Determination of Cholesterol Oxides in Dairy Products. Effect of Storage Conditions

Antonio J. Angulo,*[,†,‡] Juan M. Romera,[†] María Ramirez,[†] and Angel Gil[†,§]

Department of Research and Development, Abbott Laboratories, Granada, Spain, and Department of Food Science and Nutrition and Department of Biochemistry and Molecular Biology, University of Granada, Granada, Spain

A methodology to quantify cholesterol oxidation products (COPS) in dairy products has been established by gas−liquid chromatography (GLC). Lipids were extracted according to the Folch method and saponified at room temperature using sodium hydroxide in methanol/benzene (3:2, v/v). Cholesterol oxides were derivatized to trimethylsilyl ethers and resolved by GLC, confirming the structures of cholesterol oxides detected in selected samples by GLC/MS. This methodology allows quick, simple, and reliable quantification of free and esterified cholesterol oxidized derivatives. This method was applied to milk powders, butter oil, and isolated milk protein powders. Effects of adverse storage conditions were studied in whole and skim milk powders. Storage temperature severely affected levels of COPS even under mild storage conditions. Packaging under nitrogen retarded cholesterol oxidation. Slight differences in COPS profiles were observed between different storage conditions. 7-Ketocholesterol was the major cholesterol oxide formed. A good correlation between thiobarbituric acid reactive substances and COPS concentration was observed.

Keywords: *Cholesterol oxides; dairy products storage; lipid oxidation; TBARS*

INTRODUCTION

As an unsaturated lipid, cholesterol is susceptible to oxidation under a variety of conditions. In contact with air, it is autoxidized, forming cholesterol hydroperoxides, from which >30 secondary oxidation products, named COPS (cholesterol oxidation products), have been reported (Smith, 1992). COPS have received much scientific attention due to their undesirable implications in human health, such as inhibition of cholesterol biosynthesis, alteration of membrane function, cytotoxicity, and factoring in atherosclerosis (Smith and Johnson, 1989). Humans are capable of absorbing cholesterol oxides from foods into the bloodstream (Emanuel et al., 1991).

Foods containing cholesterol, particularly those that have been exposed to heat and air during processing or shelf life, could contain autoxidation products of cholesterol (van de Bovenkamp et al., 1988). Dairy products are fairly resistant to cholesterol autoxidation, even after a prolonged period under adverse conditions. The basis for this resistance probably stems from the following three characteristics: low transition metal content, modest cholesterol level, and an environment of highly saturated fat (Addis and Park, 1992). The drying technology exerts a major influence on the levels of cholesterol oxides in milk powders both directly after drying and upon storage (Appelqvist, 1996).

Many different methods have been used for the analysis of cholesterol oxides in foods. Actually, very few validation data have been presented by most published methods (McCluskey and Devery, 1993), and the need for a method of standardization was also recently stressed (Paniangvait et al., 1996; Appelqvist, 1996). Analytical methods to quantify COPS in foods include extraction of total lipids, followed by enrichment, separation, and detection of COPS. The most controversial step is the enrichment in cholesterol oxides, which is commonly implemented by saponification or chromatography. Hot saponification causes extensive destruction of 7-ketocholesterol, a predominant product of cholesterol oxidation (Park et al., 1996). Cold saponification requires an overnight incubation and thus 2 days for analysis. This analysis time makes it difficult to apply cold saponification in food quality control and raw material screening in food manufacturing. Cleanup column chromatography does not measure cholesterol and its oxides in the form of fatty acid esters if a previous saponification is not used. The effects of a cold transesterification as described by Zubillaga and Maerker (1988) on COPS standards have been tested but its suitability in samples has not been established.

The main purpose of this study was to develop a method for the quantitation of COPS in dairy products, which allows quick and reliable quantitation of both free and esterified COPS. This method was used to quantify cholesterol oxides in whole and skim milk powders, butter oil, and isolated casein and lactoalbumin proteins and to determine the effects of storage conditions on cholesterol and fatty acid oxidation in skim and whole milk powders.

MATERIALS AND METHODS

Reagents and Chemicals. Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from Fluka Chemie (Buchs, Switzerland). 5α-Cholestane, cholesterol, cholesteryl oleate, 7β-hydroxycholesterol, cholesterol 5,6-α-epoxide, cholestanetriol, 6-ketocholestanol, 7-ketocholesterol, 25-hydroxycholesterol, tocopherol standards, tetraethoxypropane (TEP), and fatty acid standards were from Sigma Chemical Co. (St. Louis, MO). 7α-Hydroxycholesterol was from Steraloids Inc. (Wilton, NH).

* Address correspondence to this author at Abbott Laboratories, R&D Department, Camino de Purchil 68, 18004 Granada, Spain (telephone 34-58-248655; fax 34-58-248660).

† Department of Research and Development.

‡ Department of Food Science and Nutrition.

§ Department of Biochemistry and Molecular Biology.

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Table 1. RRFs of Cholesterol Oxides

	linear regression			average RRFs		
	slope	intercept	*r*	mean	STD	CV
7α-hydroxycholesterol	1.156	0.010	0.9998	1.042	0.036	3.72
7β-hydroxycholesterol	0.971	0.001	0.9999	0.987	0.030	3.04
α-epoxide	0.977	0.040	0.9993	0.973	0.051	5.25
cholestanetriol	1.065	0.012	0.9999	1.095	0.047	5.18
6-ketocholestanol	0.958	0.002	0.9999	0.940	0.071	7.57
7-ketocholesterol	1.070	0.020	0.9999	1.099	0.022	2.46
25-hydroxycholesterol	1.042	0.027	0.9999	1.042	0.040	4.20

Samples. Whole milk powder (WMP), skim milk powder (SMP), butter oil, isolated casein protein powder (ICP), and isolated lactoalbumin protein powder (ILP) were obtained from national suppliers. Milk and protein powder were obtained by spray-drying. SMP and WMP were stored for 1 year in the dark in closed bottles at 32 and 55 °C under nitrogen or air, designated 32N, 32A, 55N, and 55A. Three replicate analyses were performed at 0, 1, 3, 6, 9, and 12 months.

Sample Pretreatment for COPS Quantification. Forty micrograms of the internal standard, 5α-cholestane, was added to 1.000 ± 0.005 g of powder sample diluted in 5 mL of water. Forty-five milliliters of Folch reagent, chloroform/methanol (2:1, v/v), was added and mixed for 45 s. The mixture was centrifuged at 1500*g* at 10 °C. After centrifugation, the lower layer was evaporated under a nitrogen stream. The residue was dissolved in 10 mL of a 4% sodium hydroxide in methanol/benzene (3:2, v/v) solution, held for 2 h at room temperature. Water (20 mL) was added, mixed, and held until two layers were formed. Ethyl ether (10 mL) was added to the mixture, which was mixed again for 30 s and centrifuged at 1500*g* for 10 min at 5 °C. After centrifugation, the lower layer was reextracted with 10 mL of ethyl ether. The upper layers were combined and washed with 5 mL of a 0.1 N sodium hydroxide solution, mixed, and centrifuged at 1000*g* for 15 min at 5 °C. The upper layer was washed with water until neutral pH was reached and evaporated under a nitrogen stream. The residue was dissolved in 200 μL of a pyridine/HMDS/TMCS (5:2:1) solution and formed trimethylsilyl (TMS) ether derivatives of the COPS after 20 min at room temperature.

Hot saponification was used as reference method to evaluate the release of esterified cholesterol from cholesteryl oleate and from hypercholesterolemic plasma, which contained about 75% of its cholesterol content in the esterified form. Forty micrograms of internal standard, 5α-cholestane, was added to 50, 100, 150, and 200 μg of cholesteryl oleate or 100 μL of plasma. They were dissolved in 2 mL of methanol, and 1 mL of 1.0 N potassium hydroxide in methanol was added. The mixture was mixed and held for 2 h at 100 °C. After cooling, 2 mL of water was added and cholesterol was extracted with 2 mL of ethyl ether. Samples were derivatized to TMS ethers as described above. A Student *t* test was used to evaluate the difference between cholesterol concentrations found in hypercholesterolemic plasma analyzed following both methods.

Chromatographic Assay/Mass Spectrometry for COPS Quantification. A gas−liquid chromatograph (Hewlett-Packard, Avon, PA) Model HP5890 with flame ionization detector was used. Data were processed with HPChem Station software (Hewlett-Packard). A 30 m × 0.2 5mm i.d. SPB-1 capillary column with 0.25 μm film thickness (Supelco Inc., Bellefonte, PA), operated with nitrogen carrier gas (column flow rate = 1 mL/min) was used for the separation of TMS ethers. The oven temperature was programmed as follows: initial temperature, 250 °C for 2 min, 2.5 °C/min to 265 °C, hold 2 min, 1 °C/min to 281 °C, and 2.5 °C/min to 300 °C. The injection and detector temperatures were held at 300 and 310 °C, respectively. Two microliters of COPS TMS derivatives was injected onto the column with a split ratio of 30:1.

A mass spectrometer system (Varian Associates Inc., Harbory City, CA) consisting of a GLC chromatograph, Model STAR3400CS, equipped with a mass selective detector, SATURN-4D, was employed to confirm the structures of cholesterol oxides detected in selected products. GC/MS analysis was performed with the same capillary column and conditions used in routine GLC analysis. Spectra were obtained by electron impact ionization within a mass range of *m*/*z* 100−600. Background subtraction and renormalization were performed. Peaks were identified by comparing their mass spectra with those of pure compounds.

Determination of Fatty Acids. To obtain the total fatty acid composition of milk powder, 350 μg of tripentadecanoine was added as internal standard to 50 mg of milk powders. Fatty acid methyl esters were formed according to the method of Lepage and Roy (1986). A gas−liquid chromatograph Model HP-5890 with a flame ionization detector was used to resolve and quantify fatty acids as methyl esters. Chromatography was performed using a 60 m × 0.3 mm i.d. SP-2330 column with 0.2 μm film thickness (Supelco Inc.). The injector and detector were maintained at 275 and 275 °C, respectively; nitrogen was used as carrier gas, and the split ratio was 29:1. Temperature programming was as follows: initial temperature, 80 °C, 15 °C/min to 165 °C, 3 °C/min to 211 °C, hold 10 min.

Determination of TBARS. Milk samples diluted to 10% (p/v) in water and 1% thiobarbituric acid solution in 5% thrichoroacetic acid in water were mixed 1:1 (v/v). The mixture was shaken and held for 1 h at 80 °C. After cooling, it was centrifuged and thiobarbituric acid reactive substances (TBARS) were quantified by fluorimetry with excitation and emission wavelength at 515 nm and 553 nm, respectively. Tetraethoxypropane (TEP) was used as standard for malondialdehyde (MDA).

RESULTS AND DISCUSSION

Isolation and Analysis of COPS. Several sources of error may be involved in the quantitative determination of cholesterol oxides in foods, such as destruction of certain compounds and generation of others during extraction, separation, and determination (Nourooz-Zadeh and Appelqvist, 1988a). For this study, the following precautions were taken: working in dim light and performing the isolation of COPS rapidly without interruption, as has been recommended by some authors (Nourooz-Zadeh and Appelqvist, 1988a).

Complete separation of COPS tested was achieved by nonpolar capillary column gas chromatography, allowing the separation of cholesterol, 7α- and 7β-hydroxycholesterol, α-epoxide, cholestanetriol, 6-ketocholestanol, 7-ketocholesterol, and 25-hydroxycholesterol standards as their TMS ethers, as has been published by other authors using a similar kind of column (Guardiola et al., 1995). 5α-Cholestane has been extensively used as internal standard in COPS analysis, although other internal standards are used when 5α-cholestane coelutes with other peaks (Lai et al., 1995). Under our conditions, there were no interfering peaks at the retention time of 5α-cholestane. The oxysterol 6-ketocholestanol was not used as internal standard because it has been described among COPS present in foods (Hwang and Maerker, 1993).

Table 1 shows the response factor relative to that of the internal standard (RRF), calculated as area ratio plotted against weight ratio. In addition, the mean RRFs obtained at different weight ratios (average

Table 2. Recovery of Cholesterol Oxides Added to Milk Powder[a]

	% recovered at addition of									
	0.2 ppm	0.5 ppm	1 ppm	2 ppm	5 ppm	10 ppm	20 ppm	100 ppm	mean	SD
7αOH	92	98	107	106	98	99	104	103	100.9	5.0
7βOH	95	96	99	102	92	101	103	103	99.4	3.3
αEPX	102	91	101	113	102	101	99	96	100.6	6.3
TRIOL	90	92	104	107	98	103	101	102	99.6	5.9
6KETO	92	95	95	106	108	111	104	92	100.4	7.7
7KETO	95	104	105	102	101	102	105	103	102.1	3.2
25OH	95	92	96	105	95	106	103	95	98.4	5.4

[a] 7αOH, 7α-hydroxycholesterol; 7βOH, 7β-hydroxycholesterol; αEPX, α-epoxide; TRIOL, cholestanetriol; 6KETO, 6-ketocholestanol; 7KETO, 7-ketocholesterol; 25OH, 25-hydroxycholesterol.

Table 3. Cholesterol Oxides in Dairy Products[a]

	CHOL	7βOH	7αOH	α-EPX	TRIOL	6K	7K	25OH	total COPS
WMP 1	1.02 ± 0.0	nd	nd	0.6 ± 0.0	0.1 ± 0.0	nd	0.4 ± 0.0	nd	1.1
WMP 2	1.04 ± 0.0	0.8 ± 0.0	2.5 ± 0.0	nd	nd	nd	2.3 ± 0.0	nd	6.8
SMP 1	0.22 ± 0.00	nd	0.1 ± 0.0	nd	nd	nd	nd	nd	0.1
SMP 2	0.23 ± 0.0	1.4 ± 0.0	2.1 ± 0.1	nd	nd	1.7 ± 0.0	2.5 ± 0.4	nd	7.7
butter oil	2.79 ± 0.05	nd	10.8 ± 0.7	nd	0.5 ± 0.0	0.5 ± 0.0	7.3 ± 0.2	nd	27.3
ILP	2.14 ± 0.80	6.2 ± 0.2	nd	0.4 ± 0.0	nd	nd	2.6 ± 0.2	nd	9.2
ICP	0.14 ± 0.00	3.8 ± 0.2	0.1 ± 0.0	0.3 ± 0.0	nd	nd	2.0 ± 0.0	nd	6.2

[a] Results express the means of three determinations ± SD for cholesterol (mg/g) and cholesterol oxides (μg/g). CHOL, cholesterol; 7βOH, 7β-hydroxycholesterol; 7αOH, 7α-hydroxycholesterol; αEPX, α-epoxide; TRIOL, cholestanetriol; 6K, 6-ketocholestanol; 7K, 7-ketocholesterol; 25OH, 25-hydroxycholesterol. nd, not detected.

response factor) are included. The response factor for each COPS may vary with different derivatization conditions and GLC performance, and it should be calculated for each individual study instead of using the value cited in the literature (Lai et al., 1995). The linearity was analyzed from 0.2 to 100 ppm of COPS standard concentration, following the recommendation of McCluskey and Devery (1993). Correlation coefficients were >0.999 for each standard curve, and the coefficient of variation (CV) for the average RRF ranged from 2.46 to 7.57%. These data support the accuracy of the method used in this study to evaluate COPS in dairy products.

To assess the recoveries of the different oxides, 40 μg of internal standard and 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100 μg of the different COPS were added in duplicate to 1 g of a WMP. The sample was processed through the entire procedure and finally analyzed by GLC. Table 2 reports the recoveries obtained, which were close to 100%, ranging from 98.4% for 25-hydroxycholesterol to 102.1% for 7-ketocholesterol. There has been some controversy about possible damage to COPS due to saponification (Park and Addis, 1992), particularly to 7-ketocholesterol and α-epoxide. Our data show that none of the oxides studied were damaged during sample processing.

The detection limit was 0.001 ppm, and limits of quantification ranged from 0.09 ppm for 7α-hydroxycholesterol to 0.03 ppm for 7-ketocholesterol.

It is necessary to take into account the cholesterol autoxidation artifacts for the accurate determination of the exent of oxidation of cholesterol in food products (Wasilchuck et al., 1992). Rose-Sallin et al. (1995) reported 7-ketocholesterol as the major COPS formed during purification procedure and 7α-hydroxycholesterol only at trace amounts. The presence of 7α-hydroxycholesterol and the absence of other COPS in fresh SMP (Table 3) may indicate that cholesterol autoxidation artifacts are not formed during our procedure.

Both the gas chromatographic separation and detector response were poorer for the GC/MS analysis than for the routine gas chromatographic analysis, as has been reported by others (van de Bovenkamp et al., 1988). All expected major MS fragments were observed and agreed favorably with published data.

Saponification has been used as an enrichment step during COPS analysis more than a method to quantify free and esterified cholesterol oxides. Some authors assume a small error by not saponifying since about 10% of cholesterol in foods is esterified (van de Bovenkamp et al., 1988). However, because autoxidation of cholesterol fatty acid esters is faster than that of free cholesterol under certain oxidation conditions (Smith, 1992), a significant amount of cholesterol oxides may exist as fatty acid esters. Therefore, the total amount of COPS in foods could be underestimated if a saponification step is not included.

To evaluate esterified cholesterol release, a standard solution of cholesteryl oleate and hypercholesterolemic plasma was quantified by our methodology and hot saponification. A good agreement was achieved between the two methods (results not presented here), with CV <2.2% and no statistical difference ($p < 0.2$), which indicate the complete release of esterified cholesterol following our methodology.

Application of COPS Analysis to Dairy Products. Table 3 shows concentrations of cholesterol and its oxides in skim (SMP1 and SMP2) and whole milk powders (WMP1 and WMP2), butter oil, and isolated milk protein powders from casein (ICP) and lactoalbumin (ILP). SMP1 and WMP1 correspond to fresh powders, and SMP2 and WMP2 were the same samples stored in closed polystyrene bags at room temperature for 6 months. Isolated milk proteins contained a residue of fat, 0.6% for ICP and 5.7% for ILP, and were exposed to several processes, including spray-drying. So, even if they were nonfat products, they contain significant amounts of COPS.

Our results show that commercial milk powders contain very low levels of COPS. The major COPS found in these samples were the oxidized C7 cholesterol derivatives, epimeric 7-hydroxycholesterol and 7-ketocholesterol. The total COPS levels found were comparable to those obtained by other authors. Sarantino et al. (1993) reported 1.2−1.7 ppm in WMP, and Rose-Sallin et al. (1995) reported from 0.26 to 1.90 ppm in

Table 4. Time Course of Non-C7 and C7 Cholesterol Oxides in SMP during 12 Months at Different Storage Conditions[a]

	0 months	1 month	3 months	6 months	9 months	12 months
			A. Non-C7 Cholesterol Oxides			
αEPX	nd					
32N		nd	0.4 ± 0.0	nd	nd	0.1 ± 0.0
32A		0.5 ± 0.0	0.7 ± 0.0	nd	nd	0.7 ± 0.0
55N		nd	0.2 ± 0.0	nd	0.1 ± 0.0	0.2 ± 0.0
55A		0.2 ± 0.0	nd	nd	nd	nd
TRIOL	nd					
32N		nd	0.3 ± 0.0	nd	0.6 ± 0.0	0.4 ± 0.0
32A		nd	0.3 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.7 ± 0.0
55N		0.2 ± 0.0	0.2 ± 0.0	4.1 ± 0.1	6.7 ± 0.4	8.4 ± 0.1
55A		0.3 ± 0.0	0.5 ± 0.0	1.2 ± 0.1	1.7 ± 0.0	1.1 ± 0.0
6KETO	nd					
32N		nd	nd	nd	0.3 ± 0.0	0.1 ± 0.0
32A		0.2 ± 0.0	nd	nd	0.2 ± 0.0	nd
55N		0.2 ± 0.0	0.2 ± 0.0	1.1 ± 0.0	1.3 ± 0.0	1.6 ± 0.0
55A		0.2 ± 0.0	nd	0.3 ± 0.0	0.3 ± 0.0	0.7 ± 0.0
			B. C7 Cholesterol Oxides			
7αOH	0.1 ± 0.0					
32N		0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	1.2 ± 0.0	0.4 ± 0.0
32A		0.2 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	1.5 ± 0.1	1.0 ± 0.0
55N		0.3 ± 0.0	0.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.0	6.0 ± 0.1
55A		0.3 ± 0.0	1.1 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	0.1 ± 0.0
7βOH	nd					
32N		0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	1.7 ± 0.0	1.1 ± 0.0
32A		0.9 ± 0.0	0.7 ± 0.0	2.4 ± 0.1	1.7 ± 0.1	2.1 ± 0.0
55N		0.5 ± 0.0	0.4 ± 0.0	1.4 ± 0.0	4.0 ± 0.3	8.2 ± 0.0
55A		1.1 ± 0.0	1.0 ± 0.0	3.8 ± 0.2	4.1 ± 0.3	3.3 ± 0.0
7KETO	nd					
32N		0.3 ± 0.0	0.3 ± 0.0	2.2 ± 0.0	3.9 ± 0.0	1.6 ± 0.1
32A		0.6 ± 0.1	1.7 ± 0.0	1.9 ± 0.0	2.6 ± 0.1	2.8 ± 0.1
55N		0.4 ± 0.0	0.7 ± 0.0	3.2 ± 0.1	7.8 ± 0.1	7.0 ± 0.2
55A		1.7 ± 0.1	2.2 ± 0.0	3.5 ± 0.2	5.3 ± 0.2	4.1 ± 0.2

[a] Results expressed as ppm of mean of three determinations.

WMP. Nourooz-Zadeh and Appelqvist (1988b) reported from undetectable amounts to 0.7 ppm of total COPS in nine different milk powders. When fresh samples were stored for 6 months, levels of COPS dramatically increased from 1.1 to 5.6 ppm in WMP and from 0.1 to 7.7 ppm in SMP (Nourooz-Zadeh and Appelqvist, 1988b).

Butter oil contained 27.3 ppm of total COPS, with 7α-hydroxycholesterol and 7-ketocholesterol as major cholesterol oxides. These results are comparable with those obtained by Nourooz-Zadeh and Appelqvist (1988a) in butter but lower than those reported by Sander et al. (1989), who found 106 ppm of total COPS in butter oil.

Casein and lactoalbumin isolated milk proteins contained levels of COPS similar to those obtained for milk powders after 6 months of storage, 6.2 and 9.2 ppm of total COPS for ICP and ILP, respectively. However, the COPS profiles obtained for milk powder and for isolated milk protein powders were quite different. In isolated milk proteins, there were low or undetectable quantities of 7α-hydroxycholesterol and 7β-hydroxycholesterol was the predominant oxide found, with a ratio of 7-ketocholesterol/7β-hydroxycholesterol equal to 0.5 (0.53 for ICP and 0.42 for ILP).

Effects of Storage Conditions on COPS Levels. The effects of storage conditions on COPS levels in SMP and WMP are shown in Tables 4 and 5. When total COPS levels were plotted against storage time, the following correlation coefficients were achieved: 0.603 for 32N, 0.878 for 32S, 0.966 for 55N, and 0.759 for 55S in SMP; 0.854 for 32N, 0.881 for 32A, 0.133 for 55N, and 0.930 for 55A in WMP.

There was a direct relationship between storage condition and cholesterol oxidation in SMP and WMP. We also observed a relationship between storage time and cholesterol oxide generation. Slight differences in COPS profiles were observed between different storage conditions, 7-ketocholesterol being the major cholesterol oxide formed.

Storage time increased C7 oxides, especially 7-ketocholesterol and 7β-hydroxycholesterol. These oxides are produced by an autoxidation process involving ground-state dioxygen ($^3O_2$) (Smith, 1992). When samples were incubated at 55 °C under nitrogen, an increase in the levels of cholestanetriol and α-epoxide also was found, which indicated that a double-oxidation mechanism, via ground-state dioxygen and hydroxyperoxide-induced free radical, may occur (Smith, 1992). We hypothesize that a relationship between COPS generation and Maillard reaction may occur during SMP storage at 55 °C under nitrogen. Samples stored at 55 °C under nitrogen had a brown appearance that samples stored in air did not have. Hydroperoxides and free radicals produced during browning reactions (Zu et al., 1996) may cause the intensive oxidation noted in SMP.

Effects of Storage Condition on Unsaturated Fatty Acids Losses. Table 6 shows unsaturated fatty acid (UFA) losses of stored WMP and SMP. When UFA losses were plotted against storage time, the following correlation coefficients were achieved: 0.364 for 32N, 0.703 for 32S, 0.776 for 55N, and 0.818 for 55S in SMP; 0.713 for 32N, 0.924 for 32A, 0.806 for 55N, and 0.976 for 55A in WMP.

UFA (18:1*n*−9 and 18:2*n*−6) losses in WMP occurred in all conditions studied, especially when packaged in air. At 32 °C under nitrogen, UFA losses only were observed after 9 months of storage. Major UFA losses were found in samples stored for 12 months at 55 °C under air, with only 39% and 45% of 18:1*n*−9 and 18:2*n*−6 remaining, respectively.

SMP stored at 32 °C suffered a 30% loss of 18:1*n*−9 during the first months of storage, rising to 51% loss in samples packaged in air after 12 months of storage.

Table 5. Time Course of Non-C7 and C7 Cholesterol Oxides in WMP during 12 Months at Different Storage Conditions[a]

	0 months	1 month	3 months	6 months	9 months	12 months
			A. Non-C7 Cholesterol Oxides			
αEPX	0.6 ± 0.0					
32N		nd	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
32A		0.5 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	1.9 ± 0.1	0.1 ± 0.0
55N		0.5 ± 0.0	0.7 ± 0.0	1.2 ± 0.1	1.7 ± 0.0	0.4 ± 0.0
55A		1.0 ± 0.0	2.5 ± 0.0	3.7 ± 0.2	7.3 ± 0.3	6.5 ± 0.1
TRIOL	0.1 ± 0.0					
32N		0.2 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	nd	nd
32A		0.1 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.8 ± 0.0	1.8 ± 0.0
55N		1.0 ± 0.0	3.9 ± 0.1	2.2 ± 0.1	1.7 ± 0.0	0.3 ± 0.0
55A		0.6 ± 0.0	1.6 ± 0.1	3.5 ± 0.2	6.9 ± 0.4	10.8 ± 0.1
6KETO	nd					
32N		nd	0.2 ± 0.0	nd	nd	nd
32A		nd	0.3 ± 0.0	nd	0.7 ± 0.0	0.3 ± 0.0
55N		0.1 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	1.2 ± 0.0	nd
55A		nd	1.0 ± 0.0	0.4 ± 0.0	0.9 ± 0.0	1.8 ± 0.1
			B. C7 Cholesterol Oxides			
7αOH	nd					
32N		0.1 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.2 ± 0.0
32A		0.8 ± 0.0	0.5 ± 0.0	1.0 ± 0.0	5.2 ± 0.0	6.9 ± 0.1
55N		0.2 ± 0.0	1.6 ± 0.1	0.7 ± 0.0	0.9 ± 0.0	0.5 ± 0.0
55A		1.3 ± 0.0	1.4 ± 0.1	6.9 ± 0.2	13.7 ± 1.0	13.1 ± 0.3
7βOH	nd					
32N		2.0 ± 0.0	0.5 ± 0.0	0.9 ± 0.0	1.6 ± 0.0	1.6 ± 0.0
32A		5.2 ± 0.2	2.3 ± 0.0	5.5 ± 0.2	11.4 ± 0.1	12.1 ± 0.0
55N		0.5 ± 0.0	2.0 ± 0.0	1.5 ± 0.0	2.1 ± 0.0	1.3 ± 0.0
55A		8.4 ± 0.1	13.0 ± 0.3	23.6 ± 1.3	45.8 ± 0.9	35.4 ± 0.2
7KETO	0.4 ± 0.0					
32N		0.2 ± 0.0	0.6 ± 0.0	1.0 ± 0.0	1.4 ± 0.0	3.2 ± 0.2
32A		4.7 ± 0.1	6.8 ± 0.2	5.0 ± 0.0	10.7 ± 0.1	11.9 ± 0.2
55N		0.6 ± 0.0	2.5 ± 0.1	1.3 ± 0.1	2.5 ± 0.1	2.1 ± 0.1
55A		13.4 ± 0.4	17.5 ± 0.9	27.6 ± 1.0	53.8 ± 1.6	53.4 ± 0.3

[a] Results expressed as ppm of mean of three determinations.

Table 6. Effect of Storage Conditions during 12 Months of Skim and Whole Milk Powders on Total COPS Concentration, TBARS Values, and UFA Losses[a]

		0 months	1 month	3 months	6 months	9 months	12 months
				A. Skim Milk Products			
32N	total COPS	0.1	0.6	1.5	2.7	3.2	3.7
	TBARS	0.2	0.2	0.3	0.3	0.7	0.4
	UFA losses	0	28	32	35	38	51
	total COPS	0.1	1.4	4.2	6.1	6.5	7.3
32A	TBARS	0.2	0.3	0.3	0.6	0.8	0.8
	UFA losses	0	30	33	38	42	51
	total COPS	0.1	1.0	2.1	11.2	21.2	31.4
55N	TBARS	0.2	1.3	3.9	6.4	9.9	11.1
	UFA losses	0	10	32	38	43	43
	total COPS	0.1	2.4	4.7	11.0	13.9	10.2
55A	TBARS	0.2	0.5	1.1	3.8	5.3	5.0
	UFA losses	0	15	37	43	48	54
				B. Whole Milk Powder			
	total COPS	1.1	1.0	2.9	3.3	3.1	5.1
32N	TBARS	0.2	0.2	0.3	1.1	1.7	0.6
	UFA losses	0	1	3	0	6	16
	total COPS	1.1	11.3	10.8	11.8	30.6	34.3
32A	TBARS	0.2	0.4	0.5	0.6	0.9	1.2
	UFA losses	0	1	3	7	18	31
	total COPS	1.1	2.9	11.2	7.7	10.1	4.6
55N	TBARS	0.2	1.4	2.2	2.1	1.6	2.0
	UFA losses	0	1	2	5	9	28
	total COPS	1.1	24.7	37.0	65.7	128.4	121.0
55A	TBARS	0.2	0.8	1.6	3.5	4.2	5.1
	UFA losses	0	3	11	20	36	58

[a] Results are expressed as ppm for total COPS, μmol/100 g for TBARS, and percentage of initial concentration for UFA losses.

Losses of 18:1*n*−9 rose to 43% and 54% after 12 months of storage at 55 °C in nitrogen and air, respectively.

Effects of Storage on TBARS Concentration. Table 6 shows TBARS values of stored WMP and SMP. When TBARS concentrations (expressed as micromoles of malondialdehyde) were plotted against storage time, the following correlation coefficients were achieved: 0.902 for 32N, 0.933 for 32S, 0.982 for 55N, and 0.912

Table 7. Correlation Coefficients among Different Lipid Parameters of WMP and SMP during 12 Months at Different Storage Conditions

	WMP			SMP		
	COPS		UFA	COPS		UFA
	TBARS	UFA	TBARS	TBARS	UFA	TBARS
32N	0.64	0.67	0.44	0.90	0.50	0.39
32A	0.91	0.89	0.95	0.84	0.80	0.66
55N	0.92	<0.1	<0.1	0.92	0.61	0.85
55A	0.92	0.86	0.90	0.93	0.81	0.78

for 55S in SMP; 0.909 for 32N, 0.967 for 32A, 0.248 for 55N, and 0.975 for 55A in WMP.

TBARS concentrations were higher in samples stored at 55 °C than at 32 °C. SMP stored at 55 °C under nitrogen showed the highest TBARS concentrations, rising from 0.2 to 11.1 μmol/100g, after 12 months of storage.

Correlation among Lipid Oxidation Parameters during Milk Powder Storage. Table 7 shows correlation coefficients among lipid oxidation parameters, COPS, TBARS concentration, and UFA losses, during storage of whole and skim milk powders. Good correlations were found between COPS and TBARS concentration, except for WMP stored at 32 °C in nitrogen. Correlation of UFA losses to the other oxidation parameters was better in samples stored in air. During milk powder storage, cholesterol seemed to suffer more extensive oxidation than UFA. These results suggest that COPS quantitation is a reliable monitor of lipid oxidation. The major advantage of COPS quantitation is precision and specificity. UFA losses did not seem to be a good parameter to monitor lipid oxidation in milk powders, likely due to the low concentration of UFA.

The method reported here provides a simple and quick means of quantifying several cholesterol oxidation products in dairy products and offers an appropriate tool for routine analysis of dairy products. Products used in food manufacturing were analyzed according to this methodology, finding detectable amounts of COPS. Storage time and temperature severely affected levels of COPS even under mild storage conditions, with a good correlation between their concentration and a classical lipid oxidation parameter, TBARS. COPS concentration is an effective monitor of lipid oxidation in milk powders.

ABBREVIATIONS USED

COPS, cholesterol oxidation products; WMP, whole milk powder; SMP, skim milk powder; ICP, isolated casein powder; ILP, isolated lactoalbumin powder; 32N, stored 32 °C in the presence of nitrogen; 32A, stored at 32 °C in the presence of air; 55N, stored at 55 °C in the presence of nitrogen; 55A, stored at 55 °C in the presence of air; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; TEP, tetraethoxypropane; TMS, trimethylsilyl; GLC, gas−liquid chromatography; RRF, relative response factor; CV, coefficient of variation; MS, mass spectrometry; UFA, unsaturated fatty acids; SD, standard deviation.

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