Quantification of Cholesterol Oxidation Products in Milk Powders Using [$^2H_7$]Cholesterol To Monitor Cholesterol Autoxidation Artifacts

Christine Rose-Sallin,[†] Anthony C. Huggett,[‡] Jacques O. Bosset,[†] Raffaele Tabacchi,[§] and Laurent B. Fay*,[‡]

Federal Dairy Research Institute, CH-3097 Liebefeld-Bern, Switzerland, Nestec Ltd., Nestlé Research Centre, Vers-Chez-les-Blanc, P.O. Box 44, CH-1000 Lausanne 26, Switzerland, and Institut de Chimie, Université de Neuchâtel, CH-2000 Neuchâtel, Switzerland

Cholesterol oxidation products (COPs) may produce adverse biological effects, and there is increasing concern regarding the potential health implications of these products in the diet. We have developed a GC/MS method allowing the quantification of COPs in commercially available milk powders and infant formulas. Since oxidation artifacts from cholesterol are essentially impossible to avoid, we have used a deuteriated cholesterol probe to precisely monitor the formation of cholesterol oxidative artifacts during all stages of sample analysis. This strategy allowed the determination of the artifact-free cholesterol oxide content of the samples. We found that freshly opened full cream powders which had been packed under inert gas contained only traces of COPs (<250 ng/g total). Infant formulas were also found to contain very low levels of COPs. Our values were considerably lower than those previously reported, especially for 7-ketocholesterol. This difference is at least in part explained by the correction of the oxidation artifacts applied to our samples. We conclude that the exposure to COPs from commercial milk powders and infant formulas, and consequently their potential health hazard, may have been previously overestimated.

Keywords: *Milk powder; cholesterol; oxidation; artifacts; GC/MS*

INTRODUCTION

Cholesterol, an unsaturated lipid, is readily susceptible to oxidation, leading to the formation of more than 30 different cholesterol oxidation products (COPs) (Smith, 1981). These products may be formed by enzymatic oxidation or autoxidation with ambient oxygen or by exposure to heat, light, or radiation (Smith, 1992). They have been detected in human plasma and in many foods of animal origin such as milk products, eggs, and meat. It has been reported that commonly used food processing and storage conditions enhance their formation (Addis and Park, 1992). For example, McCluskey and Devery (1993) recently reviewed the total levels of seven common COPs that have been reported for several cholesterol-rich foods following storage. Samples of freeze-dried chicken and beef contained 8–78 and 9–63 ppm of COPs, respectively, French fries 11–39 ppm, whole egg powder 29-211 ppm, cheddar cheese powder 6–57 ppm, canned baby foods 8–20 ppm, skim milk powder 19–61 ppm, and infant formula powders 42–86 ppm of total COPs.

There is growing interest and concern regarding the potential health implications of COPs in the diet. The effects in humans of chronic ingestion of normal dietary levels of these products are not known. However, there have been several studies performed either in animal models or in isolated cellular systems that have demonstrated the potential of these components to produce adverse effects. Several studies have implicated COPs, in particular 25-hydroxycholesterol and cholestanetriol rather than their precursor cholesterol, as a cause of atherosclerotic lesions (Imai et al., 1976; Taylor et al., 1979). α-Epoxycholesterol has been reported to be mutagenic and cytotoxic in vitro (Sevenian and Peterson, 1984; 1986) and has been detected in UV-induced skin tumors in experimental animals (Black, 1980). COPs may also exert biological effects by their influence on cell membranes (Peng and Morin, 1987) and on cellular lipid metabolism (Erickson et al., 1978).

During the past decade numerous papers have been published concerning the determination of cholesterol oxides in food products. A wide variety of different methodologies have been used, and there have been large discrepancies in the results reported. This issue presently gives rise to controversy, and the precise quantification of the COPs remains a challenging task, especially in foods containing low levels of these compounds (McCluskey and Devery, 1993). Although HPLC with UV detection has been widely used to monitor COPs in food or biological samples (Ansari and Smith, 1979; Csiky, 1982; Sallin et al., 1993a), certain COPs, such as cholestanetriol, are not detectable with this technique. The utilization of capillary GC is the current method of choice for the separation and quantification of COPs and is a particularly powerful technique when combined with GC/MS for simultaneous confirmation of their identity (Park and Addis, 1985).

The critical step in the analysis of COPs is the cleanup procedure, which should be designed to minimize the generation of artifacts. The formation of COPs by autoxidation of cholesterol and their subsequent instability require a very mild analytical methodology. The presence of air, light, peroxides in solvents, or heat treatment during the cleanup procedure can promote their formation. The activity of some of adsorbing agents such as silica gel is another possible reason for

* Author to whom correspondence should be addressed.

[†] Federal Dairy Research Institute.

[‡] Nestec Ltd.

[§] Université de Neuchâtel.

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artifact formation (Claude and Beaumont, 1966; Horvath, 1966) due to the interaction of cholesterol with silicic acid in the presence of air. Moreover, the pH of the media can influence the stability of several COPs (Kim and Nawar, 1993), in particular 7-ketocholesterol, which is very sensitive to hydrolysis in hot alkaline media (Bergström and Wintersteiner, 1941; Chicoye et al., 1968).

Validation of the analysis of COPs is crucial since any deviation from the procedure such as increases in temperature, light, and oxygen availability may lead to variations in artifact formation. Since oxidation artifacts are essentially impossible to avoid and reproducibly control, we have used a deuteriated cholesterol probe to precisely monitor the formation of cholesterol oxidative artifacts during all stages of sample analysis (Kudo et al., 1989; Wasilchuk et al., 1992). In this strategy, deuterium-labeled cholesterol is added to the samples in an amount equivalent to the endogenous cholesterol present, and thereafter individual COPs (deuterium-labeled and unlabeled) are extracted and quantified using GC/MS. The extent of oxidation occurring during the cleanup procedures can be easily determined from the deuteriated cholesterol oxides formed. Subtraction of labeled COPs from the unlabeled COPs then allows the determination of the artifact-free cholesterol oxide content of the samples. We have applied this strategy to the analysis of COPs in commercially available milk powders and infant formulas.

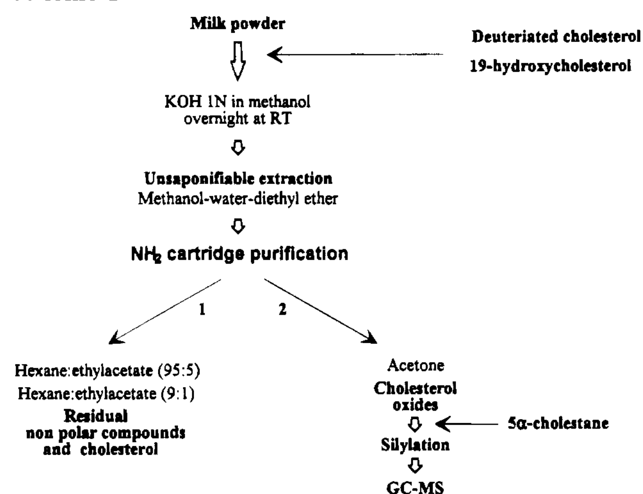
EXPERIMENTAL PROCEDURES

Reagents and Chemicals. 5-Cholesten-3β-ol (cholesterol), 5α-cholestane, 5β-cholestan-3α-ol (epicoprostanol), 5-cholestene-3β,19-diol (19-hydroxycholesterol), and α-epoxycholesterol were obtained from Sigma (St. Louis, MO). 7-Oxo-5-cholesten-3β-ol (7-ketocholesterol) was purchased from Aldrich Chemical Co. (Milwaukee, WI), and 5-cholestene-3β,25-diol (25-hydroxycholesterol), 5-cholestene-3β,7α-diol (7α-hydroxycholesterol), 5-cholestene-3β,7β-diol (7β-hydroxycholesterol), cholestane-3β,5α,6β-triol, 5-cholestene-3β,20α-diol (20α-hydroxycholesterol) were from Steraloids Inc. (Wilton, NH). [25,26,26,26,27,27,27-$^2H_7$]Cholesterol was obtained from Dr. Glaser AG (Basel, Switzerland). The silylating agents *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and *N*,*O*-bis(trimethylsilyl)acetamide (BSA) were from Fluka (Buchs, Switzerland). Pro analysis-grade solvents such as acetone, ethyl acetate, diethyl ether [deperoxidized with Molecular Sieve Deperox from Fluka according to the procedure of Burfield (1982)], methanol, pyridine, tetrahydrofuran, toluene, methylene chloride, and LiChrosolv-grade solvents such as *n*-hexane and water were purchased from Merck (Dietikon, Switzerland). Supelclean aminopropyl phase cartridges were from Supelco (Gland, Switzerland).

Samples. Four instant full cream powdered milks prepared using the same spray-drying conditions and obtained directly from the manufactures (samples A–D, 280 mg of fat/g of milk powder) and three infant formula powders purchased locally (samples E and F, 260 mg of fat/g of infant formula; sample G, 250 mg of fat/g of infant formula) were analyzed. All samples were vacuum-packed under an inert gas except for sample C, which contained 21% oxygen.

Quantification of Cholesterol in Milk Powders. One milligram of epicoprostanol (internal standard) in 10 mL of cyclohexane was added to 1 g of milk powder and 1 g of sea sand. Direct saponification of the milk powder was performed by adding 10 mL of ethanolic 1 N KOH and refluxing for 15 min at 80 °C. After the mixture cooled to room temperature, 10 mL of aqueous 1 N KOH was added, and the mixture was stirred for 15 min. The saponified mixture was then transferred into a glass tube sealed with a Teflon-coated cap and centrifuged at 2000 rpm for 15 min. A 100 mL aliquot of the organic phase containing cholesterol and epicoprostanol was withdrawn, derivatized to trimethylsilyl ethers, and analyzed by GC/FID (Dieffenbacher et al., 1994).

Scheme 1



Extraction of COPs from Milk Powder. The extraction procedure is summarized in Scheme 1. Prior to saponification, [$^2H_7$]cholesterol (in an equimolar amount to the cholesterol present in the sample as previously determined) and 500 ng of 19-hydroxycholesterol (internal standard) were added to 1 g of milk powder in a glass tube sealed with a Teflon-coated cap. Direct saponification was performed according to the method of Park and Addis (1986a) by adding 10 mL of methanolic 1 N KOH to the milk powder and stirring the mixture overnight in the dark at room temperature. Thereafter, 10 mL of LiChrosolv-grade water was added, and the unsaponifiable fraction was extracted three times with 15 mL of deperoxidized diethyl ether. The pooled diethyl ether extracts were washed once with 20 mL of 0.5 N aqueous KOH and three times with 20 mL of aqueous 0.47 N Na_2SO_4 and then dried with anhydrous Na_2SO_4. The dried extract was evaporated to dryness, and the residue was redissolved in 1 mL of hexane/ethyl acetate (95:5 v/v) prior to application onto an aminopropyl phase cartridge (previously activated with 3 mL of hexane). The residual nonpolar compounds together with the cholesterol were eluted with 6 mL of hexane/ethyl acetate (95:5 v/v) and 10 mL of hexane/ethyl acetate (9:1 v/v). The COPs fraction was eluted with 10 mL of acetone and evaporated under a stream of nitrogen. The COPs were then converted to trimethylsilyl ethers by addition of 100 mL of BSTFA, 20 mL of dried pyridine, and 2 mL of TMCS. Silylation was performed overnight at room temperature. The extract was dried under a stream of nitrogen and redissolved in 100 mL of hexane/BSA (10:1 v/v).

Thin Layer Chromatography. A control of the fractions eluted from the aminopropyl phase cartridges was performed using TLC (Sallin et al., 1993b). Kieselgel F_{254} plates 20 × 20 cm, 0.2 mm thickness, from Merck (Dietikon, Switzerland), were developed with methylene chloride/toluene/acetone (2:1:1 v/v). The COPs were revealed by their UV absorption at 254 nm or by spraying with a 50% aqueous solution of H_2SO_4 followed by heating for 10 min at 120 °C.

Gas Chromatography/Mass Spectrometry. Full scan spectra were obtained using an HP-5988 mass spectrometer connected to an HP-5890 gas chromatograph equipped with an HP-7673 autosampler (Hewlett-Packard, Geneva, Switzerland). The quantification experiments were carried out using an HP-5890 gas chromatograph equipped with an HP-7673 autosampler, a Finnigan TSQ 700 mass spectrometer, and an ICIS II data system (Finnigan, Bremen, Germany). The GC/MS conditions were as follows: DB-1 J&W fused-silica capillary column (30 m × 0.32 mm i.d., film thickness 0.25 mm); carrier gas, helium at 69 kPa; splitless injection at 280 °C. The oven temperature program was as follows: 50 °C (1 min), increased at 30 °C/min to 200 °C and then at 5 °C/min to 300 °C (2 min); interface temperature, 280 °C. The mass

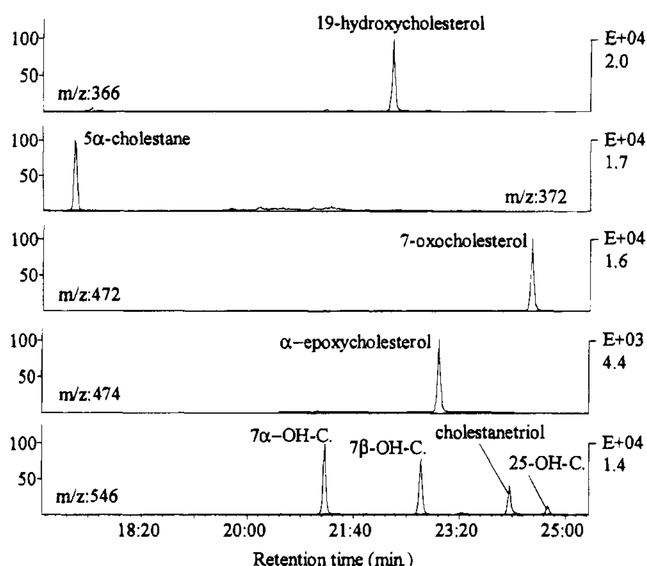


Figure 1. GC/MS traces of the standard cholesterol oxides. The compounds are chromatographed on an apolar capillary column and detected by selective ion monitoring mass spectrometry after electron impact ionization.

spectrometers were operated under electron ionization conditions with an electron energy of 70 eV and an ion source temperature of 180 °C. For quantification the ions of interest were acquired by selected ion monitoring at unit resolution.

Formation of COPs in Infant Formula Powders. The formation of COPs was examined in the three infant formula samples used under simulated normal household conditions. Samples E and G were packed in bags, while sample F was a canned product. On day 0 the packages were opened and the basal levels of COPs were analyzed as above. About 15−20 g of formula was removed each day using the spatulas provided, and the packets were subsequently closed and stored at room temperature. In the case of samples E and F, the tops of each bag were folded and closed with a clothes-peg. Further analyses of COPs content were performed at regular intervals over 3 weeks.

RESULTS AND DISCUSSION

GC/MS Analysis of Cholesterol Oxides. Overnight derivatization at room temperature allowed the quantitative silylation of the cholesterol oxides of interest without formation of byproducts. The trimethylsilyl derivatives obtained were well resolved on an apolar methylsilicone capillary column (Figure 1). Mass spectrometric detection of the compounds was achieved after electron impact ionization. The major ions obtained for each compound are summarized in Table 1. Each of these ions has been previously described (Park and Addis, 1992). All of the hydroxyl groups of the hydroxycholesterol isomers were silylated, and in the case of 7-ketocholesterol the keto group remained unchanged. The derivative of cholestanetriol was identified as the bis(trimethylsilyl) ether (Park and Addis, 1989). 20α-Hydroxycholesterol was not quantitatively silylated and produced two derivatives, which were identified as 3β,20α-bis[(trimethylsilyl)oxy]-cholest-5-ene (20α-hydroxycholesterol diTMS, first peak) and 3β-[(trimethylsilyl)oxy]-20α-hydroxy-cholest-5-ene (20α-hydroxycholesterol monoTMS, second peak). For this reason 20α-hydroxycholesterol was not quantified in milk powders. All of these identifications were confirmed using positive chemical ionization mass spectrometry with ammonia as reagent gas.

The quantification of the COPs in the samples was achieved by monitoring the molecular ion of each compound except for cholestane-3β,5α,6β-triol and 19-hydroxycholesterol, for which the fragments $[M - H_2O]^+$ and $[M - (2 \times 90)]^+$, respectively, were acquired. The deuteriated cholesterol oxides were monitored at 7 mass units higher than for the corresponding unlabeled compounds except for 25-hydroxycholesterol, for which the shift was only 6 mass units ([$^2H_6$]-25-hydroxycholesterol). The quantitation of the COPs using 19-hydroxycholesterol as internal standard (Pie et al., 1990) was linear over a range from 20 pg to 20 ng (pure compounds injected).

Purification of Cholesterol Oxides from Milk Powders. In milk powder, sterols constitute a minor part of the total lipid extract and cholesterol oxides are present mainly in the free form (Nourooz-Zadeh and Appelqvist, 1988). Triglycerides and phospholipids were removed from the lipids by a cold saponification applied directly to the milk powder. A lipid extraction was not performed prior to the saponification as this did not improve the purity of the final extract. The mild conditions employed for the saponification minimized artifact formation and the decomposition of the COPs, especially the 7-ketocholesterol (Park and Addis, 1986b). After diethyl ether extraction, the cholesterol oxides were purified using a solid phase aminopropyl extraction cartridge which allows the removal of cholesterol and nonpolar compounds (see Figure 2).

Determination of Cholesterol Oxide Artifacts in Milk Powders. Figure 3 presents a typical chromatogram obtained from a milk powder extract showing the simultaneous detection of cholesterol oxides and the corresponding deuteriated analogues. Each compound is quantified using the internal standard, and the amount of the endogenous COPs present in the milk sample is calculated by subtracting the amount of deuteriated COPs from the unlabeled compounds. The general formula for this calculation is

$$X = U - (LC_u/C_d)$$

where X is the amount of cholesterol oxide in the milk powder, U is the sample amount of the unlabeled cholesterol oxide, L is the sample amount of the deuteriated cholesterol oxide, C_u is the sample amount of endogenous unlabeled cholesterol, and C_d is the amount of deuteriated cholesterol added to the sample.

This calculation is based on the fact that no interferences were found between the deuteriated and the corresponding unlabeled oxides as presented in Figure 4, which shows the mass spectra of labeled and unlabeled 7-ketocholesterol and 7β-hydroxycholesterol.

Table 2 gives an average of the levels of cholesterol oxides formed artifactually in the milk powders analyzed following our method. It highlights the importance of the correction of the cholesterol autoxidation during the purification procedure. During our mild purification procedure about 2‰ of cholesterol is autoxidized. This value shows the necessity to take into account the cholesterol autoxidation artifacts for the accurate determination of the extent of the oxidation of cholesterol in food products (Walsilchuk et al., 1992). However, such a correction introduces a large source of variation in the final value of the cholesterol oxide level, especially when low amounts of COPs are measured (see also Table 4).

Validation of the Method. The recovery of the method was checked using a solution of pure cholesterol oxides and also using milk powders spiked with COPs. The results of the recoveries of the individual COPs

Table 1. Characteristic Ions in the Electron Impact Mass Spectra of the TMS Derivatives of the Cholesterol Oxides of Interest[a]

cholesterol oxide	$M^{\bullet+}$	$[M - CH_3]^+$	$[M - 90]^+$	other ions
5α-cholestane	372 (29)	357 (25)		262 (8); 218 (55); 217 (100)
7α-hydroxycholesterol	546 (4)	531 (2)	456 (100)	129 (7)
7β-hydroxycholesterol	546 (4)	531 (2)	456 (100)	129 (6)
α-epoxycholesterol	474 (100)	459 (17)	384 (54)	446 (5); 369 (10); 366 (33)
cholestane-3β,5α,6β-triol	564 (<1)	531 (11)		546 (47); 456 (67); 403 (100); 321 (47); 129 (59)
25-hydroxycholesterol	546 (2)		456 (3)	271 (7); 131 (100); 129 (14)
7-ketocholesterol	472 (100)	457 (12)	382 (22)	455 (11); 367 (43); 129 (82)
19-hydroxycholesterol		531 (<1)	456 (11)	366 (67); 353 (100)

[a] 5α-Cholestane and 19-hydroxycholesterol are, respectively, external and internal standards for the quantification.

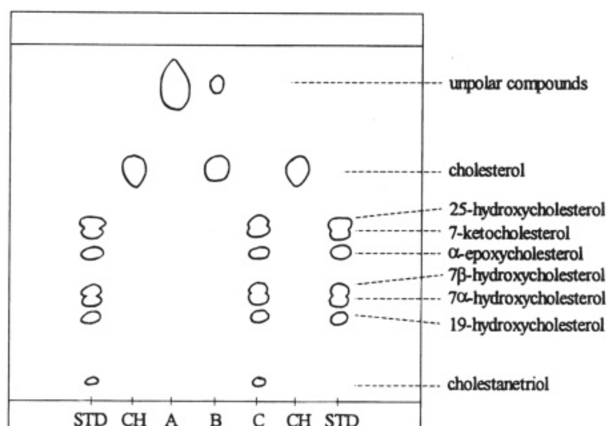


Figure 2. TLC separation of a milk powder extract purified with an amino phase extraction cartridge. (STD) standard solution of COPs; (CH) standard solution of cholesterol; (A) nonpolar fraction eluted with hexane/ethyl acetate (95:5 v/v); (B) cholesterol-containing nonpolar fraction eluted with hexane/ethyl acetate (9:1 v/v); (C) COPs eluted with acetone.

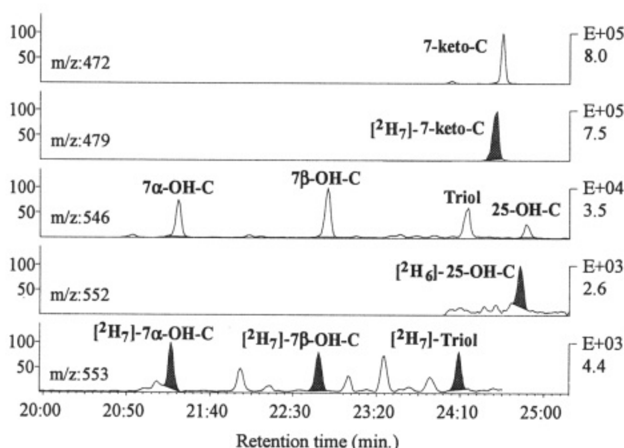


Figure 3. Typical GC/MS traces obtained from a milk powder extract. The COPs and their deuteriated analogs are simultaneously monitored and quantified using 19-hydroxycholesterol as internal standard.

following workup through the complete procedure are given in Table 3. The recoveries of the different COPs from a standard solution containing 500 ng of each ranged from 80 to 99%. 7-Ketocholesterol, which has been reported to be easily decomposed during cleanup (Van de Bovenkamp et al., 1988), is quantitatively purified using our mild sample preparation conditions. The recovery of spiked COPs from the milk powders was examined over the concentration range 200−1000 ng/g. At a concentration of 200 ng/g the compounds are difficult to quantitatively purify (recoveries from 52 to 88%). At concentrations of 500 and 1000 ng/g acceptable recoveries for each of the COPs investigated were obtained. α-Epoxycholesterol was not accurately purified using our method and therefore was not quantified in the milk powder samples.

The repeatability of the method is given in Table 4. These results were obtained after quantification of pure standards that were silylated and also following analysis of fresh and old abused milk powders containing low and relatively high levels, respectively, of cholesterol oxides. Large coefficients of variation were observed, particularly with the milk powder containing low amounts of cholesterol oxides. This may at least in part be due to decomposition of the compounds during the injection step, which was performed in splitless mode at an elevated temperature (Van de Bovenkamp et al., 1988). Another explanation could be found in the closeness of the level of COPs found in the milk powders with the limit of detection of the method (see Table 4).

Determination of Cholesterol Oxides in Milk Powders. The results (Table 5) demonstrate that the commercial milk powders and infant formulas analyzed contain very low levels of COPs. The major COPs found in these samples were 7α-hydroxycholesterol and 7β-hydroxycholesterol. Although 7-ketocholesterol was the major oxidized compound detected before artifact correction, essentially all of this was due to oxidation of cholesterol during sample cleanup (about 1.5 mg of 7-ketocholesterol/g of cholesterol).

The freshly opened full cream powders that had been packed under inert gas (samples A and B) contained only traces of COPs (<250 ng/g of powder), even after storage of the unopened cans for 1 year (sample B). However, the level of COPs was substantially greater in the full cream powder which had been opened 1 year previously (sample D) and also in the freshly opened can (sample C), which was not vacuum-packed under an inert gas. These values are comparable to the results obtained by Nourooz-Zadeh and Appelqvist (1988), who demonstrated that fresh milk powders produced by low or medium heat spray-drying contain very low amounts of COPs. These authors reported from undetectable amounts to 0.7 mg/g of total COPs in nine different milk powders. Moreover, their COP values obtained for milk stored in paper cans during 1 year (1.8−3.5 mg/g of milk powder) are also in good agreement with our results (samples C and D containing, respectively, 1.9 and 1.5 mg/g of milk powder).

Two of the freshly opened infant formulas (samples E and F) contained small amounts of COPs (<350 ng/g). In contrast, the third infant formula (sample G) tested contained about 1200 ng/g of total COPs. Since the cholesterol content of these infant formulas is low, the extent of cholesterol oxidation in sample G (0.52%) is higher than that occurring in all of the full cream milk powders tested when expressed as a percentage of total cholesterol content. Nevertheless, the levels of COPs measured in the infant formulas are considerably lower than the values previously reported by Sander et

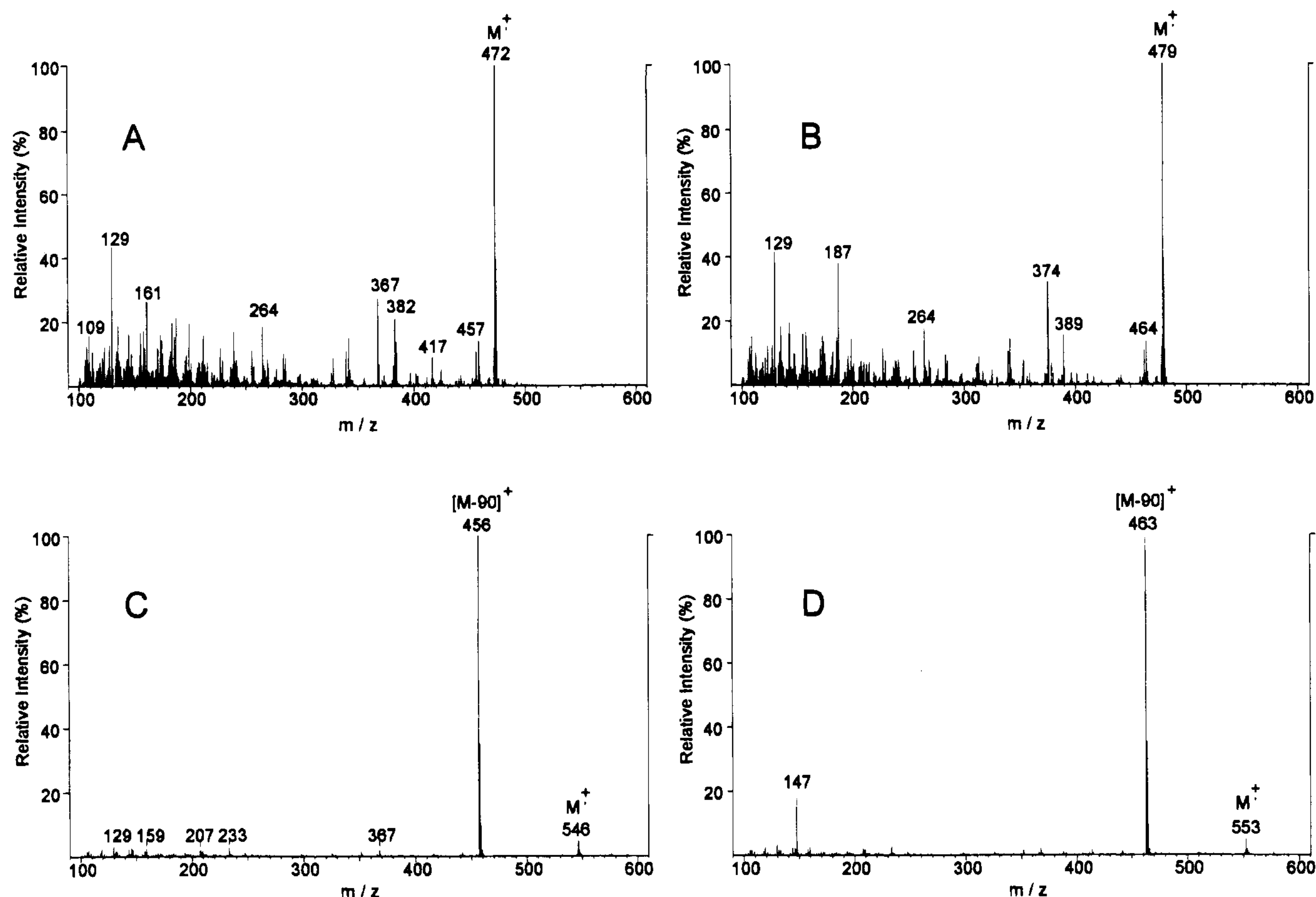


Figure 4. Mass spectra of the TMS derivatives of 7-ketocholesterol and 7β-hydroxycholesterol and their corresponding deuteriated analogues identified in the milk powders after the purification procedure: (A) electron impact (EI) mass spectrum of 7-ketocholesterol; (B) EI mass spectrum of [25,26,26,26,27,27,27-$^2H_7$]-7-ketocholesterol; (C) EI mass spectrum of 7β-hydroxycholesterol; (D) EI mass spectrum of [25,26,26,26,27,27,27-$^2H_7$]-7β-hydroxycholesterol.

Table 2. Determination of the Labeled Cholesterol Oxides Formed during the Purification Procedure[a]

labeled cholesterol oxide	ng/g of milk powder
7α-hydroxycholesterol	<10
7β-hydroxycholesterol	80 ± 30
cholestane-3β,5α,6β-triol	10 ± 5
7-ketocholesterol	1490 ± 380
25-hydroxycholesterol	340 ± 140
total COPs	1920

[a] The results are the mean of 20 determinations and are expressed in ng/g of milk powder. The analyzed milk powder contains 1 mg of cholesterol/g of milk powder.

Table 3. Recovery of Cholesterol Oxides from a Mixture of Pure Compounds and from Milk Powders[a]

	mean percentage recovery			
	standard compounds	added to milk powders		
COP	500 ng each	200 ng/g	500 ng/g	1000 ng/g
7α-hydroxycholesterol	95	59	72	124
7β-hydroxycholesterol	97	71	96	80
cholestane-3β,5α,6β-triol	80	52	103	95
7-ketocholesterol	99	88	108	83
25-hydroxycholesterol	98	60	96	86

[a] A mixture of pure compounds and three samples of milk powders spiked with 200, 500, and 1000 ng/g of milk powder for each cholesterol oxide were analyzed. The results are the mean of three determinations and are expressed as percentages.

al. (1989), especially for 7-ketocholesterol. This discrepancy could be at least in part explained by the correction of the oxidation artifacts applied to our samples. In addition, the COPs reported by these authors could not be unambiguously identified and it is likely that the elevated levels are also due to cochromatographing components.

Table 4. Repeatability of the Cholesterol Oxide Quantification from a Mixture of Pure Compounds and from Milk Powders[a]

	coefficient of variation (%)			
	pure compounds		milk powders	
COP	100 pg each	50 ng each	fresh[b]	old[c]
7α-hydroxycholesterol	11	4	26	6
7β-hydroxycholesterol	13	3	27	7
cholestane-3β,5α,6β-triol	22	4	34	23
7-ketocholesterol	4	3	21	17
25-hydroxycholesterol	9	7	24	25

[a] Pure compounds and two samples of milk powders were analyzed. The coefficients of variation were calculated from three determinations (interassay CVs) and are expressed as percentages. [b] Fresh milk powder containing a total amount of 1100 ng/g of cholesterol oxides before subtraction of the deuteriated oxides. [c] Old milk powder containing a total amount of 4100 ng/g of cholesterol oxides before subtraction of the deuteriated oxides.

The formation of COPs in infant formulas used and stored in conditions simulating normal household use is summarized in Figure 5. The level of COPs increased during the daily utilization of the formulas. Although there was little cholesterol oxidation during the first 10 days of use, thereafter the level of total COPs increased, particularly in samples E and G. It should be noted that in normal household use infant formula powder is usually used up within about 10 days of opening the packet. The formation of COPs in these infant formulas

Table 5. Concentration of Cholesterol Oxides in Milk Powders and Infant Formulas Stored under Different Conditions[a]

sample	storage time, opening date	cholesterol (mg/g of milk powder) and its oxides (ng/g of milk powder)						total COPs	total COPs/cholesterol (%)
		cholesterol	7-keto	7α-hydroxy	7β-hydroxy	cholestanetriol	25-hydroxy		
full cream									
powder A	3 months old, freshly opened	0.94	<100[b]	81 ± 18	32 ± 25	10 ± 12	132 ± 108	**255**	**0.03**
powder B	1 year old, freshly opened	0.79	<100	135 ± 19	107 ± 18	<10	<100	**242**	**0.03**
powder C	1 year old, freshly opened	0.73	<100	674 ± 48	1158 ± 49	63 ± 12	<100	**1895**	**0.26**
powder D	1 year old, 1 year opened	0.89	<100	587 ± 41	879 ± 80	24 ± 14	<100	**1490**	**0.17**
infant formula									
E	freshly opened	0.65	<100	214 ± 56	130 ± 41	<10	<100	**344**	**0.05**
F	1 year old, freshly opened	0.75	<100	94 ± 6	61 ± 4	<10	<100	**155**	**0.02**
G	freshly opened	0.24	296 ± 47	317 ± 12	628 ± 38	<10	<100	**1244**	**0.52**

[a] Each value represents the mean of three determinations and is expressed in ng/g of milk powder. The values for cholesterol are expressed in mg/g of milk powder. [b] Below the detection limit of the method, which is 100 ng/g of milk powder for 7-ketocholesterol, α-epoxycholesterol, and 25-hydroxycholesterol and 10 ng/g of milk powder for cholestanetriol and 7α- and 7β-hydroxycholesterol.

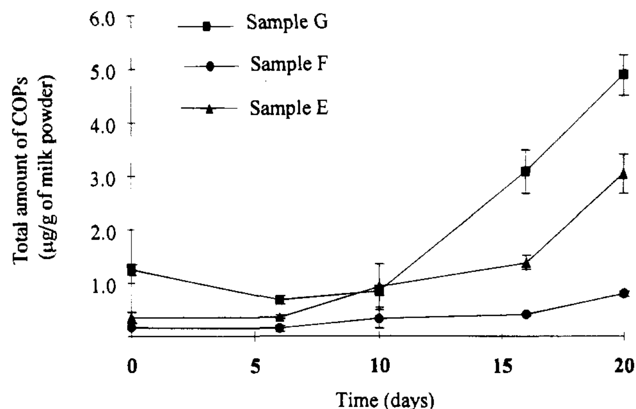


Figure 5. Formation of COPs in milk infant formulas used in conditions corresponding to those normally applied at home (room temperature and opened packages). The samples were opened (day 0), and then aliquots of powder were taken up and analyzed after 6, 10, 16, and 20 days. The results represent the total amount of COPs and are the mean of three determinations.

seems to be accelerated in comparison with the 1 year old milk powder (sample D opened for 1 year and containing 1490 ng/g of COPs). This may be related to the presence of unsaturated fats (Osada et al., 1993) and/or iron in the infant formula preparations. The reasons for the varying amounts of COPs formed in the different formulas cannot be determined from this very limited study. The major difference between samples E and F is that sample E, which was more sensitive to oxidation, is packed in aluminum foil bags, while sample F is packed in cans. These preliminary studies demonstrate the need for further investigations into the formation of COPs in infant formulas.

Conclusion. Previous reports of high levels (6–211 ppm) of COPs measured in different foodstuffs together with the observation that these oxidation products are likely to be absorbed from the gastrointestinal tract have led to concern regarding the potential health effects of cholesterol oxides (McCluskey and Devrey, 1993). However, COPs are also produced endogenously in the body through oxidation of cholesterol during normal metabolism and also as a result of oxidant stress (Smith and Johnson, 1989). The relative importance of dietary COPs versus endogenously formed COPs has not been established and requires further investigation so that the potential health impact of COPs in foodstuffs can be evaluated. Nevertheless, our results demonstrate that exposure to COPs from commercial milk powders and infant formulas is unlikely to represent an important source of COPs. Thus, the potential health hazard of COPs in milk powders and infant formulas needs to be reassessed in light of the present data, since it is likely that the risk/exposure data may have been previously overestimated.

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