Stability of Sterols in Phytosterol-Enriched Milk under Different Heating Conditions

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Commercially available phytosterol-enriched milk was subjected to usual and drastic heating conditions to evaluate the stability of the sterols at different treatments. Products showed 422.2 mg of phytosterols/ 100 g of milk and 132 μg of sterol oxidation products (SOPs)/g of fat (277 μg of SOPs/100 g of milk). Schaal oven conditions (24 h/65 °C, equivalent to 1 month of storage at room temperature) reduced the phytosterol content by only 4%. Drastic heating treatments (2 min of microwave heating at 900 W or 15 min of electrical heating at 90 °C) led to a 60% decrease of total phytosterol content, with a significant increase of TBARs. The oxysterol amount under those conditions (which was higher in microwave-treated samples) was lower than expected, probably because of the degradation of the oxidation products. Usual heating conditions (1.5 min of microwaves) maintained phytosterol content on physiologically active values (301 mg/100 g of milk) with oxidation percentages around 0.12–0.40% for phytosterols and 1.13% for cholesterol.

INTRODUCTION

Cholesterol is the main sterol in animal-derived products, and it is well established that increased serum cholesterol concentrations are positively and causally related to the risk of coronary disease (*1*). Phytosterols are natural plant sterols, β-sitosterol, stigmasterol, and campesterol being the most common ones. These compounds play an important role in the control of cholesterol serum levels (*2*), and their consumption at certain levels has been demonstrated to be useful for the treatment of hypercholesterolemia. Some studies have shown that a daily intake of 2 g of sterols or stanols could lead to a 25% reduction of the cardiovascular risk (*3*). Although most of the studies are made with spreads (*4, 5*), it has been also proved that plant sterol esters, when provided in low-fat phytosterol-enriched milk, are effective in lowering total and LDL-cholesterol (*6*).

Sterols are well-known to be susceptible to oxidation by reactive oxygen species, light, UV light, ionizing radiation, chemical catalysts, lipid hydroxyperoxides, and enzymatic reactions, leading to the formation of sterol oxidation products (SOPs) (*7−9*). Studies carried out in humans reveal that cholesterol oxidation products (COPs) could be absorbed from the diet (*10*). According to Plat et al. (*11*), the concentration of serum oxyphytosterols (POPs) in healthy control subjects is below the limit of detection, whereas other papers show the existence of noticeable quantities of these compounds in healthy volunteers (*12*). The toxicological effects of COPs have been well documented because of their wide range of adverse biological effects related to cytotoxicity, apoptosis, mutagenesis,

and carcinogenesis, the fact that they have been suggested to induce the development of atherosclerosis being especially important (*13, 14*). POPs are structurally related to COPs, so they are suspected to cause health damages comparable to COPs. There are only a few studies regarding the cytotoxic effects of POPs, which seem to be less severe than those observed for COPs (*15*), and there is no evidence of genotoxic effect in vivo for some of them (*16*). Anyway, it is clear that phytosterol oxidation products do not exert the beneficial effects of phytosterols, so it seems to be very convenient to know the content of sterol oxidation products in foods, especially those which have been subjected to technological or cooking processes, considering that heating treatments are known to generate oxidation processes.

Several studies about COP formation and content in different types of foods including milk and dairy products have been carried out (*17−19*). The findings obtained in recent years led to the conclusion that fresh milk and milk products contain little or no COPs, and their formation occurs under extreme conditions such as high temperatures for a long period or prolonged storage at high temperatures (*20, 21*). On the other hand, some research work attempting to find a proper methodology for the identification and quantification of POPs in vegetable oils, potato chips, and French fries (*22, 23*), in which phytosterols are naturally present, and milk products (*24*) and enriched spreads (*25*) has been performed.

Commercial spreadable fats, margarines, milks, and yogurts formulated with esterified phytosterols are available on the market, and only a few papers have dealt with the evaluation of the content, the stability of sterols, and the factors affecting the formation of SOPs in model systems or in these types of

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product (*26−28*). Furthermore, Cercaci et al. (*8*) showed that phytosterols are more prone to oxidation in oil in water emulsions than in bulk fat systems, as the oxidative stress is high in the emulsion droplets.

The aim of this study was to determine how different types and intensities of heat treatments may affect the intensity of lipid oxidation and especially the sterol content in phytosterol-enriched milks.

MATERIALS AND METHODS

Samples. Commercial milk enriched with phytosterols was purchased from the supermarket. Nutritional labeling on packages gives the following information: low-fat milk (98%), plant oils (sunflower and corn oil, 1.5%), sterol esters (0.5%), emulsifier (E-435), stabilizer (sodium phosphates and polyphosphates), vitamins E, A, and D. Nutritional content per 100 g serving: energy, 48 kcal/ 201 kJ; protein, 3.2 g; carbohydrate, 4.7 g (of which sugars are 4.7 g); fat (excluding phytosterols), 1.8 g; PUFA, 0.25 g; MUFA, 0.5 g; SFA, 1.05 g; plant sterols, 0.3 g; sodium, 0.15 g.

Reagents. 7α-Hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxycholesterol, 3,5,6-cholestanetriol, 25-hydroxycholesterol, 7-ketocholesterol (also named 7-oxocholesterol), and 19-hydroxycholesterol were purchased from Steraloids (Wilton, NH). The sterols were from Fluka (Buchs, Switzerland): stigmasterol (pure) and the mixture 60% β-sitosterol and 30% campesterol. Tri-Sil reagent was obtained from Pierce (Rockford, IL). Hexane for gas chromatography was from Merck & Co., Inc. (Whitehouse Station, NJ). Sep-Pak Vac 6 cm³ silica 1 g cartridges were obtained from Waters (Milford, MA). Acetone, ammonium, chloroform, diethyl ether, methanol, hexane, sodium sulfate anhydrous, petroleum ether, potassium hydroxide, and trichloroacetic acid were obtained from Panreac (Barcelona, Spain). Ethanol was from Oppac (Noain, Navarra, Spain). Cholesterol, 5α-cholestane, and 2-thiobarbituric acid were purchased from Sigma-Aldrich Chemical (Steinheim, Germany). Whatman 3 filters were obtained from Whatman (Maidstine, Kent, U.K.).

Accelerated Stability Test. To estimate the susceptibility of fat to oxidation, the sample was subjected to an accelerated oxidation test under standardized conditions. Phytosterol-enriched milk (240 mL) was added to a 250 mL flask, which was kept during 24 h in an oven under Schaal oven test conditions (65 °C). It has been established that 1 day of storage under this condition is equivalent to 1 month of storage at room temperature (*29*).

Cooking Procedures. Phytosterol-enriched milk was subjected to the following treatments: Two batches of phytosterol-enriched milk (240 mL) added to 250 mL glasses were heated in a Whirpool microwave oven (Whirpool Corp., Norrköping, Sweden) at 900 W during 1.5 and 2 min, respectively. A third batch (240 mL), added to a 250 mL glass, was cooked on an electrical heating plate unit for 15 min. Before heating, samples were kept at room temperature, resulting in a mean core temperature of the milk samples of 22.51 ± 0.66. Internal temperature of samples was measured with a digital thermometer after every heating treatment (51 J/7 RS 614-299, Fluke), resulting in the following mean values: 69 °C for the 1.30 min microwave heated samples, 83 °C for the 2 min microwave heated samples, and 87 °C for the electrically heated samples. Every heating treatment was applied in triplicate, and analysis of every sample was made in duplicate.

Fat Extraction. Phytosterol-enriched milk was previously lyophilized to yield a good extraction of the fat content needed for the analysis of SOPs. Lyophilization was performed on a freeze-dryer-cryodo (Telstar, Barcelona, Spain) with refrigerating compressor and vacuum pump. Milk lyophilized samples were kept in hermetical recipients and stored at −20 °C until their analysis. Fat extraction was made by using the Röse−Gottlieb method according to the Association of Official Analytical Chemists (AOAC) method (*30*).

Fat Content. Quantitative analysis of the fat content was determined by using the Gerber method according to the AOAC method (*31*).

Thiobarbituric Acid Reactive Substances (TBARs). The measurement of TBARs was performed using a slight modification of the method originally developed by King (*32*). Phytosterol-enriched milk (17.6 mL) was prewarmed to 30 °C using a Tembloc (P Selecta,

Barcelona, Spain). After that, milk samples were precipitated by the addition of 1 mL of 1 g mL⁻¹ trichloroacetic acid (TCA) and 2 mL of 95% ethanol. Following 5 min of incubation at 30 °C, the precipitate was removed by filtration through a Whatman 3 filter paper, and 1.4% 2-thiobarbituric acid solution (1 mL) was added to the resulting filtrate (4 mL). This was then incubated for 60 min at 80 °C in the Tembloc and cooled, and the absorbance was read at 531 nm using a Perkin-Elmer spectrophotometer (Lambda 5-UV−vis, Paris, France). Results are shown in milligrams of malonaldialdehyde per kilogram of sample (parts per million, ppm). Quantification was performed by using a calibration curve with tetraethoxypropane (TEP) as standard for malondialdehyde. Recovery (percent) of the entire method was found to be 95.9 ± 1.8%, using an addition level of 0.13 ppm of TEP, and this was taken into account to express final results.

Sterols. Sterol compounds were extracted and derivatized to form trimethylsilyl (TMS) ethers according to the method of Kovacs et al. (*33*). Gas chromatography−FID analysis was performed on a Perkin-Elmer Autosystem XL gas chromatograph equipped with an HP1 column (30 m × 0.25 mm × 0.1 μm). The carrier gas was hydrogen. The oven was programmed with an initial temperature of 210 °C, heated to 270 °C at a rate of 10 °C/min, raised to 290 °C at rate of 2 °C/min, and, finally, maintained at 290 °C during 10 min. The temperature of both the injector port and the detector was 285 °C. Sterols were identified by comparing their retention time with those of the standards derivatized in the same manner as the samples. Quantification was done by using pure α-cholestane as an internal standard, which was added to the sample as a solution (2 mg/mL) prior to the extraction procedure. A Perkin-Elmer Turbochrom software program was used for integration and quantification.

SOPs. The methodology used was that described in Menéndez-Carreño et al. (*34*) that enables the quantification of COPs and POPs simultaneously. Briefly, the extracted fat (0.5 g), 19-hydroxycholesterol (20 μg/ml, 1 mL) as internal standard, and 1 N KOH solution in methanol (10 mL) were subjected to a cold saponification at room temperature for 20 h, in darkness and under continuous agitation in an orbital shaker (Rotaterm, P Selecta, Barcelona, Spain) at 100 rpm. The unsaponifiable material was extracted with diethyl ether and purified by silica SPE according to method III described in Guardiola et al. (*35*). COPs were finally eluted from the cartridge with acetone. SOPs were derivatized to TMS ethers according to a modified version of the method described by Dutta and Appelqvist (*22*). After the solvent had been dried, Tri-Sil reagent (400 μL) was added, and the tubes were kept at 60 °C for 45 min. The solvent was evaporated under a stream of nitrogen, and the TMS-ether derivates were dissolved in hexane (400 μL). Dissolved samples were filtrated prior to GC-MS analysis to avoid the damage of the capillary column. Gas chromatography−mass spectrometry analysis was performed on a GC 6890N Hewlett-Packard coupled to a 5975 mass selective detector (Agilent Technologies, Inc., Palo Alto, CA), following the conditions described in Menéndez-Carreño et al. (*34*). Identification of the peaks was made by the characteristic ion fragmentation of the standard substances and by comparison of their retention times with those of compounds previously synthesized (*25*); the quantification was made using a select ion monitoring program using the internal standard method and the corresponding calibration curves for each SOP (*34*).

To evaluate the recoveries of the different oxides during the analytical procedure of SOP determination in phytosterol-enriched milk, three spiking levels were tested. A known mixture of standard compounds (1 mL, 0.01 μg mL⁻¹, 1.25 μg mL⁻¹, 40 μg mL⁻¹) was added to milk fat (0.5 g). The fat samples were processed following the entire procedure above, and finally SOPs were analyzed by GC-MS. The recovery was calculated according to

$$\%R = \frac{M_{fc} - M_c}{M_f} \times 100 \qquad (1)$$

where $\%R$ is percent recovery, M_{fc} is the raw amount in μg of compound determined in the fortified sample, M_c is the raw amount in μg of compound in the fortified material, and M_f is the fortification amount in μg.

Table 1 reports the recoveries obtained, which ranged between 96.3% for 5,6α-epoxycholesterol and 105.1% for 25-hydroxycholes-

Sterols in Heated Phytosterol-Enriched Milk

J. Agric. Food Chem., Vol. 56, No. 21, 2008 **9999**

Table 1. Recovery Data at Three Spiking Levels and Final Mean Values for Every Sterol Analyzed

oxycholesterol	sample (μg mL^{-1})	spiked sample (0.01 μg mL^{-1})		spiked sample (1.25 μg mL^{-1})		spiked sample (40 μg mL^{-1})		recovery mean (%)	CV (%)
		data (μg mL^{-1})	% recovery	data (μg mL^{-1})	% recovery	data (μg mL^{-1})	% recovery		
7α-hydroxycholesterol	0.005	0.015	96.4	1.305	104.0	40.620	101.5	100.6	3.8
7β-hydroxycholesterol	0.005	0.014	97.1	1.257	100.2	41.453	103.6	100.3	3.2
5,6β-epoxycholesterol	0.005	0.016	104.8	1.355	108.0	39.992	100.0	104.2	3.9
5,6α-epoxycholesterol	0.003	0.012	95.5	1.166	93.1	40.135	100.3	96.3	3.8
cholestanetriol	0.003	0.014	102.8	1.243	99.2	37.974	94.9	99.0	4.0
25-hydroxycholesterol	nd	0.010	102.9	1.291	103.3	43.663	109.2	105.1	3.3
7-ketocholesterol	0.005	0.015	97.4	1.245	99.2	42.649	106.6	101.0	4.8

Table 2. Sterol Content in Control Milk Samples and Those Treated under Different Heating Conditions (Milligrams per 100 g of Milk) ($n = 6$)[a]

sterol	control	Schaal oven (65 °C, 24 h)	microwave (900 W, 1.5 min)	microwave (900 W, 2 min)	electrical heating (15 min)
cholesterol	17.6 ± 0.0d	17.3 ± 0.0d	14.7 ± 0.1c	9.9 ± 0.2a	10.5 ± 0.1b
campesterol	68.7 ± 0.2e	66.1 ± 0.2d	50.3 ± 1.5c	23.3 ± 0.7a	26.9 ± 0.4b
campestanol	10.9 ± 0.0e	10.7 ± 0.0d	7.6 ± 0.2c	8.1 ± 0.2a	8.5 ± 0.2b
stigmasterol	11.3 ± 0.0d	11.1 ± 0.0d	8.1 ± 0.4b	7.4 ± 0.2a	8.5 ± 0.4c
sitosterol	288.9 ± 0.5e	277.1 ± 0.3d	203.2 ± 0.3c	85.8 ± 3.5a	101.7 ± 0.5b
sitostanol	42.3 ± 0.2e	39.9 ± 0.1d	31.5 ± 0.1c	18.2 ± 0.9a	21.9 ± 0.7b
Σ phytosterols	422.2 ± 0.8e	404.8 ± 0.5d	301.2 ± 4.5c	142.8 ± 5.1a	167.5 ± 1.2b
Σ sterols	439.7 + 0.8e	422.1 ± 0.55d	315.9 ± 4.4c	152.7 ± 4.875a	178.0 ± 1.3b

[a] Within the same row, different letters indicate significant differences among treatments ($p < 0.05$). nd, not detected.

terol, with an average of 100.9%. According to these values, it can be stated that the analytical procedure applied does not alter the stability of SOPs during the analysis.

The percentage of oxidation of sterols was calculated by taking into account the total content of the oxyderivatives of each sterol after each heat treatment in relation to the content of the corresponding sterol after this treatment (sterol oxiderivatives/sterol) × 100.

Statistical Analysis. All results are expressed as mean ± standard deviation of the mean. The differences between the groups were evaluated by one-way ANOVA, and the Tukey b post hoc test was applied when suitable (SPSS 15.0 packages for Windows, Chicago, IL). A level of probability at $p < 0.05$ was set as statistically significant. A Pearson correlation test was performed between TBARs and oxysterol content in the phytosterol-enriched milks subjected to the different heating treatments.

RESULTS AND DISCUSSION

Results shown in the tables for each condition studied are the mean of the six results obtained. **Table 2** shows the amount of the different sterols analyzed summing to 422.15 mg/100 g of milk for phytosterols in untreated samples (control). β-Sitosterol was the most abundant sterol, representing 68.45% of the total phytosterols. The Commission Decision of March 31, 2004 (*36*), specifies the percentages of the different sterols of the mixtures used to enrich milk and yogurt products, allowing values up to 80% for β-sitosterol. Campesterol and stigmasterol were present at 16.27 and 22.68%, respectively. The only stanols detected were sitostanol (10.03%) and campestanol (2.59%).

The stability of phytosterols during the shelf life of milk seemed to be guaranteed by the antioxidants (such as vitamin E) included in the formulation. Those data were confirmed by the results obtained for the samples treated under Schaal oven conditions, equivalent to 1 month of storage at room temperature, showing values 4% lower than the control samples for total phytosterol content. In contrast, as could have been expected, the application of heat treatment affected the stability of sterols. Thanh et al. (*37*) analyzed the stability of phytosterols in vegetable oils and found that heating at 200 °C led to a 50−60% decrease of phytosterols. A decrease of phytosterols has also been found in the present work. Electrical heating of

milk during 15 min at 90 °C led to a 60% decrease of total phytosterol (**Table 2**). β-Sitosterol and campesterol were the most affected ones, decreasing 65 and 61%, respectively, followed by sitostanol, which decreased 48%, and stigmasterol and campestanol, which decreased 25 and 22%, respectively. The application of microwaves during 2 min gave rise to similar results, confirming that a drastic heating treatment significantly decreased the presence of phytosterols. When the microwave application time was reduced to 1.5 min, the negative effect on the stability of sterols was also reduced, with percentages of reduction around 30% compared to control. The usual cooking conditions for milk are normally those corresponding to this last treatment, or equivalent.

Cholesterol was similarly affected. Thus, drastic heat treatments (microwave during 2 min or electrical heating during 15 min) resulted in decreases of around 40%, whereas a usual heating (microwave during 1.5 min) gave rise to a 16% cholesterol reduction.

The main mechanism for phytosterol degradation is known to be the oxidation process. Other mechanisms are also involved, which affect phytosterols at a similar intensity as cholesterol. Regarding TBARs data, the Schaal oven conditions did not increase lipid oxidation, values (0.08 ppm) similar to those observed for control samples being obtained (**Table 3**). These results were in agreement with the fact that the stability of sterols was well guaranteed during the shelf life of the product. The heat treatment produced, as expected, a significant increase of TBARs according to the following sequence: microwave 2 min > electrical heating > microwave 1.5 min, obtaining the following values, respectively, 0.23, 0.16, and 0.14 ppm. A negative and significant Pearson correlation between TBARs and total phytosterols was found (-0.95; $p < 0.001$). On the contrary, significant positive correlations between TBARs and total COPs (0.75; $p < 0.001$) and total POPs (0.57; $p < 0.01$) were obtained. Previous papers have demonstrated that the formation of COPs is a good index to detect slight oxidation processes (*17*). Furthermore, the formation of epoxidized sterol derivatives proceeds readily in contact with fatty acid hydroperoxides, thus explaining the correlation between the second

Table 3. Oxysterol Content (Micrograms per Gram of Fat) in Control Milk Samples and Those Treated under Different Heating Conditions ($n = 6$)[a]

	control	Schaal oven (65 °C, 24 h)	microwave (900 W, 1.5 min)	microwave (900 W, 2 min)	electrical heating (15 min)
7α-hydroxycholesterol	5.38 ± 0.21d	2.69 ± 0.53b	5.61 ± 0.23d	4.68 ± 0.20c	2.25 ± 0.15a
7β-hydroxycholesterol	5.09 ± 0.55a	14.25 ± 1.30e	10.80 ± 0.37c	12.17 ± 0.31d	7.76 ± 0.12b
5,6β-epoxycholesterol	5.98 ± 0.55a	6.12 ± 0.25a	10.46 ± 0.49b	11.24 ± 1.83b	9.39 ± 0.51b
5,6α-epoxycholesterol	4.74 ± 0.21b	1.70 ± 0.17a	7.80 ± 1.41d	9.25 ± 0.43e	6.30 ± 0.27c
cholestanetriol	5.92 ± 0.33c	2.14 ± 0.35a	5.64 ± 0.50c	5.65 ± 0.04c	3.01 ± 0.18b
25-hydroxycholesterol	nd	nd	5.82 ± 0.58a	4.74 ± 4.57a	3.02 ± 0.31a
7-ketocholesterol	5.16 ± 0.16a	7.59 ± 0.65ab	32.82 ± 3.10c	33.43 ± 0.37c	9.14 ± 0.48b
total oxycholesterols	32.27 ± 0.85a	34.49 ± 2.51a	78.94 ± 1.50c	81.16 ± 5.02c	40.87 ± 0.41b
7α-hydroxysitosterol	2.98 ± 0.12a	12.95 ± 0.53d	7.65 ± 1.17b	8.89 ± 0.33c	8.18 ± 0.15c
7β-hydroxysitosterol	18.56 ± 1.85a	21.00 ± 1.50b	34.88 ± 0.72d	26.86 ± 0.90c	18.15 ± 0.98a
5,6β-epoxysitosterol	10.12 ± 0.93b	8.74 ± 0.50a	19.35 ± 0.09d	12.36 ± 0.65c	11.04 ± 0.34b
5,6α-epoxysitosterol	9.03 ± 0.48a	7.55 ± 0.64a	17.29 ± 0.97c	12.48 ± 1.46b	11.96 ± 1.01b
sitostanetriol	2.36 ± 0.68a	6.74 ± 0.13b	13.76 ± 0.76d	12.87 ± 0.22d	10.55 ± 0.60c
25-hydroxysitosterol	nd	nd	nd	nd	nd
7-ketositosterol	13.38 ± 1.15a	18.00 ± 1.68b	25.09 ± 1.21d	19.09 ± 0.55bc	21.14 ± 1.50c
total oxysitosterols	56.43 ± 4.62a	74.98 ± 4.14b	118.02 ± 2.25d	92.25 ± 2.37c	81.02 ± 1.36b
7α-hydroxycampesterol	3.84 ± 0.41a	5.90 ± 0.32b	9.63 ± 0.45d	7.85 ± 0.23c	6.72 ± 0.18b
7β-hydroxycampesterol	7.96 ± 0.69a	14.52 ± 0.74b	17.31 ± 0.91c	15.86 ± 0.25b	14.48 ± 0.17b
5,6β-epoxycampesterol	7.67 ± 0.39a	13.12 ± 0.96b	18.55 ± 1.17c	13.19 ± 2.10b	15.25 ± 0.64b
5,6α-epoxycampesterol	7.35 ± 0.19a	9.40 ± 0.80ab	14.11 ± 1.23b	9.93 ± 5.15ab	9.59 ± 0.41ab
campestanetriol	2.03 ± 0.17a	1.78 ± 0.08a	6.58 ± 0.43d	4.99 ± 0.10c	3.28 ± 0.24b
25-hydroxycampesterol	nd	nd	nd	nd	nd
7-ketocampesterol	11.97 ± 1.01a	21.61 ± 1.65b	29.51 ± 0.66c	28.47 ± 0.69c	20.61 ± 1.16b
total oxycampesterols	40.83 ± 2.63a	66.33 ± 4.09b	95.70 ± 0.99d	80.29 ± 3.08c	69.93 ± 1.36b
7α-hydroxystigmasterol	0.28 ± 0.03a	1.11 ± 0.04d	1.01 ± 0.02c	0.97 ± 0.05c	0.90 ± 0.04b
7β-hydroxystigmasterol	2.51 ± 0.10a	4.47 ± 0.21b	11.04 ± 0.50d	10.74 ± 1.27d	7.79 ± 0.05c
5,6β-epoxystigmasterol	nd	nd	nd	nd	nd
5,6α-epoxystigmasterol	nd	nd	nd	n.d	nd
stigmastanetriol	nd	nd	nd	nd	nd
25-hydroxystigmasterol	nd	nd	nd	nd	nd
7-ketostigmasterol	nd	nd	nd	nd	nd
total oxystigmasterols	2.79 ± 0.09a	5.58 ± 0.23b	12.05 ± 0.21d	11.71 ± 0.25d	8.69 ± 0.07c

[a] Within the same row, different letters indicate significant differences among treatments ($p < 0.05$). nd:, not detected. Detection limit is below 3.09 ng mL^{-1}, and quantification limit is below 7.76 ng mL^{-1}. For detailed values for each compound, see ref *34*.

oxidation products detected by TBARs and the sterol oxidation products (*38*).

Table 3 presents results for every oxysterol found in the samples analyzed in this work. The amounts of total COPs and POPs in control samples were 32 and 100 μg/g of fat, respectively. These values imply intakes of COPs and POPs around 67 and 210 μg/100 g of milk, respectively. Soupas et al. (*39*), analyzing the oxidative stability of phytosterols in nonfat heat-treated milk enriched with 0.5% phytosteryl esters during its storage in different conditions (room temperature and 4 °C), found values between 203 and 131 μg of oxidized POPs/100 g of milk. In previous works with phytosterol-enriched spreads, values around 105.7 μg/g of fat were obtained (*25*). Other papers showed no (*40*) or only a small formation of COPs (*41*) in milk heated under pasteurization or ultrahigh-temperature conditions. Scopesi et al. (*42*) found levels of 7-ketocholesterol in adapted milk formulas of about 3.6 μg/g of fat, and 4.76 μg/g fat was found for this compound in liquid growth milks (*43*).

Schaal oven conditions, which did not affect TBARs, led to an increase of total oxysterol derivates in relation to the amount found in control samples, although in the case of cholesterol, it was not statistically significant. These results are again in agreement with the fact that oxysterols are sensitive markers of oxidation processes.

Samples subjected to microwave conditions during 1.5 min showed the highest oxysterol level (304.71 μg/g of fat), greater than those shown by the samples microwaved for 2 min (265 μg/g of fat) and by the electrically heated samples (200 μg/g of fat). A possible explanation of these results could be the degradation of sterols into other compounds and the destruction of sterol oxides after their formation owing to the use of prolonged drastic heating conditions. Oehrl et al. (*44*) found that, as the temperature increased, fewer phytosterol oxides were recovered, implying that the extreme heat treatments resulted in the total degradation of sterol complexes. Sterols may have also been broken down to components smaller than oxides, which were not detectable by GC-MS. These authors also stated that the greatest degradation of the oxides occurred in the most saturated oils, resulting in lower recovery of both intact sterols and sterol oxides. Kim and Nawar and Chien et al. (*45, 46*) analyzed the parameters influencing cholesterol oxidation and pointed out that total altered cholesterol did not correspond to the sum of the oxidation products usually detected by GC-MS, being also formed polymers and/or other compounds of relatively high molecular weight. Furthermore, heating sterols and their oxidation products can generate other oxygenated compounds such as conjugated dienes and trienes (*47*). Dehydration processes can also lead to the formation of this type of

Sterols in Heated Phytosterol-Enriched Milk

J. Agric. Food Chem., Vol. 56, No. 21, 2008 **10001**

Table 4. Oxidation Percentages Obtained for Each Type of Sterol in Control Milk Samples and Those Treated under Different Heating Conditions ($n = 6$)

sterol type	control	Schaal oven (65 °C, 24 h)	microwave (900 W, 1.5 min)	microwave (900 W, 2 min)	electrical heating (15 min)
total COPs	0.39	0.42	1.13	1.73	0.81
total oxysitosterols	0.04	0.06	0.12	0.22	0.17
total oxycampesterols	0.12	0.21	0.40	0.72	0.55
total oxystigmasterols	0.05	0.10	0.31	0.33	0.21

compound, such as occurs to hydroxyphytosterols during bleaching of vegetable oils at 80 °C (*48*).

Cercaci et al. (*8*) pointed out that the order of susceptibility to oxidation was stigmasterol > cholesterol > β-sitosterol in oil in water emulsions, due to their differences in their surface activity. In milk-based infant foods, the extent of stigmasterol oxidation (2.9%) was higher than that of cholesterol (1.9%) and β-sitosterol (1.4%) (*49*). In our work, the oxidation percentages obtained for each type of sterol (oxysterols/sterols × 100) (**Table 4**) showed that β-sitosterol and stigmasterol were the compounds least affected by the oxidation process, cholesterol being the one with the highest oxidation percentages in every sample. Maeker (*50*) also pointed out that cholesterol oxidizes at a higher rate than phytosterols. Anyway, the highest amounts of POPs in every analyzed sample were derived from β-sitosterol, as happened in samples analyzed by Tabee et al. (*51*) due to, as those authors pointed out, the fact that this sterol was the most abundant one in all of the samples. The low oxidation percentages found for the drastically heated samples can be explained by the previously cited hypothesis of oxyderivative degradation.

By comparison of total COPs and POPs in samples heated for 2 min by microwaves (81 and 184 μg/g of fat, respectively) and electrically heated samples (41 and 160 μg/g of fat, respectively) an enhancement of the oxidation process by microwave heating could be pointed out. Oxidation percentages were also higher in microwaved samples. Echarte et al. (*18*) also observed a higher effect of microwave heating than other treatments in COP formation in meat patties. Greater alterations in microwave-heated samples of edible fats were detected in comparison to their corresponding samples heated in a conventional oven (*52, 53*). A similar conclusion was drawn by Farag et al. (*54*), working with refined cottonseed, who pointed out that samples heated by microwave developed rancidity twice as quickly as samples produced by conventional heating.

In summary, commercial phytosterol-enriched milk constitutes an adequate phytosterol source even when it is heated for consumption. However, when the heating conditions are too drastic, there is a significant decrease of sterol content without a corresponding increase of SOPs, probably because the oxidation products are also broken down. This work reveals the importance of the cooking conditions in the stability of sterols, further studies being necessary to control the optimal conditions to guarantee the adequate intake of phytosterols.

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10002 *J. Agric. Food Chem.,* Vol. 56, No. 21, 2008

Menéndez-Carreño et al.

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