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Stability of Plant Sterols in Ingredients Used in Functional Foods

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ABSTRACT: The content of plant sterol (PS) and their oxidation products (POPs) in eight ingredients used to enrich functional foods was studied. A gas chromatographic (GC) technique with mass-spectrometric detection was used for identification, while GC with a flame ionization detector (GC-FID) was used for quantification. β-Sitosterol was the most abundant phytosterol, and the main POPs found were derived from this compound (7α/β-hydroxysitosterol, 7-ketositosterol, and sitostanetriol). The total amount of POPs found in the ingredients ranged from 29.03 to 110.02 μg/100 g PS. The β-sitosterol oxidation rates ranged from 10 to 50 μg β-sitosterol oxides/100 g of β-sitosterol. In view of this low rate of oxidation in the ingredients tested, it can be concluded that the PS remain stable in these ingredients. Significant correlations ($p < 0.01$) were found between total oxysitosterols versus β-sitosterol contents ($R^2 = 86.5\%$) and between total POPs and total PS ($R^2 = 81.6\%$).

KEYWORDS: Plant sterol oxidation products, POPs, phytosterol stability, gas chromatography−mass spectrometry, functional ingredients

INTRODUCTION

Plant sterols (PS: phytosterols/-stanols) are natural constituents of plants and have many essential functions in plant cells, in a way similar to cholesterol (cholest-5-en-3β-ol) in animal cells. Structurally, they are very similar to cholesterol but have a much lower intestinal absorption rate in humans. PS consumption (2 g/day) results in an approximately 9% reduction in LDL-cholesterol. PS also have beneficial effects upon other lipid parameters.[1] In the European Union, the use of PS and their esters in different food matrices is regulated by different Commission Decisions.[2,3] Recently, the European Commission authorized as a health claim that "plant sterols and plant stanol esters have been shown to lower/reduce blood cholesterol. High cholesterol is a risk factor in the development of coronary heart disease."[4]

A variety of commercial foods have been enriched with free or esterified PS, spreads being the first (and still the most common) commercial application of PS-enriched food.

Like all unsaturated lipids, PS are liable to oxidation, giving rise to plant sterol oxidation products (POPs). To date, data on the biological effects of POPs are still scarce and sometimes contradictory. In some studies, no deleterious biological effects have been observed, with no reported POP genotoxicity. In other cases, however, POPs have been suggested to have cytotoxic effects, these being less severe than in the case of cholesterol oxidation products (COPs).[5]

The major sources of PS for current functional foods and dietary supplements are tall oil and vegetable oil deodorizer distillate. Tall oil is a byproduct of the Kraft pulping of wood to make paper and contains a mixture of PS. The latter are extracted from tall oil soap, a residue from the paper pulping process, or from tall oil pitch. Vegetable oil processing involves refining, one of the stages of which is deodorization. The lower molecular weight compounds are eliminated in the deodorizer distillate by-product fraction. Free sterols are a major component (15−30%) of the contents of the deodorizer distillate fraction.[6]

Obtainment of the deodorizer distillate by conventional or physical refining involves high temperatures (180−220 and 240−250 °C, respectively),[7] and separation of the sterol fraction from the distillate involves different processes that can reach temperatures ranging from 150 to 240 °C.[8] Both conditions are favorable for POPs formation.[9] POPs have been quantified in by-product fractions from chemical refining and by-product distillate fractions from physical refining of edible fats and oils (limit of quantification 0.01 mg/100 g), for their use in animal feed formulations.[10] However, industrial processing (chemical refining and deodorization) caused no increase in POPs of β-sitosterol ((24*R*)-ethylcholest-5-en-3β-ol) in soybean oil at a detection level of 0.2 ppm,[11] and no POPs (from β-sitosterol and campesterol [(24*R*)-methylcholest-5-en-3β-ol]) were detected in rapeseed oil at a detection level of 1 μg/g oil.[12]

Contents of POPs in different crude and refined vegetable oils have been reported.[9,13−17] Once the PS are extracted, they are processed to be later incorporated into different food matrices. Therefore, PS storage, processing, and storage once PS are incorporated in different food matrices are stages susceptible for POPs formation. For this reason, PS ingredients manufacturers usually use a wide variety of delivery systems for the protection of the PS from degradation during processing and storage.[18] Emulsion-based technologies and spray drying are currently the most common approaches employed for microencapsulation and delivery of functional lipophilic components into food.[19]

To our knowledge, only one study has been published on the evaluation of the oxidation of PS in different vegetable oils added as microcrystalline PS suspensions prepared from wood-based fractions.[16]

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Table 1. Ingredients Analyzed: Physical States, Composition, and Characteristics Indicated by the Manufacturer

ingredient	source	sterols purity (manufacturer)	other compounds	physical state
1	soy (free sterols)	min 25%	malt dextrin, inulin, citric acid, preservatives	liquid emulsion
2	pine tree (tall-oil) (free sterols)	min 25%	malt dextrin, inulin, citric acid, preservatives	liquid emulsion
3	pine tree (tall-oil) (free sterols)	68−75%	malt dextrin, inulin, sucrose ester	powder
4	soy (free sterols)	68−75%	malt dextrin, inulin, sucrose ester	powder
5	soybean, rapeseed, sunflower, corn oil (sterol esters)	43.9%	glucose syrup, sodium caseinate, tocopherol-rich extracts, ascorbyl palmitate, sodium ascorbate	spray dry powder
6	soybean, rapeseed, sunflower, corn oil (free esterols)	86−92%	skimmed milk powder	spray dry powder
7	sunflower, tall-oils (free sterols)	12%	water, emulsifier, stabilizer, potassium sorbate, ascorbyl palmitate, tocopherols	liquid emulsion
8	high oleic sunflower, tall-oils (free sterols)	30%	antioxidants	oil paste

Because of the scant information available on POPs formation and contents in PS sources used for the enrichment of foods, and considering that different ingredients can be used as sources of PS, we designed the present study to identify and quantify the PS and their oxidation products in eight ingredients currently used for the enrichment of foods with PS.

■ MATERIALS AND METHODS

Samples. Eight commercially available ingredients containing PS were used for this study. They were presented as esterified or free plant sterols from different sources (pine, soybean, rapeseed, soybean, corn, and sunflower oils), and in different physical states (powder, oil paste, and liquid emulsion). A description of their composition and other characteristics is provided in Table 1.

Chemicals. For the standards of PS, 5β-cholestan-3α-ol (epicoprostanol) (purity ≥95%) was used as internal standard (IS) in phytosterol determination, (24*S*)-ethylcholest-5,22-dien-3β-ol (stigmasterol) (purity 95%), (24*R*)-ethylcholest-5-en-3β-ol (β-sitosterol) (purity 95%), and 24α-ethyl-5α-cholestan-3β-ol (stigmastanol) (purity 97.4%) were from Sigma Chemical Co. [St. Louis, MO]. (24*S*)-Methylcholest-5,22-dien-3β-ol (brassicasterol) (purity 98.6%) and (24*R*)-methylcholest-5-en-3β-ol (campesterol) (purity 98.6%) were purchased from Steraloids [Newport, RI], and (24*R*)-ethylcholest-5-en-3β-ol (β-sitosterol) (purity 60% β-sitosterol and 30% campesterol) was from Fluka [Buchs, Switzerland].

5α-Cholest-5-en-3β,19-diol (19-hydroxycholesterol) (purity 95%) from Sigma Chemical Co. was used as IS in POPs determination. Cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) (purity 98.6%), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol) (purity 95%), 5α,6α-epoxycholestan-3β-ol (α-epoxycholesterol) (purity 80%), 5β,6β-epoxycholestan-3β-ol (β-epoxycholesterol) (purity 98%), and cholestane-3β,5α,6β-triol (cholestanetriol) (no purity information) from Sigma Chemical Co. were used as standards of COPs. (24*S*)-Ethylcholest-5,22-dien-3β-ol-7-one (7-ketostigmasterol) (purity 98.6%) was from Steraloids.

All reagents were analytical grade. Chloroform, diethyl ether, methanol, anhydrous sodium sulfate, acetone, 2-propanol, and anhydrous pyridine were purchased from Merck & Co., Inc. [Whitehouse Station, NJ]. Potassium hydroxide was purchased from Poch, S.A. [Sowinskiego, Poland], potassium chloride was from Panreac [Barcelona, Spain], and *n*-hexane was from J.T. Baker [Deventer, The Netherlands]. The silylating reagents hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from Fluka and Carlo Erba [Rodano, Italy], respectively. Ultrapure water was obtained by means of a Millipore Q water purification system [Milford, MA]. The Si-SPE (silica solid-phase extraction) cartridges (3 mL/500 mg) were purchased from Supelco [Bellefonte, PA]. Silica-gel thin-layer chromatography (TLC) plates (20 cm × 20 cm × 0.25 mm film thickness) were purchased from Merck & Co., Inc.

Determination of Plant Sterols. A weight of ingredient that yields approximately 30 mg of PS was taken. Saponification was performed according to the method described by Piironen et al.,[20] with slight modifications. Briefly, IS was added (2000 μg of epicoprostanol) to the ingredient, and hot saponification (3 mL of 1 N KOH in methanol 65 °C during 1 h) was performed. The nonsaponifiable material was extracted with diethyl ether. 1/10 of the unsaponifiable material was subjected to derivatization with HMDS/TMCS in anhydrous pyridine (2:1:5) for 20 min at 40 °C. The trimethylsilyl ether (TMSE) derivatives obtained were dissolved in *n*-hexane, filtrated using a Millipore [Milford, MA] syringe-driven Millex-FH (1 mL, 0.45 μm) filter unit, and evaporated with nitrogen. The TMSE derivatives were dissolved in 250 μL of *n*-hexane for chromatographic analysis. For quantification, 1 μL was injected in a GC−FID (AutoSystem XL, Perkin-Elmer [Nortwalk CT]) equipped with a CP-Sil 8 low bleed/MS (50 m × 0.25 mm × 0.25 μm film thickness) capillary column (Chrompack-Varian [Middelburg, The Netherlands]). The oven temperature was initially held at 280 °C for 20 min, raised to 290 at 0.7 °C/min, and held for 5 min, then raised to 320 °C at rate of 30 °C/min and held for 45 min. The carrier gas was hydrogen (0.7 mL/min). The temperature of both the injector port and the detector was 325 °C. A split ratio of 1:10 was applied. For PS identification, 1 μL was injected into a GC−mass spectrometer (MS) (Trace GC-Ultra ITQ ion trap 900, Thermo Scientific [Waltham, MA]). Analysis was performed on a capillary TR-5 ms SGC capillary column (30 m × 0.25 mm id × 0.25 μm) (Thermo Scientific), using hydrogen as the carrier gas (1 mL/min). The oven temperature was initially held at 250 °C for 1 min, raised to 280 at 0.8 °C/min, and raised to 310 at 5 °C/min and held for 5 min. The programmable temperature vaporization (PTV) injector was set at ballistic heating from 50 to 260 °C at a 1:10 split ratio. The filament emission current was 70 eV, the ion source temperature was 250 °C, and the scan range was 50−650 *m*/*z*.

For quantification, calibration curves of brassicasterol (9.86−59.16 μg), campesterol (98−553 μg), stigmasterol (9.5−99.75 μg), and β-sitosterol (703−2394 μg) containing 200 μg of IS (epicoprostanol) were performed.

Quantification of phytostanols was performed using the response factor calculated for stigmastanol versus epicoprostanol (IS).

Analytical Parameters. Limits of detection (LOD) and quantification (LOQ) for phytosterol determination were calculated. The LOD was calculated as 3 times the standard deviation of the noise, and the LOQ as 10 times the standard deviation of the noise, expressed in quantity, by

Table 2. Identification of Plant Sterol Oxides by GC−MS: Relative Retention Time (RRT), Precursor and Product Ions, and Optimized Collision Energy (CE)

plant sterol oxides	RRT[a]	precursor ion	CE	product ions
		β-Sitosterol		
7α-hydroxy	1.168	484	2.9	470, 469, 379, 233
7β-hydroxy	1.45	484	2.9	470, 469, 379, 233
α-epoxy	1.52	396	2	381, 367, 255, 213
β-epoxy	1.56	396	2	381, 367, 255, 213
triol	1.79	431	2.3	343, 341, 301
7-keto	1.95	500	3.2	483, 410, 395
		Campesterol		
7α-hydroxy	1.14	470	2.9	456, 455, 442
7β-hydroxy	1.27	470	2.9	456, 455, 442
7-keto	1.73	486	3.3	485, 471, 470, 469, 381
		Stigmasterol		
7α-hydroxy	0.89	482	2.9	468, 467, 439, 327
7β-hydroxy	1.09	482	2.9	468, 467, 439, 327
α-epoxy	1.21	500	2.5	486, 485, 481, 456, 410
β-epoxy	1.23	500	2.5	485, 471, 457, 443, 410, 395, 382.
7-keto	1.57	498	3	482, 409, 408, 394, 393

[a] With respect to 19-hydroxycholesterol (IS), 19.7 min.

running five blanks subjected to the same derivatization procedure as the study samples.

The accuracy of the method was evaluated by recovery assays. The PS content of three aliquots of one ingredient and another three aliquots added with 59.16 μg of brassicasterol, 19.95 μg of stigmasterol, and 1596 μg of β-sitosterol standards (contents similar to those found in the ingredient) was determined. Recovery was calculated as:

$$\%\text{recovery} = \frac{\mu\text{g PS in spiked ingredients} - \mu\text{g PS in not spiked ingredients}}{\mu\text{g spiked PS}} \times 100$$

Precision was calculated as the relative standard deviation of three replicates of one ingredient.

Determination of Phytosterol Oxides. *POPs Obtained by Thermo-oxidation.* As standards of POPs are not commercially available, except for 7-ketostigmasterol, they were obtained by thermo-oxidation using the method described by Conchillo et al.[21] to identify POPs in the eight ingredients and to optimize the GC methods. Standard solutions: 1 mg/mL of β-sitosterol (purity 60%); 0.8 mg/mL campesterol; 1 mg/mL stigmasterol were prepared, and each of them was placed in a different Petri dish. After the solvent was evaporated with nitrogen, they were heated at 150 °C for 2 h in an oven.

POPs derived from thermo-oxidation of β-sitosterol were separated by silica gel (TLC) according to their degree of oxidation and compared to a mixture of commercial standards of COPs.[21] The corresponding bands from different β-sitosterol oxides were extracted three times with chloroform.

The POPs obtained by thermo-oxidation from campesterol and stigmasterol were purified by SPE according to the method described by Guardiola et al.[22] Briefly, a 0.5 g silica cartridge was activated by 5 mL of *n*-hexane. The POPs obtained were dissolved in 100 μL of *n*-hexane: diethyl ether (95:5 v/v) and loaded onto the column. Nonoxidized phytosterols were eluted with different mixtures of *n*-hexane:ether, and finally the POPs were eluted out of the cartridge with acetone.

The different TLC POP fractions from β-sitosterol, as well as the purified solutions from campesterol and stigmasterol, were subjected to silylation according to the same procedure applied to PS. The TMSE derivatives obtained were dissolved in 50 μL of *n*-hexane, and 1 μL was injected into a GC−FID using the same instrumental conditions as for PS, except that the last holding time at 320 °C was lengthened by 20 min.

One microliter was also injected into the GC−MS. Identification of the POPs obtained from the TLC fractions from β-sitosterol, as well as the SPE purified solutions from campesterol and stigmasterol, was performed on the basis of the resulting fragmentation patterns and retention times used in previous studies[23] consulted in the literature,[13,15,24,25] as well as from the TLC elution pattern of POPs according to Conchillo et al.[21] The relative retention times, precursor and product ions, and optimized energy collision of the POPs identified by GC−MS are summarized in Table 2.

Application to Ingredients. The procedure for POPs analysis was adapted from the method proposed by García-Llatas et al.,[23] with minor modifications. A weight of ingredient that provides approximately 30 mg of PS was taken. IS (9.82 μg of 19-hydroxycholesterol) was added to the sample. Cold saponification at room temperature, in darkness, and under continuous agitation in an orbital shaker (IKA KS26, Stauffen, Germany) at 150 rpm was performed (10 mL of 1 N KOH in methanol for 18−20 h). The unsaponifiable material was extracted with diethyl ether, and 1/2 of the unsaponifiable material was subjected to purification by silica SPE according to ref 22. POPs were eluted with acetone. The purified fraction was then subjected to silylation. The TMSE derivatives obtained were dissolved in 40 μL of *n*-hexane, and 1 μL was injected into the GC−MS for identification and the GC−FID for quantification purposes. Identification of different oxides was performed as described before.

Quantification of POPs was performed using the response factors calculated for their analogue COPs: 7β-hydroxycholesterol for hydroxyphytosterols, α-epoxycholesterol for epoxyphytosterols, cholestanetriol for triol-derivatives, and 7-ketocholesterol for ketophytosterols. The response factor for 7-ketoestigmasterol was calculated and compared to that calculated for 7-ketocholesterol, to confirm the response factors of COPs and POPs.

Analytical Parameters. The LOD and LOQ for phytosterol oxides were calculated.

The LOD was calculated as 3 times the standard deviation of the noise, and the LOQ as 10 times the standard deviation of the noise, expressed in quantity, by running five blanks subjected to the same derivatization procedure as the study samples.

The accuracy of the method was evaluated by recovery assays. Three aliquots of an ingredient were spiked with 2.08 μg of 7β-hydroxycholesterol, 0.92 μg of α-epoxycholesterol, and 4.58 μg of 7-ketocholesterol standards (contents similar to those respective POPs found in the ingredient) were determined. Recovery was calculated as:

$$\%\text{recovery} = \frac{\mu\text{g COPs in spiked ingredients}}{\mu\text{g spiked COPs}} \times 100$$

Precision was calculated as the relative standard deviation of three replicates.

Statistics. An ANOVA test ($p < 0.05$) was applied to total PS and individual PS contents among ingredients. The same evaluation was applied to total POPs and individual oxysitosterol contents among ingredients. When significant differences existed, a Tukey test was performed.

Regression analyses were made to evaluate the total POPs contents in ingredients versus total PS and total oxysitosterols versus β-sitosterol contents.

Table 3. Plant Sterols and Plant Sterol Oxides Determination: Quantification Method and Analytical Parameters[a]

			quantification[b]	LOD (ng in assay)	LOQ (ng in assay)	precision[c]
plant sterols	brassicasterol	regression equation	$y = 1.28x + 0.003$ $R^2 = 0.9961$	0.34	1.12	1.23
	campesterol		$y = 1.313x + 0.002$ $R^2 = 0.9993$	0.45	1.51	1.36
	stigmasterol		$y = 0.999x + 0.007$ $R^2 = 0.9978$	0.81	2.68	1.72
	β-sitosterol		$y = 0.94x - 0.478$ $R^2 = 0.9848$	0.73	2.43	2.09
	sitostanol	response factor[d]	1.12	0.59	1.97	1.79
	stigmastanol		1.12	0.81	2.68	1.84
	campestanol		1.12	1.83	6.09	1.15
plant sterol oxides	7α-OH-S	response factor[e]	1.14	4.99	16.62	9.19
	7β-OH-S			6.17	20.55	3.67
	Triol-S		2.58	0.81	2.68	2.48
	7-K-S		1.15	2.33	7.76	3.00
	7-K-C			3.12	10.40	24.60

[a] 7α-OH-S, 7α-hydroxysitosterol; 7β-OH-S, 7β-hydroxysitosterol; Triol-S, sitostanetriol; 7-K-S, 7-ketositosterol; 7-K-C, 7-ketocampesterol. [b] y = analyte area/IS area, x = analyte content (μg)/IS content (μg), R^2 = correlation coefficient. LOD = limit of detection. LOQ = limit of quantification. [c] Expressed as relative standard deviation of three replicates. [d] Corresponds to stigmastanol. [e] Corresponds to COPs.

Table 4. Plant Sterol Contents (g/100 g of Ingredient)[a]

ingredients	brassicasterol	campesterol	campestanol	stigmasterol	stigmastanol	β-sitosterol	sitostanol	total PS
1	0.23 ± 0.022	5.91 ± 0.561	0.15 ± 0.013	6.61 ± 0.621	0.17 ± 0.015	8.99 ± 0.920	0.32 ± 0.118	22.37 ± 2.271
	(1.02 ± 0.007) a	(26.43 ± 0.175) a	(0.65 ± 0.008) a	(29.55 ± 0.222) a	(0.75 ± 0.007)	(40.19 ± 0.035) a	(1.41 ± 0.384) a	
2	0.01 ± 0.001	1.88 ± 0.200	0.26 ± 0.043	0.16 ± 0.014	ND	15.97 ± 1.746	2.68 ± 0.457	20.94 ± 2.263
	(0.06 ± 0.002) b	(8.98 ± 0.136) b	(1.22 ± 0.145) b	(0.74 ± 0.033) b		(76.25 ± 1.545) b	(12.75 ± 1.513) b	
3	0.03 ± 0.001	5.06 ± 0.329	0.85 ± 0.067	0.58 ± 0.046	ND	55.94 ± 3.663	8.48 ± 0.583	70.93 ± 4.684
	(0.04 ± 0.001) b,c	(7.13 ± 0.007) c	(1.20 ± 0.024) b	(0.82 ± 0.023) b		(78.86 ± 0.120) c	(11.95 ± 0.072) b,c	
4	0.58 ± 0.012	17.02 ± 0.373	0.51 ± 0.006	17.86 ± 0.361	0.47 ± 0.009	29.95 ± 0.673	1.63 ± 0.029	68.02 ± 1.440
	(0.85 ± 0.008) d	(25.03 ± 0.056) d	(0.76 ± 0.014) a	(26.26 ± 0.081) c	(0.69 ± 0.012)	(44.03 ± 0.169) d	(2.39 ± 0.013) a	
5	1.08 ± 0.129	7.71 ± 0.998	0.40 ± 0.053	0.61 ± 0.040	ND	31.17 ± 4.732	3.97 ± 0.542	44.93 ± 6.491
	(2.41 ± 0.071) e	(17.19 ± 0.302) e	(0.89 ± 0.013) c	(1.37 ± 0.108) d		(69.31 ± 0.554) e	(8.83 ± 0.068) d	
6	1.73 ± 0.026	10.87 ± 0.181	0.90 ± 0.020	0.93 ± 0.020	ND	63.51 ± 1.627	8.47 ± 0.175	86.40 ± 2.017
	(2.00 ± 0.038) f	(12.58 ± 0.117) f	(1.04 ± 0.005) d	(1.08 ± 0.008) e		(73.50 ± 0.171) f	(9.81 ± 0.077) d	
7	ND	0.95 ± 0.131	0.15 ± 0.023	0.11 ± 0.012	ND	10.42 ± 1.954	1.43 ± 0.147	13.06 ± 2.261
		(7.28 ± 0.245) c	(1.15 ± 0.038) b	(0.86 ± 0.096) b		(79.66 ± 1.092) c	(11.04 ± 0.761) c	
8	0.01 ± 0.001	2.34 ± 0.114	0.37 ± 0.016	0.27 ± 0.005	ND	25.40 ± 1.608	3.66 ± 0.214	32.05 ± 1.953
	(0.04 ± 0.001) b,c	(7.30 ± 0.086) c	(1.15 ± 0.021) b	(0.85 ± 0.050) b		(79.25 ± 0.182) c	(11.42 ± 0.032) c	

[a] Values in parentheses are expressed as g/100 g of plant sterols. Values are expressed as mean ± standard deviation of three replicates. ND: not detected. Different letters, in the same column, indicate significant differences ($p < 0.05$).

The Statgraphics Plus version 5.1 [Rockville, MD] statistical package was used throughout.

RESULTS AND DISCUSSION

Plant Sterols. *Quantification and Analytical Parameters.* Calibration curves and linear regression coefficients obtained for the different phytosterols, the response factors for phytostanols, and the analytical parameter results are reported in Table 3.

Accuracy, estimated by recovery assays and expressed in percentage as the mean ± standard deviation of three replicates, was 125.41 ± 3.20 for brassicasterol, 114.82 ± 12.65 for stigmasterol, and 105.18 ± 19.34 for β-sitosterol.

The analytical parameters obtained showed that the method was useful for PS determination in the ingredients studied.

The PS contents from the ingredients analyzed are summarized in Table 4. The PS totals ranged from 13.06 to 86.4 g/100 g of ingredient. Significant differences ($p < 0.05$) were found in total PS contents among all ingredients (see Table 4). Phytostanol contents were detected at amounts always lower than the respective phytosterols. The content of total phytosterols matched that declared in the information provided by the manufacturer. All ingredients (see Table 4) complied with current legislation regarding PS percentages: <80% β-sitosterol, <40% campesterol, <30% stigmasterol, <3% brassicasterol, <15% sitostanol, <5% campestanol, <3% other sterols/stanols.[2]

The total content and profile of the PS ingredients differed according to the sample considered. When each PS was considered individually, significant differences ($p < 0.05$) were found among ingredients (see Table 4). β-Sitosterol was the most abundant phytosterol, followed by campesterol and stigmasterol. The highest contents (over 10-fold higher than other ingredients) of stigmasterol in ingredients 1 and 4 may be attributed to their origin: soybean oil (the only oil in their

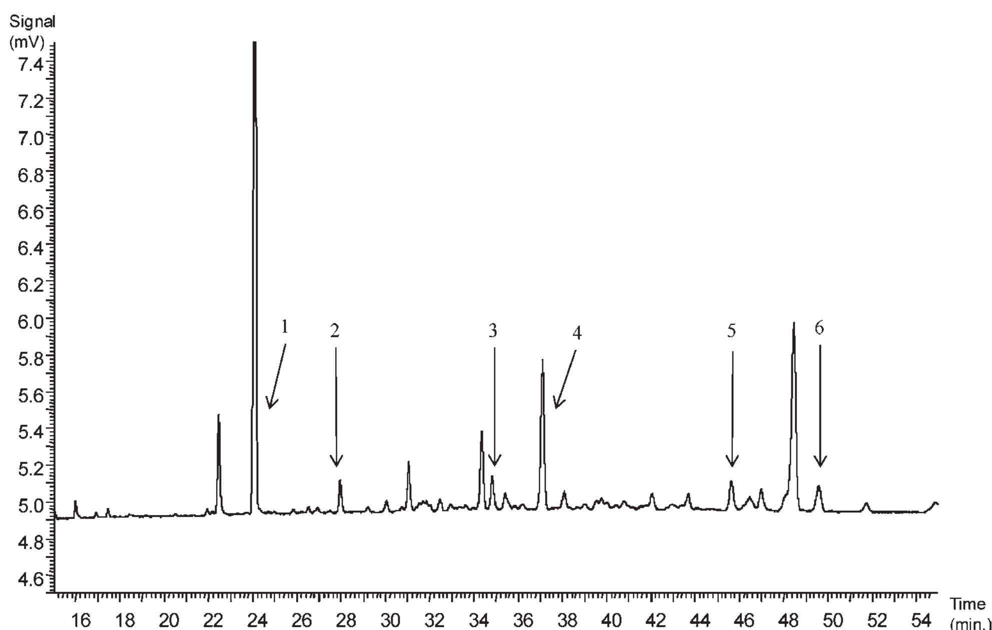

Figure 1. GC–FID chromatogram of POPs from ingredient number 3. The following POPs are identified: (1) 19-hydroxycholesterol (5α-cholest-5-en-3*β*,19-diol) (IS), (2) 7α-hydroxysitosterol ((24*R*)-ethylcholest-5-ene-3*β*,7α-diol), (3) 7*β*-hydroxysitosterol ((24*R*)-ethylcholest-5-ene-3*β*,7*β*-diol), (4) 6α-hydroxysitostanol ((24*R*)-ethylcholestane-3*β*,6α-diol), (5) sitostanetriol ((24*R*)-ethylcholestane-3*β*,5α,6*β*-triol), and (6) 7-ketositosterol ((24*R*)-ethylcholest-5-en-3*β*-ol-7-one).

composition) (see Table 1). It has been indicated that the stigmasterol content in soybean oil is greater than that in other edible plant or vegetable oils,[26,27] although the content varies according to the soybean genotype involved.[26]

Brassicasterol was the least abundant sterol in the analyzed ingredients. It has been suggested that in rapeseed oil (*Brassica* species) the content is higher than in other types of oils.[26−28] This explains the high brassicasterol contents in ingredients 5 and 6 that have rapeseed oil in their composition.

It has been reported that on comparing tall oils versus vegetable oils, the former have higher sitostanol levels and lower stigmasterol and brassicasterol levels.[29] This coincides with the ranges found in our ingredients: ingredients 2 and 3 (see Table 1), which entirely come from tall oil, and ingredients 7 and 8, which have tall oil in their composition, generally presented the lowest contents of brassicasterol and stigmasterol, and higher contents of sitostanol (see Table 4).

Phytosterol Oxides. *Quantification and Analytical Parameters.* The COPs response factors, together with the analytical parameters of POPs, are reported in Table 3. As expected, the respons factor of 7-ketostigmasterol (Rf = 1.12) was similar to that of 7-ketocholesterol (Rf = 1.15).

The LOD and LOQ obtained allowed the quantification of POPs found in the ingredients, and they were lower than those obtained by other authors (in the order of 0.05 μg).[13,30]

Accuracy, estimated by recovery assays and expressed in percentage as the mean ± standard deviation of five replicates, was 116.27 ± 12.57 for 7*β*-hydroxycholesterol, 114.26 ± 19.85 for α-epoxycholesterol, and 111.02 ± 2.49 for 7-ketocholesterol.

The identification of POPs was performed by GC–MS. 7α-Hydroxysitosterol ((24*R*)-ethylcholest-5-ene-3*β*,7α-diol), 7*β*-hydroxysitosterol ((24*R*)-ethylcholest-5-ene-3 *β*,7*β*-diol), sitostanetriol ((24*R*)-ethylcholestane-3*β*,5α,6*β*-triol), and 7-ketositosterol ((24*R*)-ethylcholest-5-en-3*β*-ol-7-one), among others, were identified in all ingredients. These POPs were also clearly separated by GC–FID in all ingredients, as shown in Figure 1 (the peaks observed at 22.8 and 30.9 min correspond to silica compounds from the SPE step, as was confirmed by the corresponding blanks). In addition, 7-ketocampesterol ((24*R*)-methylcholest-5-en-3*β*-ol-7-one) was identified only in ingredient 4, which was, besides ingredient 1, the ingredients with the highest campesterol content. The differences in the presence or not of 7-ketocampesterol between ingredients 1 and 4 could be attributed to the different efficacy of the delivery system (microencapsulated liquid emulsion in ingredient 1 vs microencapsulated powder in ingredient 4).

In all of the ingredients, the following POPs were also identified: 6-hydroxysitosterol (retention time (RT) = 32.3 min); 25-hydroxysitosterol ((24*R*)-ethylcholest 5-ene-3*β*,25-diol) (RT = 35.5 min), 6α-hydroxysitostanol ((24*R*)-ethylcholestane-3*β*, 6α-diol) (RT = 37 min), α/*β* epoxycampesterol ((24*R*)-5α/*β*, 6α/*β*-epoxy-24-methylcholestan-3*β*-ol) (RT = 39.2 min), and α/*β* epoxysitosterol ((24*R*)-5α/*β*,6α/*β*-epoxy-24-ethylcholestan-3*β*-ol) (RT = 43.7 min). It should be noted that brassicasterol and stigmasterol oxides were not found in any ingredient.

POPs contents from the ingredients analyzed are shown in Table 5. The total amount of POPs found ranged from 1.70 to 27.12 μg/100 g of ingredient or from 7.61 to 31.39 μg/100 g of PS. Regarding the total *β*-sitosterol oxide contents, significant differences ($p < 0.05$) were found among all ingredients, as indicated in Table 5.

Among the eight ingredients tested, the percentage of oxidized relative to total *β*-sitosterol was <0.000046%. These oxidation percentages are of the same order as those reported by other authors (0−0.3% of oxidized *β*-sitosterol) when assaying a triplamitin matrix or rapeseed oil spiked with *β*-sitosterol free, esterified, or sitostanol standard.[14,31] However, percentages are much lower than those published by Zhang et al.[15] in sunflower

Table 5. Phytosterol Oxidation Products (μg/100 g Ingredient)[a]

ingredients	7α-hydroxysitosterol	7β-hydroxysitosterol	7-ketocampesterol	sitostanetriol	7-ketositosterol	total POPs
1	0.87 ± 0.153	0.09 ± 0.045	ND	0.35 ± 0.057	0.40 ± 0.071	1.70 ± 0.164
	(3.87 ± 0.683) a,b	(0.42 ± 0.201) a		(1.56 ± 0.255) a	(1.77 ± 0.317) a	(7.61 ± 0.734) a
2	0.59 ± 0.055	0.72 ± 0.034	ND	0.61 ± 0.021	0.96 ± 0.142	2.89 ± 0.238
	(2.81 ± 0.258) a,c	(3.41 ± 0.161) b		(2.87 ± 0.099) b	(4.56 ± 0.672) b	(13.66 ± 1.128) b
3	1.22 ± 0.140	1.30 ± 0.048	ND	3.74 ± 0.093	1.58 ± 0.160	7.84 ± 0.404
	(1.72 ± 0.197) d	(1.83 ± 0.067) c		(5.27 ± 0.131) c	(2.23 ± 0.226) a,c	(11.05 ± 0.570) b
4	2.76 ± 0.379	1.73 ± 0.341	1.787 ± 0.440	2.99 ± 0.517	6.39 ± 1.298	15.66 ± 2.848
	(4.06 ± 0.558) b,e	(2.54 ± 0.501) d	(2.628 ± 0.646)	(4.39 ± 0.760) d	(9.40 ± 1.908) d	(23.02 ± 4.187) c
5	0.96 ± 0.115	0.58 ± 0.052	ND	1.90 ± 0.136	1.42 ± 0.175	4.86 ± 0.070
	(2.14 ± 0.255) c,d	(1.20 ± 0.115) c		(4.22 ± 0.304) d	(3.16 ± 0.389) c	(10.82 ± 0.155) b
6	6.59 ± 0.657	5.78 ± 0.262	ND	4.63 ± 0.396	10.12 ± 0.304	27.12 ± 0.644
	(7.63 ± 0.760) f	(6.69 ± 0.303) e		(5.36 ± 0.458) c	(11.71 ± 0.352) e	(31.39 ± 0.745) d
7	0.67 ± 0.073	0.61 ± 0.103	ND	0.58 ± 0.044	1.01 ± 0.046	2.86 ± 0.210
	(5.10 ± 0.556) e	(4.65 ± 0.789) f		(4.41 ± 0.335) d	(7.69 ± 0.352) f	(21.85 ± 1.609) c
8	1.53 ± 0.345	1.59 ± 0.079	ND	1.41 ± 0.068	2.56 ± 0.155	7.08 ± 0.505
	(4.77 ± 1.078) b,e	(4.95 ± 0.247) f		(4.39 ± 0.213)d	(8.00 ± 0.485) f	(22.10 ± 1.575) c

[a] Values in parentheses are expressed as μg/100 g plant sterols. Values are expressed as mean ± standard deviation of three replicates. ND: not detected. Different letters, in the same column, indicate significant differences ($p < 0.05$).

oil (2% of β-sitosterol oxidized), or those obtained by Grandgirard et al.,[32] with 0.08% POPs in PS-enriched spread.

The identification and quantification of oxides of β-sitosterol (7α/β hydroxy, α/β epoxy, and 7-keto) in microcrystalline phytosterol (from wood-based fractions) suspension in different fats/oils, stored at 4 °C during 12 months, have been evaluated by Soupas et al.,[16] the recorded oxidation rates of β-sitosterol ranging between 0.01% and 0.05%. Thus, the formation of POPs did not seem to be a limiting factor for the manufacture and subsequent storage of these products.

From the results obtained (low rate of oxidation) in the ingredients tested, we can conclude that the PS remain stable in these ingredients.

When each oxysitosterol was considered individually and expressed as μg/100 g PS, significant differences ($p < 0.05$) were found among ingredients (Table 5).

The large differences in total POPs contents and in the different oxide profiles found among the ingredients can be explained in terms of different obtainment processes, compositions, and physical states of the ingredients (powder, liquid) and the PS encapsulant materials, concentration, and chemical form (free or esterified to fatty acid). Because, these ingredients were acquired from different manufacturers, it is likely there were differences in the production processes used. In addition, we do not know whether they underwent storage before or after refining, or what the conditions of storage may have been. However, antioxidant presence does not seem to be related to lesser PS oxidation, because ingredients 5 and 8 present a high difference among them in POPs contents, ingredient 8 being one of the most oxidized samples (Table 5).

The main POPs found in the ingredients analyzed were derived from β-sitosterol (7α/β-hydroxysitosterol, 7-ketositosterol, and sitostanetriol). The presence of oxides originating only from β-sitosterol is attributed to the fact that it is the predominant sterol in all of the ingredients analyzed. Higher amounts of oxysitosterols have been detected in the refining of oils fractions, yielding greater amounts of β-sitosterol in comparison with the quantities of other sterols present.[10]

A significant correlation ($p < 0.01$) was found between total oxysitosterols versus β-sitosterol contents according to a double inverse model: total POPs = 1/(7.36825 + 4118.44/β-sitosterol), with correlation coefficient (R^2) = 86.5%. When total POPs and total PS are considered, significant correlation was also obtained ($p < 0.01$), the best adjustment corresponding to the exponential model (R^2 = 81.6%): total POPs = exp(−6.40025 + 0.029054 × PS).

7-Keto and 7-hydroxy derivatives were present in higher amounts than triol derivatives from β-sitosterol. This oxidative behavior agrees with the observations of previous studies of vegetable oils.[12] It has been suggested that in vegetable oils 7α-hydroxy, 7β-hydroxy, and 7-ketositosterol are the main oxides in the first stage of oxidation and start to decrease when other oxides are formed.[17] This suggests that the eight ingredients tested are all in the first oxidation stage. Additional studies in different food matrices would be useful for studying the oxidation behavior of each ingredient.

Sitostanetriol was found to be present in similar amounts in ingredients 3−8, while in ingredients 1 and 2 it was less abundant. These contents do not seem to be related to the source of PS. Triol derivatives have been detected in by-product obtained from chemical or physical refining of edible fats and oils.[10]

In ingredients 1 and 4 composed entirely of soy, 7α-hydroxy, 7-keto, and triol were the main POPs, followed by 7β-hydroxy. In the ingredients 5−8 that contain sunflower oil, 7-keto was the main POP, followed by 7β-hydroxy and 7α-hydroxy in similar amounts, except for ingredient 5, where the 7-hydroxy and 7-keto contents were lower and sitostanetriol was the most abundant. These profiles fall in the ranges found for sunflower oils.[13,15,17,24] A significant correlation ($p < 0.01$) between β-sitosterol and sitostanetriol contents was found according to a logarithmic model: Sitostanetriol = −0.440782 + 1.40817 × ln(β-sitosterol), with R^2 = 59.8%.

All of the ingredients analyzed in our study had undergone a refining process. It has been shown that during the process of bleaching, 7-ketositosterol is generated from 7-hydroxysitosterol.[24]

The deodorization does not appear to affect the total POP contents.[33] In this same sense, Rudzińska et al.[34] reported that in degumming, neutralization and bleaching of rapeseed oil increase the POP contents, the predominant representatives being the 7β-hydroxy derivatives and α-epoxy derivatives. Subsequent storage of the refined oil appears to have less of an effect upon POPs content. Bortolomeazzi et al.[13] studied the influence of steps of the refining process on the stability of POPs contents from crude sunflower oil rich in POPs: in bleaching, the 7-keto derivative proved to be the most stable, whereas the 7α/β-hydroxy derivatives were more stable on the deodorization step.

We have not detected epoxy derivatives in the ingredients analyzed. In sunflower oil, epoxy derivatives from β-sitosterol and campesterol were quantified by Dutta;[24] however, Zhang et al.[17] did not observe any epoxy and triol derivatives in sunflower, soybean, and rapeseed oils. In tripalmitin spiked with 1% β-sitosterol, only epoxy derivatives were detected when free β-sitosterol was added.[14]

Enrichment of foods with PS (saturated or unsaturated) is difficult, because PS are insoluble in water and only poorly soluble in dietary fats. Esterification of PS with fatty acids increases their lipid solubility in the fat phase of foods.[16] In our samples, only ingredient 5 contains esterified PS and constitutes one of the samples with the lowest oxidation rate (10.8 μg POPs/100 g PS) (Table 5). Ingredients 5 and 6, which come from the same PS source, have the same physical state but differ in PS esterification (Table 1); they have very different oxidation levels, oxidation being lower for esterified PS (ingredient 5). In this same sense, Soupas et al.[14] found a lower proportion of POPs in esterified phytosterols added to tripalmitin matrix than when free phytosterols are used for tripalmitin enrichment.

In conclusion, in view of the low content of POPs in the analyzed ingredients, we see that the PS remain stable in the samples. It should be noted that the PS source ingredients for food enrichment can have different origins, with different obtainment methods and storage conditions. These variables are not always known; as a result, we consider it of interest to evaluate the oxidation state of PS present in the ingredients used by the food industry as a source of PS for food enrichment.

■ ABBREVIATIONS USED

ANOVA, analysis of variance; COPs, cholesterol oxidation products; EU, European Union; GC, gas chromatographic; GC−FID, gas chromatography−flame ionization detector; GC−MS, gas chromatography−mass spectrum detector; HMDS, hexamethyldisilazane; IS, internal standard; LDL-cholesterol, low density lipoprotein-cholesterol; LOD, limits of detection; LOQ, limits of quantification; POPs, plant sterol oxidation products; PS, plant sterols; PTV injector, programmable temperature vaporization injector; R^2, correlation coefficient; Rf, response factor; RT, retention time; Si-SPE, silica solid-phase extraction; TLC, thin-layer chromatography; TMCS, trimethylchlorosilane; TMSE, trimethylsilyl ether.

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