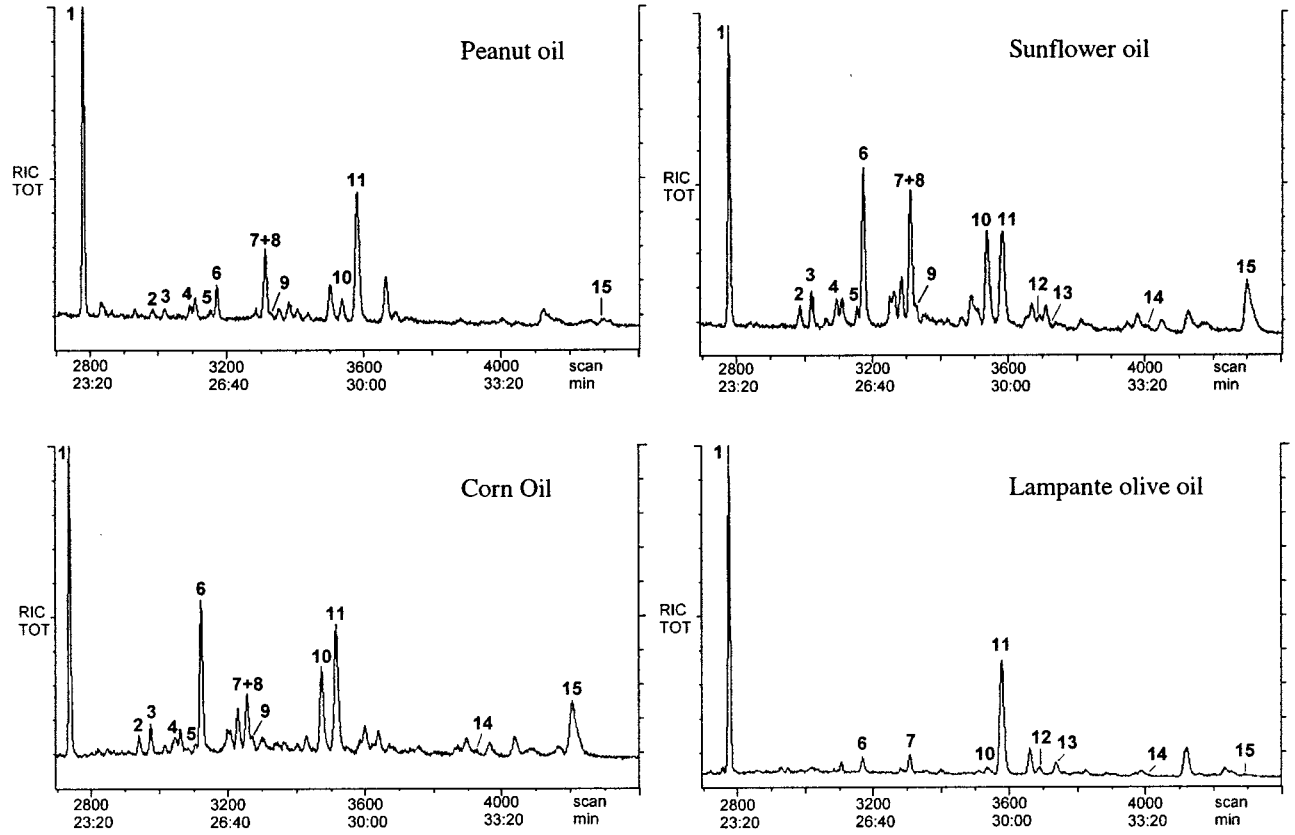

Figure 2. GC-MS chromatograms (total ion current) of phytosterol oxides containing fractions of some crude oils. All compounds were analyzed as TMS derivatives. Peaks: (1) 7α-OH-cholesterol (I.S.); (2) 7α-OH-campesterol; (3) 7α-OH-stigmasterol; (4) campesterol; (5) stigmasterol; (6) 7α-OH-β-sitosterol; (7) β-sitosterol; (8) 7β-OH-campesterol; (9) 7β-OH-stigmasterol; (10) 7β-OH-β-sitosterol; (11) 7-ketocholesterol (I.S.); (12) 5β,6β-epoxy-β-sitosterol; (13) 5α,6α-epoxy-β-sitosterol; (14) 5,6-dihydroxy-β-sitosterol; (15) 7-keto-β-sitosterol.

campesterol, stigmasterol, and β-sitosterol and 7-keto-β-sitosterol were present in almost all of the oils analyzed, whereas only traces of the epoxy and dihydroxy derivatives of β-sitosterol were detected in some oils. Residues of the phytosterols were also present in all of the chromatograms. The purification by SPE did not completely eliminate the sterols, and in all of the fractions containing the phytosterol oxidation products there were residues of campesterol, stigmasterol, and mainly β-sitosterol, which is the more abundant sterol present in the oils analyzed. The main interference was due to the coelution of 7β-OH-campesterol (peak 8) and β-sitosterol (peak 7), but the use of the single ion for quantitation overcame the problem.

Quantitative Analysis. Quantitative analysis of the phytosterol oxidation products in the oil sample was carried out using two internal standards. The relative response factors (RRF) were calculated only for the standard compounds 7α-OH-β-sitosterol and 7α-OH-campesterol, and the results were very similar, 0.854 and 0.857, respectively. The same RRF was used for the corresponding 7β-OH epimers. For the 7-hydroxy derivatives of stigmasterol, the RRF of 7α-OH-β-sitosterol was used. Similarly for 7-keto-β-sitosterol, for which a standard was not available, an RRF = 1 with respect to 7-ketocholesterol was used. The determination was linear over a range from 0.3 to 1000 ng for 7α-OH-β-sitosterol and 7α-OH-campesterol, with a detection limit of 0.1 ppm in the oil, calculated as the ratio between 3 times the standard deviation of the noise and the sensitivity.

The repeatability was evaluated by analyzing the same sample of sunflower oil eight times. The average results and the percent coefficients of variation (CV%) are reported in **Table 1** together with the repeatability results obtained by carrying out the analysis by replacing the silica SPE column with an aminopropyl phase column and applying a published method of purification (*25*). The aminopropyl phase is widely used for sample purification in the determination of cholesterol oxidation products in food. **Table 1** shows that the CV% are relatively low and that there are not significant differences between the two methods of purification.

Table 1. Repeatability Results

	silica gel SPE		aminopropyl SPE	
oxysterol	ppm[a] ± SD	CV%	ppm[b] ± SD	CV%
7α-OH-β-sitosterol	14.8 ± 1.16	7.81	13.9 ± 0.84	6.04
7β-OH-β-sitosterol	10.6 ± 1.14	10.82	12.8 ± 1.81	14.15
7α-OH-campesterol	1.8 ± 0.13	7.14	1.8 ± 0.12	6.60
7β-OH-campesterol	1.4 ± 0.12	8.49	1.4 ± 0.21	15.08
7α-OH-stigmasterol	2.4 ± 0.18	7.46	2.6 ± 0.17	6.50
7β-OH-stigmasterol	1.7 ± 0.18	10.85	1.9 ± 0.20	10.47
7-keto-β-sitosterol	34.8 ± 3.28	9.42	30.4 ± 2.43	8.00

[a] Means of eight analyses. [b] Means of six analyses.

In **Tables 2−5** are reported the concentrations of the phytosterol oxidation products found in the crude oils analyzed. The 7α-OH and 7β-OH derivatives of β-sitosterol, campesterol, and stigmasterol and 7-keto-β-sitosterol were the phytosterol oxidation products present in the highest concentration, and only these compounds have been quantitated. The 7-OH epimers and the 7-keto derivatives are, in fact, the first products of the decomposition of the 7α- and 7β-hydroperoxides formed by autoxidation. The hydroxy derivatives of β-sitosterol were present in larger concentrations among the phytosterol oxidation products, this sterol being the most abundant in all of the oils

Table 2. Concentration (Parts per Million)[a] of Phytosterol Oxides in Peanut Oils

	sample				
oxysterol	PE1	PE2	PE3	PE4	PE5
7α-OH-β-sitosterol	1.0	1.5	0.9	2.7	2.2
7β-OH-β-sitosterol	0.7	1.2	0.8	1.9	1.8
7α-OH-campesterol	0.3	0.4	0.3	0.7	0.6
7β-OH-campesterol	0.2	0.4	0.2	0.5	0.5
7α-OH-stigmasterol	0.3	0.3	0.2	0.6	0.6
7β-OH-stigmasterol	0.2	0.3	0.2	0.5	0.4
7-keto-β-sitosterol	<0.1	<0.1	<0.1	2.7	<0.1
total	2.7	4.1	2.6	9.6	6.1

[a] Means of two analyses.

Table 3. Concentration (Parts per Million)[a] of Phytosterol Oxides in Sunflower Oils

	sample				
oxysterol	SF1	SF2	SF3	SF4	SF5
7α-OH-β-sitosterol	14.8	14.7	9.8	1.2	1.9
7β-OH-β-sitosterol	10.6	10.3	7.3	0.8	1.1
7α-OH-campesterol	1.8	1.9	1.3	0.3	0.6
7β-OH-campesterol	1.4	1.5	1.2	0.2	0.4
7α-OH-stigmasterol	2.4	3.0	1.9	0.2	0.3
7β-OH-stigmasterol	1.7	1.9	1.4	0.1	0.2
7-keto-β-sitosterol	34.8	29.9	14.6	3.2	<0.1
total	67.5	63.2	37.5	6.0	4.5

[a] Means of two analyses.

Table 4. Concentration (Parts per Million)[a] of Phytosterol Oxides in Corn Oils

	sample					
oxysterol	CO1	CO2	CO3	CO4	CO5	CO6
7α-OH-β-sitosterol	2.2	2.0	1.8	2.0	15.3	12.4
7β-OH-β-sitosterol	1.5	1.1	1.3	1.4	10.4	8.9
7α-OH-campesterol	0.6	0.7	0.6	0.6	1.9	1.5
7β-OH-campesterol	0.5	<0.1	0.5	<0.1	1.6	1.3
7α-OH-stigmasterol	0.3	0.3	0.4	0.3	3.0	2.4
7β-OH-stigmasterol	0.2	<0.1	0.2	<0.1	2.0	1.7
7-keto-β-sitosterol	3.0	<0.1	<0.1	<0.1	22.3	29.6
total	8.3	4.1	4.8	4.3	60.1	57.8

[a] Means of two analyses.

Table 5. Concentration (Parts per Million)[a] of Phytosterol Oxides in Lampante Olive Oils and Palm Nut Oil

	sample			
oxysterol	LOO2	LOO5	LOO6	PN
7α-OH-β-sitosterol	1.0	1.1	1.0	1.3
7β-OH-β-sitosterol	0.4	0.6	0.5	1.3
7α-OH-campesterol	0.1	<0.1	<0.1	0.2
7β-OH-campesterol	<0.1	<0.1	<0.1	0.2
7α-OH-stigmasterol	<0.1	<0.1	<0.1	0.3
7β-OH-stigmasterol	<0.1	<0.1	<0.1	0.3
7-keto-β-sitosterol	<0.1	<0.1	1.0	1.9
total	1.5	1.7	2.5	5.5

[a] Means of two analyses.

analyzed, and for it only the 7-keto, the epoxy, and the dihydroxy derivatives were found. The concentrations of phytosterol oxides in the oils were quite variable among the different samples of the same type of oil as well between the different oils. Sunflower and maize were the oils with the larger amount of total phytosterol oxidation products with values in the range from 4.5 to 67.5 ppm and from 4.1 to 60.1 ppm, respectively. Lower concentrations, between 2.7 and 9.6 ppm, were present in the peanut oils and in the palm nut oil, 5.5 ppm. In the case of the lampante olive oils, only three samples of the six analyzed contained phytosterol oxidation products, and these were in the low concentration range, 1.5−2.5 ppm. No detectable levels of phytosterol oxides were found in the samples of palm and coconut oils. The only previous quantitative data on phytosterol oxide contents in crude vegetable oils were reported by Nourooz-Zadeh and Appelqvist (*22*). These authors, studying the effect of refining and storage, reported no detectable levels of β-sitosterol oxides in a crude soybean oil at a detection limit of 0.2 ppm. In the case of refined oils, Dutta (*21*) reported total phytosterol oxides contents of 41.0, 39.9, and 46.7 ppm in a palm/rapeseed oil blend, a sunflower oil, and a high-oleic sunflower oil, respectively, before frying. These values included also the epoxy and dihydroxy derivatives. As with our results, the same author found the 7-keto derivative of β-sitosterol only, in the range of 1.6−14.1 ppm, whereas the 7-hydroxy derivatives ranges were between 1.3 and 7.7 ppm for β-sitosterol, between 0.5 and 2.8 ppm for campesterol, and between 0.4 and 0.7 ppm for stigmasterol. These values lie in the ranges found for the samples of sunflower oils analyzed in the present study. The large differences in phytosterol oxides contents found among the oil samples can be explained in terms of fatty acid composition, with the palm nut, peanut, olive, palm, and coconut oils being the less unsaturated and thus less prone to oxidation, as well as in terms of the different conditions and times of storage of the raw materials and of the oils before the refining process.

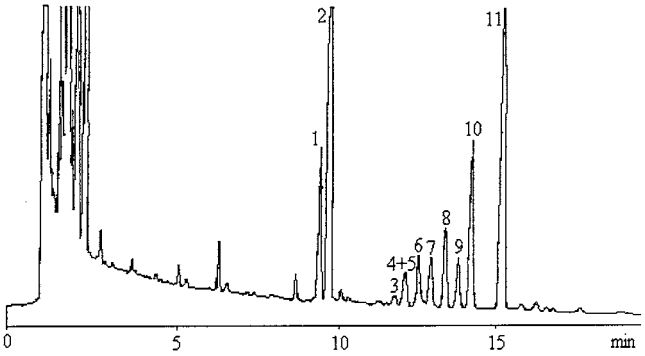

Figure 3. GC chromatogram of the hydrocarbon fraction of sunflower oil after bleaching with 2% acidic earths. Peaks: (1) 2,4-cholestadiene; (2) 3,5-cholestadiene (I.S.); (3) 2,4-campestadiene; (4) 3,5-campestadiene; (5) 2,4,22-stigmastatriene; (6) 3,5,22-stigmastatriene; (7) 2,4,6-campestatriene; (8) 2,4,6,22-stigmastatetraene; (9) 2,4-stigmastadiene; (10) 3,5-stigmastadiene; (11) 2,4,6-stigmastatriene.

Bleaching of the Sunflower Oil. A crude sunflower oil (sample SF1), rich in hydroxyphytosterols, was used for the bleaching and deodorization tests. The quantitative determination of steradienes from the dehydration of sterols and steratrienes from the dehydration of hydroxysterols was performed with the internal standard method by using cholest-3,5-diene as I.S. and considering 1 to be the RRF at the FID detector (*16*).

The GC chromatogram (Carbowax column) of the steroidal hydrocarbon fraction of the sunflower oil bleached with acidic earths is shown in **Figure 3**. The compounds were identified on the bases of the mass spectrometric data reported in the literature (*7*, *8*, *16*). The concentrations of steratrienes in the sunflower oil before and after bleaching together with the percentage of dehydration, on a molar basis, of the hydroxysterols, are reported in **Table 6**. In the calculation of the

Table 6. Concentrations (Parts per Million ± SD)[a] of Steratrienes and Steradienes and Percent Dehydration (%D) of Phytosterol Oxides in Sunflower Oil before and after Bleaching

	initial	1% acidic earths		2% acidic earths		1% neutral earths		2% neutral earths	
steroidal hydrocarbon	ppm	ppm	%D	ppm	%D	ppm	%D	ppm	%D
2,4,6-stigmastatriene	2.2 ± 0.25	9.1 ± 0.28	29.4 ± 2.49	11.6 ± 0.88	40.1 ± 4.69	3.7 ± 0.19	6.4 ± 1.42	5.1 ± 0.16	12.2 ± 1.52
2,4,6-campestatriene	0.4 ± 0.05	1.3 ± 0.16	32.5 ± 6.01	1.6 ± 0.10	41.4 ± 4.35	0.6 ± 0.12	6.3 ± 4.52	0.8 ± 0.02	13.3 ± 2.06
2,4,6,22-stigmastatetraene	0.4 ± 0.04	1.7 ± 0.09	32.5 ± 3.39	2.1 ± 0.17	44.7 ± 5.36	0.8 ± 0.05	8.0 ± 1.78	1.0 ± 0.06	15.5 ± 2.10
stigmastadienes[b]	0.5 ± 0.15	2.5 ± 0.22	0.1 ± 0.01	5.5 ± 1.32	0.2 ± 0.06	0.8 ± 0.07	0.01 ± 0.01	1.1 ± 0.09	0.03 ± 0.01

[a] Means of three analyses. [b] 2,4-Stigmastadiene + 3,5-stigmastadiene.

Table 7. Percent Recovery (± SD) of Phytosterol Oxides in Sunflower Oil after Bleaching and Deodorization

	bleaching				deodorization
phytosterol oxide	1% acidic earths	2% acidic earths	1% neutral earths	2% neutral earths	180 °C
7α-OH-β-sitosterol	11.8 ± 2.88	5.8 ± 1.55	30.4 ± 4.18	10.6 ± 0.87	99.6 ± 9.15
7β-OH-β-sitosterol	9.1 ± 1.88	nd[a]	29.7 ± 4.64	15.9 ± 1.94	102.2 ± 17.04
7α-OH-campesterol	10.8 ± 3.18	nd	29.5 ± 5.09	11.9 ± 1.17	99.9 ± 8.04
7β-OH-campesterol	nd	nd	27.6 ± 6.22	15.5 ± 1.70	110.1 ± 17.95
7α-OH-stigmasterol	7.5 ± 2.56	nd	27.8 ± 4.38	12.2 ± 0.95	106.1 ± 12.44
7β-OH-stigmasterol	nd	nd	27.6 ± 4.33	18.2 ± 2.45	109.8 ± 18.84
7-keto-β-sitosterol	76.7 ± 7.89	63.5 ± 13.32	79.9 ± 8.70	83.2 ± 9.15	83.0 ± 10.72

[a] nd, not determined; in the samples, after bleaching, the hydroxyphytosterols were present only at trace levels.

percentage of dehydration the sum of the two 7α- and 7β-OH epimers was considered because by dehydration they give rise to the same steratriene (*16*). In the same table the concentration of the steradienes derived from the dehydration of β-sitosterol is also reported; in this case the percent dehydration refers to β-sitosterol.

In the sample bleached with acidic earths (1%) there was a significant increase of the steratrienes derived by dehydration of the hydroxy sterols with an average dehydration of ∼30%. Similarly, at 2% of acidic earths the steratrienes concentration increased further with an average dehydration of ∼42%. In the case of the oil bleached with neutral earths, the formation of steratrienes was lower, with average dehydrations of about 7 and 14% at earths levels of 1 and 2%, respectively. These results clearly show the effect of the acidity of the earths on the dehydration of the hydroxysterols.

Regarding the steradienes and in particular the 2,4- and 3,5-stigmastadienes derived by dehydration of β-sitosterol, the concentration of which was of 2264 ppm, the results showed a similar trend with a higher concentration of stigmastadienes formed in the case of bleaching with acidic earths. The basic difference lies in the dehydration percentages, with values that are much higher for the hydroxysterols with respect to the sterols, confirming the higher rate of dehydration of the former (*16*).

After bleaching, the oils were further analyzed for their residual content of hydroxysterols and 7-keto-β-sitosterol. Recoveries of the hydroxysterols (**Table 7**) after bleaching with acidic earths were very low and, in particular, at the level of 2% of earths, only ∼6% of the initial 7α-OH-β-sitosterol was left in the oil. In the case of the bleaching with neutral earths, recoveries of the hydroxysterols were higher, with average values of 29 and 14% at earths levels of 1 and 2%, respectively. Recoveries of 7-keto-β-sitosterol were, on the contrary, similar for the different bleaching conditions with an average value of 76%. 7-Keto-β-sitosterol is then more stable with respect to the hydroxysterols in this process.

The recovery of the hydroxysterols and 7-keto-β-sitosterol and steratrienes formed did not account for the initial amounts of these compounds in the oil before bleaching. This fact can be explained by the occurrence of other more complex degradation reactions as well by the adsorption of these compounds on the earths.

Deodorization of Sunflower Oil. The same sunflower oil used for the bleaching test was heated at 180 °C, under vacuum, to simulate the process of deodorization. The temperature of 180 °C is at the lower limit of the temperatures used in this process, which lie generally between 180 and 240 °C.

There were no significant differences between the trienes and dienes concentrations before and after deodorization, indicating that heating at 180 °C for 1 h was a condition not sufficient for the dehydration of either the hydroxysterols or sterols. Moreover, recoveries of the hydroxysterols (**Table 7**) were quantitative with no decomposition reactions, whereas the recovery of 7-keto-β-sitosterol was ∼80%, similar to the recoveries obtained after bleaching. Other authors reported the formation of steradienes at a deodorization temperature of 270 °C (*8*) and above 175−200 °C (*9*). It is reasonable to hypothesize that dehydration of the hydroxysterols would occur at higher temperatures.

Bleaching of Extra Virgin Olive Oil in the Presence of 7-Ketocholesterol, α-Epoxycholesterol, and Dihydroxycholesterol. It was hypothesized that the formation of the steratrienes was derived only by dehydration of the hydroxysterols as shown by Bortolomeazzi et al. (*16*) in model systems, and no mention was made of the presence of 7-keto-β-sitosterol in the sunflower oil. The high recovery of this compound after bleaching seems, however, to exclude its role as a possible precursor of the steratrienes. Moreover, other authors reported the presence in vegetable oils of the epoxy and dihydroxy derivatives of phytosterols, compounds that were present only at low concentration in the sunflower oil used in this study. To verify the possible formation of steratrienes also from the other principal sterol oxides, bleaching experiments with acidic earths were carried out using a model system composed of extra virgin olive oil fortified with 100 ppm of 7-ketocholesterol, α-epoxycholesterol, and dihydroxycholesterol. The oxidation products of cholesterol were chosen because of their availability, and an extra virgin olive oil was used as matrix because of the very low content of cholesterol (<0.1 ppm) and the even lower content of the corresponding oxidation products. It is moreover

reasonable to hypothesize a similar behavior regarding chemical reactions involving the ring system of the molecule, which is the same for the cholesterol and phytosterol oxides. This has been already verified for reactions such as autoxidation, photooxidation, and dehydration.

The bleaching experiments were carried out with 1% acidic earths at 80 °C for 1 h, and the steratriene quantified was 2,4,6-cholestatriene. In the oil bleached in the presence of 7-ketocholesterol there was no formation of 2,4,6-cholestatriene, whereas small amounts, 0.3 and 0.5 ppm, respectively, were formed during the bleaching in the presence of α-epoxycholesterol and dihydroxycholesterol. These results showed that decomposition with formation of a triene derivative is not an important reaction for these compounds. Recovery of 7-ketocholesterol was ∼60%, similar to that found for 7-keto-β-sitosterol in the sunflower oil. No epoxycholesterol was found in the oil after bleaching and only ∼37% of dihydroxycholesterol, indicating a complete decomposition of the epoxy derivative with ring opening and a partial decomposition of the dihydroxy derivative. Similarly, in the case of the dihydroxycholesterol there was partial decomposition with a recovery of 31%.

In almost all of the oils analyzed phytosterol oxides were present and, in particular, the 7α- and 7β-hydroxy derivatives of the principal sterols and 7-keto-β-sitosterol. The hydroxysterols were confirmed as precursors of the steratrienes found in the refined oils, whereas no formation of these hydrocarbons occurred during the bleaching in the case of the other phytosterol oxides. Among the different phytosterol oxides, the 7-keto derivative was the more stable, whereas the hydroxy, epoxy, and dihydroxy derivatives undergo a more or less major decomposition.

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