Cholesterol Oxides in Swedish Foods and Food Ingredients: Butter and Cheese

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Fresh butter manufactured by batch or continuous technologies in Sweden contained only traces of 7-ketocholesterol at the detection limit (0.1 ppm in the lipids) and no detectable levels of other common cholesterol oxides. Storage at 4°C for up to four mo caused an increase in the levels of the isomeric 5,6-epoxycholestanols, the epimeric 7-hydroxycholesterols, 20α-hydroxycholesterol and 7-ketocholesterol as revealed by Lipidex chromatography, TEAP-Lipidex chromatography and GLC. Heating of butter for 10 min at the temperature interval 150–200°C under conditions similar to that in shallow-pan kitchen frying caused a gradual increase in the total amount of cholesterol oxides from 0 to 2.5 ppm, while at 200°C the total amount of cholesterol oxides was 1.6 ppm. Butter heated five min at 200°C also had less cholesterol oxides than that heated at 190°C for 10 min. Industrially manufactured butter oil and cream oil contained only low levels of some of the cholesterol oxides. Further, three types of processed cheese (soft-melted, hard-melted and grated cheese) were investigated. The soft-melted and the hard-melted cheese types contained only traces of some of the oxides, while the grated cheese samples had 0.5–2.2 ppm of the total cholesterol oxides in the lipids.

Free and esterified cholesterol are minor components of the total lipids (about 0.4%) in dairy products, and about 90% of the total cholesterol is unesterified. Cholesterol undergoes autoxidation in the presence of molecular oxygen by a free radical reaction predominantly at position C-7, and a number of oxidation products are formed (1). Recently, these oxidation products of cholesterol have received considerable attention because of their involvement in developing artery diseases (2,3).

Cholesterol oxides have been shown to have adverse effects in both in vivo and in vitro studies (4–7). Biological effects which can be related directly or indirectly to the cytotoxicity are: angiotoxicity (8–11) and inhibition of cholesterol biosynthesis at the 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase (12–14). Another hazard is the health risks associated with the intake of the suspected carcinogenic 5,6α-epoxy-5α-cholestan-3β-ol (15). Recently, it was shown that the isomeric 5,6-epoxycholestanols have mutagenic properties (16–18).

Deterioration of dairy products as a result of the autoxidation of cholesterol has not received much attention in comparison to that focused on dehydrated egg products. In a few recent papers, the presence of some of the cholesterol oxides in butter and cheese has been reported (19–25). The quantitative data available are very incomplete because either few experiments were done or unspecified samples were used (19,21,22). Further, it has been demonstrated that cholesterol oxidation in butter is accelerated at higher temperatures (19,22). Because of lack of information concerning the experimental details used by Csiky (19) and the excessively long heating times used by Fischer et al. (22), it was deemed necessary to design a well-controlled heating experiment.

The purpose of the present study was to obtain quantitative data on the levels of eight important oxidation products from free cholesterol, the isomeric 5,6-epoxycholestanols, the epimeric 7-hydroxycholesterols, 7-keto cholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol and cholestanetriol, in (i) some dairy products expected to provide important sources of exogenous cholesterol oxides as related to processing technologies and storage, and (ii) heated butter in the temperature interval 150–200°C under conditions similar to those used in home frying.

MATERIALS

The reference compounds were obtained from Sigma Chemical Co., St. Louis, Missouri, or Steraloids Inc., Wilton, New Hampshire, except the 5,6β-epoxy-5α cholestan-3β-ol, which was supplied by Peter Eneroth, Karolinska Institute, Sweden. The solvents were purchased from E. Merck, Darmstadt, Federal Republic of Germany, except the 1,2-dichloroethane, which was obtained from Fischer Scientific, Fair Lawn, New Jersey. Tri-Sil was obtained from Pierce Chemical, Rockford, Illinois. Sep-Pak silica cartridges were purchased from Waters Associates, Mildford, Massachusetts, and the Lipidex 5000 was obtained from Packard Instrument Inc., Downers Grove, Illinois.

Food samples. Normal salted butter (salt content 0.4–0.7%) was obtained immediately after production in 1985 through the Swedish Dairies' Association, Stockholm, Sweden. Butter oil and cream oil were provided by Arla Dairy, Götene, Sweden. Hard-melted cheese was obtained from Arla Dairy, Nyköping, Sweden, and soft-melted cheese from Nedre Norrlands Producentförening, Östersund, Sweden. All these samples were kept in a dark room at 4°C until analysis. Grated cheese (Rivosto) was purchased from local groceries and/or local supermarkets and was kept at room temperature, about 18°C, until analysis.

Preparation of triethylaminohydroxypropyl-Lipidex (TEAP-Lipidex). TEAP-Lipidex was synthesized from Lipidex-5000 as described elsewhere (26). Briefly, a chlorohydroxy derivative of Lipidex-5000 was prepared using boron trifluoride and epichlorohydrin. A 15% substitution was obtained as measured by weighing. The gel was then reacted with triethylamine and the gel capacity determined by titration. The ion exchange capacity was 0.7 meq/g gel. The gel was converted into acetate form by washing with 10% acetic acid in 72% ethanol and stored in ethanol at −20°C. Prior to column packing, the gel was converted into hydroxyl form by washing with 0.3 NaOH in 72% ethanol.

Column packing. The procedure is described in detail in a previous paper (26). For gel-filtration, an appropriate amount of Lipidex-5000 was freed from the storage solvent, ethanol, and was washed consecutively by 20%,

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50% and 99% aqueous ethanol under careful stirring at 70°C. Then, the gel was washed with excess of the actual chromatographic solvent and allowed to swell in the solvent for about two hr. Finally, the slurry was poured into the column and pressure was applied to achieve a uniform packing. For TEAP-Lipidex, the gel was allowed to swell in the actual chromatographic solvent for about 30 min, the slurry was poured into a column with solvent reservoir and pressure was applied to get a uniform packing.

Lipid extraction, butter. A five-g sample of fresh or stored butter was transferred into a separatory funnel, 90 ml hexane/isopropanol (3:2, v/v) (HIP) and 60 ml 0.47 M Na_2SO_4 were added. The mixture was shaken vigorously, allowed to settle, and the upper layer, mainly hexane, was transferred into an evaporation flask. The lipids were dried with a rotary evaporator at 35°C. About 500 mg of the lipid extract was transferred into a glass-stoppered test tube and weighed. The sample was kept at 4°C until analysis. Heated butter was melted in a water bath at 50°C and centrifugted at 4000 rpm for 10 min to remove the water before weighing. Butter oil and cream oil were melted on a water bath at 50°C before weighing.

Soft-melted cheese and grated cheese. A 20-g sample of soft melted cheese or a five-g sample of grated cheese was transferred into a separatory funnel and 90 ml HIP (3:2, v/v) was added. The mixture was shaken vigorously for three min and centrifuged at 4000 rpm for five min. The supernatant was transferred into another separatory funnel, 60 ml 0.47 M Na_2SO_4 was added and the mixture was agitated. The remaining procedure was as described for butter.

Hard-melted cheese. A 20-g sample was transferred into a beaker, 40 ml HIP (3:2, v/v) was added and the content was homogenized with an Ultra Turrax Homogenizer. The sample was transferred into a separatory funnel, another 50 ml HIP was added and the mixture was shaken vigorously for about three min. The mixture was centrifuged at 4000 rpm and the supernatant was treated as described for soft-melted and grated cheese.

Isolation and enrichment. The cholesterol oxides were concentrated as described elsewhere (27). Briefly, the sample, max. 500 mg lipids, was dissolved in one ml hexane/1,2-dichloroethane (9:1, v/v) (H/DC), applied on a prewashed Sep-Pak silica cartridge and the column was washed with eight ml H/DC (9:1, v/v) to remove the hydrocarbons, sterol esters and the major part of the triacylglycerols. The remaining triacylglycerols, cholesterol and more polar lipids were eluted by 12 ml methanol/1,2-dichloroethane (1:1, v/v) (M/DC) and dried in a rotary evaporator at 35°C. The residue was transferred into a glass-stoppered test tube and the solvent removed under a stream of nitrogen. The sample was redissolved in 0.3 ml H/DC (9:1, v/v) and the Lipidex-5000 column was charged. The cholesterol oxide fraction was collected according to (26), dried in vacuo and the residue transferred into a glass-stoppered test tube. The solvent was removed under a stream of nitrogen and the sample was redissolved in 0.1 ml H/DC (3:7, v/v). The cholesterol oxide fraction was applied onto a TEAP-Lipidex column and fractions were collected as described elsewhere (26).

Saponification. Cholestane (about 0.2 μg) was added to the cholestane-triol fraction, and the solvent was removed. The sample was saponified with one ml 2 M NaOH in 95% ethanol by shaking for one hr at 80°C. The unsaponified lipids were extracted three times with four ml diethyl ether, and the combined extract was then washed twice with water (26).

Derivatization, GLC and GLC/MS. Cholestane (about 0.2 μg) was added to the cholesterol oxide fractions from TEAP-Lipidex chromatography and the solvent removed under a stream of nitrogen. The sterol oxides were derivatized to TMS ethers by adding 0.1 ml Tri-Sil, followed by heating for 30 min ato 60°C. The samples were dried under nitrogen and the residue was redissolved in 0.1 ml hexane. One-two μl of the sample were injected into the GLC.

The GLC was performed with a Varian 3700 equipped with a flame ionization detector (FID) and a falling needle injector. The cholesterol oxide derivatives were separated on a chemically bonded methyl silicone column, 25 m × 0.2 mm, film thickness 0.2 u (Quadrex Corp., New Haven, Connecticut). Helium was used as carrier gas. Operation conditions were column temperature 258°C, detector temperature 310°C and inlet pressure 12 psi. The peaks were recorded using a Hp 3390 A Computing Integrator (Hewlett Packard, Avondale, Pennsylvania).

To confirm the identities of GLC peaks tentatively identified by RRT values, mass spectrometry was used. The analysis was performed using a Finnigan 4000 GLC/MS (San José, California), equipped with a falling needle injector and the previously mentioned chemically bonded methyl silicone capillary column. Operation conditions were column temperature 258°C, interface temperature 258°C and ion source temperature 258°C. The spectra were obtained using electron impact ionization at 40 eV and a scan rate of one sec/scan.

The cholesterol oxides were identified by checking the relative retention time values of the TMS derivatives of the components in the TEAP fractions of the sample with that of eight authentic autoxidation products of cholesterol analyzed in this system. Appropriate TEAP fractions were selected for GC/MS confirmation. Cholestane was used as an internal standard and the quantification of the cholesterol oxides was based on peak area measurement. The values are means of duplicate analysis (enrichment and GLC).

Heating experiment. The experiment was designed to investigate the generation of cholesterol oxides at a temperature interval (150–200°C) and at frying times which are commonly used in regular pan-frying. The study was performed with a shallow frying pan (23 cm in diameter), mimicking ordinary kitchen equipment as closely as possible but with temperature control and registration of the temperature profile vs time. The temperature was adjusted and the frying pan allowed to stand for about 10 min before the start of the experiment, in order to obtain the desired temperature. An 80-g sample of two-mo-old butter was transferred into the frying pan where a butter layer of about 1.5 mm was obtained. The thermo element was placed about 0.5 mm from the bottom of the frying pan. After the desired frying time, the sample was recovered, and the pan was cleaned and allowed to be heated for five min before the next experiment. The frying experiments were performed in duplicate. For cholesterol oxide analysis, an aliquot of the heated butter was transferred into a glass tube with teflon cap and kept in an icebox during transport and at −20°C

at the laboratory until analysis. The residual butter was then transferred into a brown flask with a plastic cap and kept in a refrigerator until analysis of peroxide value and anisidine value.

RESULTS AND DISCUSSION

Several sources of errors 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. These problems are discussed in detail in a previous paper (26). When performing this investigation the following precautions were taken: working in dim light and performing the isolation of the cholesterol oxides (Sep-Pak enrichment, gel filtration and TEAP chromatography) rapidly without interruption.

Fresh and stored butter. Freshly made butter samples were obtained immediately after production at different dairies in Sweden. The samples were manufactured by the traditional batch or modern continuous technologies. The analyses indicated that fresh butter manufactured by these two technologies from milk of cows grazing during the summer season contained only traces of 7-ketocholesterol at the detection limit 0.1 ppm in the lipids (Table 1). It must be mentioned that the cholestane-triol fraction from TEAP-Lipidex chromatography was not analyzed due to the absence of any isomeric 5,6-epoxycholestanols in these samples. One of these samples (batch 1) stored at 4°C for two mo had slightly increased levels of some oxides, viz, the isomeric 5,6-epoxycholestanols, the epimeric 7-hydroxycholesterols, 7-ketocholesterol and 20α-hydroxycholesterol (Table 1). The ratios of the 5β-,6β-epoxy to 5α,6α-epoxycholestanol and 7β-hydroxy to 7α-hydroxycholesterol were both slightly higher than 1/1.

Moreover, a number of one-month-old butter samples (batches 3–6) were analyzed (Table 1), and two of the samples were re-analyzed after a further three months. The samples were manufactured from milk of cows fed on concentrated feed mixes and/or silage during the winter. All these samples contained variable levels of cholesterol oxides, and the concentration of cholesterol oxides increased slightly with storage time. The ratios of the 5β,6β-epoxy to 5α,6α-epoxycholestanol and 7β-hydroxy to 7α-hydroxycholesterol in the four-month-old butter samples were about 1/1. It is noteworthy that the one-month-old samples contained no detectable levels of the common 7-derivatives of cholesterols but detectable levels of the suspected carcinogenic compound 5α,6α-epoxycholestanol. Churned butter contained about 5% air by volume; it was suggested by Luby et al. (24) this level is enough to initiate the autoxidation of cholesterol at the same rate at the surface as in the interior of the block. Hence, one may conclude that sampling errors due to the differences in the autoxidation rate at the surface and inside the butter block cannot make a substantial contribution to the differences observed in the levels of cholesterol oxides between the fresh and the stored butter samples. Sampling in this study was made at various places in the 500-g consumer packages of butter protected by aluminum foil wrappings.

A comparison of the content of cholesterol oxides in a two-month-old butter sample from milk of cows grazing to those of two stored butter samples (4 mo old) from milk of cows fed on prepared feed during the winter revealed no differences. Hence, the variation in the sum and the level of the individual cholesterol oxides probably is related to the storage time rather than to different feeding systems and/or manufacturing technologies. However, the effect of feed type on cholesterol oxidation in milk fat needs to be studied further. These data are the first to give a more comprehensive picture of cholesterol oxides in butter.

Previousy, Csiky (19) used HPLC to quantify some autoxidation products of cholesterol, viz, 4-hydroxycholesterol, the epimeric 7-hydroxycholesterols, 7-ketocholesterol and 25-hydroxycholesterol in butter. A single unspecified sample had about 2.6 ppm of 25-hydroxycho-

TABLE 1

Content of Cholesterol Oxides in Butter

Storage time	Dairy[b]	Batch	Month produced	Churning technology	Cholesterol oxides, ppm in lipids[a]							
					5α-,6α-epoxy	5β-,6β-epoxy	7-keto	7α-hydroxy	7β-hydroxy	20α-hydroxy	25-hydroxy	5β,6α-dihydroxy
8 days	A	1	July	Continuous	ND[c]	ND	TR[d]	ND	ND	ND	ND	NA[e]
2 months	A	1	July	Continuous	0.3	0.5	0.2	0.2	0.3	0.4	ND	NA
10 days	B	2	July	Batch	ND	ND	TR	ND	ND	ND	ND	NA
1 month	B	3	April	Continuous	0.1	ND	TR	TR	TR	ND	ND	NA
1 month	C	4	April	Continuous	0.3	ND	TR	TR	TR	ND	ND	NA
1 month	D	5	April	Batch	0.4	ND	TR	TR	TR	ND	ND	ND
4 months	D	5	April	Batch	0.6	0.8	0.5	0.3	0.2	0.6	TR	ND
1 month	E	6	May	Batch	0.4	ND	TR	ND	ND	ND	ND	ND
4 months	E	6	May	Batch	1.0	1.2	0.6	0.3	0.3	0.9	ND	ND

[a]Fat content is 80% by weight.

[b]A, the Kattarp Dairy; B, Borgholm Dairy; C, Katrineholm Dairy; D, Götene Dairy; E, Umeå Dairy.

[c]Not detected.

[d]Traces (<0.1 ppm in lipids), detection limit 0.1 ppm in lipid.

[e]Not analyzed because of the low level of the isomeric 5,6-epoxycholestanol.

lesterol in the lipids, but the identity of that HPLC peak was not verified by other means. Three samples from a cold storage depot (14 days, 2 mo and 18 mo old) were analyzed by Fischer et al. (22). Only traces of some of the predominant oxidation products of cholesterol were detected. All aforementioned eight oxides were analyzed, but the isomeric 5,6-epoxycholestanols were not separated. The total lipids were saponified, which means that the analytical data by Fischer et al. (22) included also any esterified sterol oxides and that some artifacts were generated. The formation of cholesterol oxides was also studied by Luby et al. (24,25) during the fluorescent light exposure of unpackaged butter. The epimeric 7-hydroxycholesterols were detected after relatively long exposure time (about 15 days). The cholesterol oxides were found to be concentrated at the surface and proportional to the light absorbed as measured by the intensities of the spots on their thin layer chromatograms.

Heated butter. The butter temperature, heating time, peroxide value (PV) and anisidine value (AV) are presented in Table 2. The PV (essentially fatty acyl peroxides) increased slightly when a two-month-old butter sample was heated at 150°C for 10 min compared to the unheated control. Further, a gradual increase in the PV was noticed up to 190°C. Likewise, the AV, a measure of secondary oxidation products, rose in a parallel fashion (Table 2).

Butter heated at 150°C for 10 min, as in the control (a 2-month-old butter), contained no detectable levels of any of the cholesterol oxides at the detection limit 0.1 ppm, while after 10 min at 160°C significant levels of some oxides were observed. Further, the heated butter developed increased levels of some the cholesterol oxides, viz 5β-,6β-epoxycholestanol, 7-ketocholesterol and the epimeric 7-hydroxycholesterols with heating temperature up to 190°C. The level of 5α,6α-epoxycholestanol was found to be essentially unchanged during the heating experiment (Table 3). In a few cases, quantifiable levels of 25-hydroxycholesterol were found while the 20α-hydroxycholesterol and cholestane-triol were not observed at all in these samples (Table 3). The ratios of 5β-,6β-epoxy to 5α-,6α-epoxycholestanol and 7β-hydroxy to 7α-hydroxycholesterol in heated butter at 190°C were 6/1 and 1/1, respectively, compared to about 1/1 in unheated butter.

At 200°C, the PV, AV and the total amount of cholesterol oxides were lower than at 190°C when the butter was heated for 10 min (Tables 2 and 3). The level of 7-ketocholesterol was drastically lower, but the change in the levels of other oxides was not significant. The isomeric 5,6-epoxycholestanols together with 20α-hydroxycholesterol were the major products from the autoxidation of cholesterol in butter heated for 10 min at 200°C rather than the epimeric 7-hydroxycholesterols and 7-ketocholesterol. The ratio of 5β-,6β-epoxy to 5α-,6α-epoxycholestanol was about 1/1, and the ratio of 7β-hydroxy to 7α-hydroxycholesterol was 2/1.

Because frying at 200°C for 10 min is not common in actual kitchen work, an experiment at 200°C for five min was undertaken. The analytical results (Table 3) showed

TABLE 2

Peroxide Value (PV) and Anisidine Values (AV) in Unheated and Heated Butter[a]

Heating temperature (°C)	Heating time (min)	PV[b] (g eq/kg)	AV[c] (g eq/kg)
Control	0	0.1	0.1
150	10	0.2	1.5
160	10	0.3	1.4
170	10	0.6	4.1
180	10	0.6	5.5
190	10	0.6	9.6
200	5	0.3	10.4
200	10	0.5	8.5

[a]The values are means of duplicate sampling and analysis. The sample was a 2-month-old butter, manufactured by continuous technology at the Katrineholm Dairy.

[b]According to IUPAC No. 2:501 (*Standard Methods for the Analysis of Oils, Fats and Derivatives*, C. Paquot and A. Hautfenne, 1987, 7th edn., Blackwell Sci. Publ., Oxford).

[c]According to IUPAC No. 2:504 (*Standard Methods for the Analysis of Oils, Fats and Derivatives*, C. Paquot and A. Hautfenne, 1987, 7th edn., Blackwell Sci. Publ., Oxford).

TABLE 3

Content of Cholesterol Oxides in Unheated and Heated Butter

Heating temp (°C)	Heating time (min)	Cholesterol oxides,[a] ppm in lipids						
		5α,6α-epoxy	5β,6β-epoxy	7-keto	7α-hydroxy	7β-hydroxy	20α-hydroxy	25-hydroxy
Unheated	0	0.2	TR[b]	ND[c]	ND	ND	ND	0.1
150	10	ND	ND	ND	ND	ND	ND	ND
160	10	0.3	0.2	TR	ND	ND	ND	TR
170	10	0.2	0.1	0.4	0.1	0.2	ND	ND
180	10	0.2	0.4	0.5	0.2	0.1	ND	0.2
190	10	0.1	0.6	0.9	0.3	0.4	ND	ND
200	5	NQ[d]	0.2	NQ	0.1	0.1	ND	ND
200	10	0.3	0.4	0.2	0.1	0.2	0.4	ND

[a]5β,6α-dihydroxycholesterol (cholestane-triol) was not detected.

[b]Traces (<0.1 ppm), detection limit 0.1 ppm in lipids.

[c]Not detected.

[d]Not quantifiable, interference made the quantitation impossible.

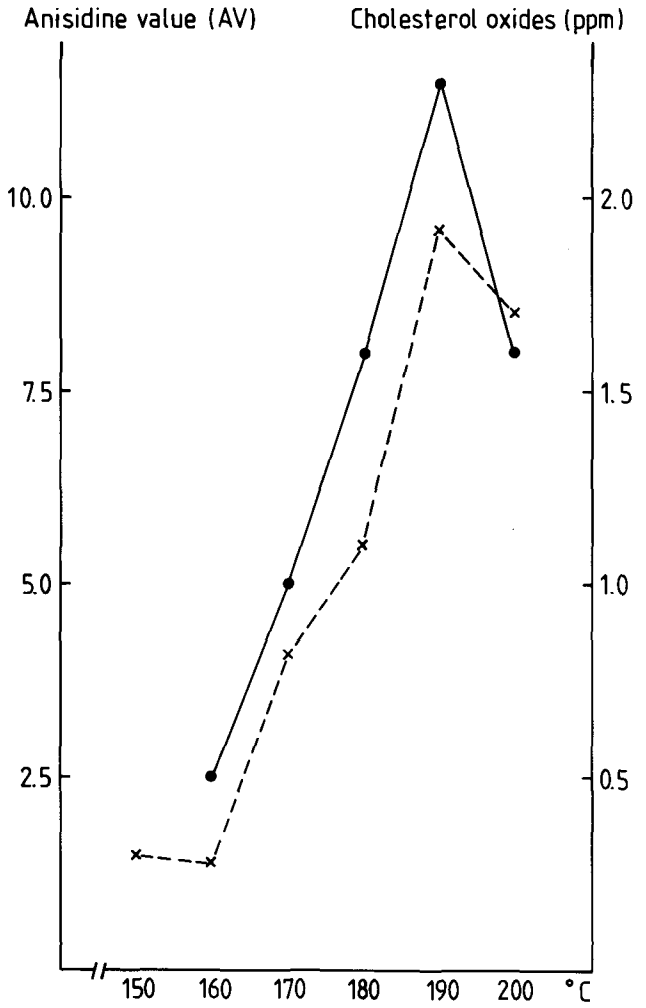

FIG. 1. The anisidine value and the content of cholesterol oxides (sum of all components quantified by GLC) in butter heated to different temperatures for 10 min. Anisidine value, ×————×; sum of the levels of cholesterol oxides, ●——●.

that the levels of individual cholesterol oxides were lower after five min at 200°C than those in butter heated at 190 and 200°C for 10 min. On the other hand, the AV was higher after 5 min at 200°C than those obtained in butter heated at 190 and at 200°C for 10 min. It is highly noteworthy that the AV rose in parallel to that of the sum of analyzed cholesterol oxides (Fig. 1).

Csiky (19) quantified some of the autoxidation products of cholesterol during heating of a one-g butter sample at 180°C for both five and 10 min in an open glass vessel. He found that about 4% of the cholesterol underwent autoxidation. In the short time heating, the 25-hydroxycholesterol and 7α-hydroxycholesterol were the major products followed by 7β-hydroxycholesterol and 7-ketocholesterol. In the long time heating, the epimeric 7-hydroxycholesterols were the major components, followed by 7-ketocholesterol, while the 25-hydroxycholesterol was not detected. Fischer et al. (22) found increased levels of total cholesterol oxides when butter was heated at 170°C for 24 and 72 hr. The isomeric 5,6-epoxycholestanols were the major products, followed by 7β-hydroxy and 7α-hydroxycholesterols. The 7-ketocholesterol was not detected because it was completely destroyed during the saponification. However, a comparison of the data reported in the present study with those of Fischer et al. (22) is not relevant because the surface to volume ratio and heating times used by them mirror conditions in deep fat frying and not those in pan frying.

Butter oil and cream oil. Other dairy products which received attention in the present investigation were butter oil and cream oil, because these products are exposed to heat during manufacturing from frozen butter and fresh cream, respectively, and they are ingredients in a very popular Swedish low fat spread.

Two different batches of freshly-made butter oil were investigated. The butter oils were manufactured from a four-month-old frozen butter. The frozen butter was transported directly from the storage depot to a melting device, and the melted butter was transferred to a storage tank where the proteins agglomerate. The melted butter was then transported to a separator where the oil was concentrated to more than 99%. The remaining water was removed by in vacuo drying and the dried oil transported to a buffer tank before packing. The analytical results (Table 4) showed that the samples contained about 0.2–0.3 ppm of 7β-hydroxycholesterol and traces of 5β,6β-epoxycholestanol. It must be noted that the identity of the 5β-,6β-epoxycholestanol in butter oil was confirmed only by spiking the actual TEAP-fractions with authentic 5β-,6β-epoxycholestanol. One of the two batches of frozen butter was available and analyzed after prolonged storage (4 mo later). No detectable levels of any of the eight "typical" oxides were observed.

Two different batches of freshly made cream oil were analyzed. The cream oil was manufactured according to the emulsion breaking method. The cream was concentrated and the oil droplets broken. Then, the emulsion was transported to a separator where the oil was concentrated. The samples contained measurable levels of the 7β-hydroxycholesterol, 7-ketocholesterol and traces of 7α-hydroxycholesterol (Table 4).

Hence, it appears that neither butter oil nor cream oil manufactured by the present Swedish technologies make an essential contribution to the level of cholesterol oxides in the low fat spread directly after production. However, because it also contains a vegetable oil, studies on changes in this product with storage have to await the completion of ongoing methodological studies on the isolation and quantitative determination of plant sterol oxides.

Melted cheese. Two different assortments of commercial melted cheese were analyzed for cholesterol oxides. Melted cheese generally is manufactured from cheeses with shape, color and texture defects or cheeses with unexpected defects in storage quality. The cheese is transferred to a scraper device and cleaned before cutting and grinding, further transferred into a melting pot where ingredients are added and the mixture is heated under continuous stirring. The melted cheese is transferred into a stainless steel container and transported to a packing machine. Melted cheese can be divided into two classes, (i) soft cheese with low pH 5.4–5.8 and high water content max. 70%, and (ii) hard cheese with high pH 5.8–6.4 and low water content max. 50%. Analyses of three different batches of soft cheese (6, 12 and 18 mo old, respectively) and two batches of hard cheese (one 3 mo and

TABLE 4

Content of Cholesterol Oxides in Butter and Cream Oils

Product	Batch	Cholesterol oxides,[a,b] ppm in lipids				
		5α-,6α-epoxy	5β-,6β-epoxy	7-keto	7α-hydroxy	7β-hydroxy
Butter oil	1	ND[c]	TR[d]	ND	ND	0.2
Butter oil	2	ND	TR	ND	ND	0.3
Cream oil	1	ND	ND	0.2	TR	0.3
Cream oil	2	ND	ND	0.4	TR	0.2

[a]5β,6α-Dihydroxycholestanol (cholestane-triol) was not analyzed.

[b]20α-Hydroxy and 25-hydroxycholesterol were not detected in these samples.

[c]Not detected.

[d]Traces (<0.1 ppm in lipids), detection limit 0.1 ppm in lipid.

TABLE 5

Content of Cholesterol Oxides in Various Cheese Types

Products	Storage time (mo)	Fat content % by weight	Cholesterol oxides,[a] ppm in lipids				
			5α-,6α-epoxy	5β-,6β-epoxy	7-keto	7α-hydroxy	7β-hydroxy
Hard melted	2	13	TR[b]	TR	ND[c]	TR	ND
Hard melted	4	13	TR	TR	ND	TR	ND
Soft melted	6	8	ND	ND	ND	ND	ND
Soft melted	12	8	TR	ND	ND	ND	ND
Soft melted	18	8	TR	ND	ND	ND	0.2
Grated I	4	28	TR	TR	0.2	ND	0.3
Grated I	4+4	28	0.2	0.2	ND	0.5	0.6
Grated I	4+8	28	TR	0.3	TR	0.5	0.8
Grated II[d]	9	28	0.4	ND	0.2	0.8	0.8
Grated III	12	28	0.5	NQ[e]	0.4	0.3	0.6

[a]5β,6α-Dihydroxycholesterol (cholestane-triol) was not analyzed because of the low figures for the isomeric 5,6-epoxycholestanols.

[b]Traces (<0.1 ppm in lipids), detection limit 0.1 ppm in lipid.

[c]Not detected.

[d]A trace of 25-hydroxycholesterol was detected in this sample.

[e]Not quantifiable because of an interfering compound.

another 4 mo old) showed that these samples contained only traces of some of the oxides (Table 3). This report seems to be the first to give quantitative data on cholesterol oxides on melted cheese. Melted cheese obviously appears to be a minor source of such oxides.

Grated cheese. Three small packages of grated cheese (Rivosto) were investigated. According to the label the age was from four mo up to 12 mo. These samples, marked I, II and III, contained quantifiable levels of the isomeric 5,6-epoxycholestanols, the epimeric 7-hydroxycholesterols and 7-ketocholesterol (0.5–2.2 ppm of total cholesterol oxides in lipids, which means about 0.2–0.7 ppm in the samples). Further storage of the four-month-old sample of grated cheese in a sealed bag at room temperature in the laboratory for four and eight mo caused a slight increase in the concentration of some of the oxidation derivatives of cholesterol (Table 5). The ratio of 7β-hydroxy to 7α-hydroxycholesterol was about 1/1. It should be remarked that the samples do not represent a single original batch, but each is manufactured independently. However, the findings in the present study suggest that the occurrence of cholesterol oxides in Swedish grated cheese could reflect different drying parameters and/or drying technologies rather than storage effects. Obviously, grated cheese on a weight basis is a more important source of cholesterol oxides than hard melted and soft melted cheese.

Peng et al. (20) isolated and tentatively identified some of the cholesterol oxides, viz, the C-7 oxidation derivatives of cholesterol and also cholestane-triol in Parmesan cheese. Finocchario et al. (21), using TLC analysis, screened eight grated cheese samples and found that four were "positive." The positive samples were re-analyzed and the cholesterol oxides estimated by TLC analysis using the spot area method. The analytical results showed that these samples contained 20–110 ppm 5,6-epoxycholestanols, 0–10 ppm of each of the 7-hydroxycholesterols in lipids. It must be emphasized that both the

workup procedure and the quantification method used by Finocchiaro et al. (21) must likely result in too high figures. Three grated cheese samples, analyzed by Fischer et al. (22), contained 4.5–10.3 ppm of the cholesterol oxides in the lipids, which means about 1.5–3.4 ppm in the samples. A comparison of the quantitative data presented herein to those of Fischer et al. (22) is of little significance due to the important methodological differences discussed under "Butter."

ACKNOWLEDGMENTS

This work was supported by Grant 610/82 D 16:2 of the Swedish Council for Agriculture and Forestery Research. The Swedish Dairies' Association, the Arla Dairies and the Nedre Norrlands Producentförening supplied the samples. The Margarine Company AB, Helsingborg, Sweden supported the heating experiment (Anna-Clara Pihlgren) and analysis of the peroxide value and anisidine value (Hans Gran). Lena Killingmo provided technical assistance, and Ulf Bondesson assisted in the GC-MS analysis.

REFERENCES

1. Maerker, G., *J. Am. Oil Chem. Soc. 64*:388 (1987).
2. Addis, P.B., *Food Chem. Toxic. 24*:1021 (1986).
3. Jacobson, M.S., *Lancet 19*: (1987).
4. Peng, S.K., P. Tham and C.B. Taylor, *Am. J. Clin. Nutr. 32*:1033 (1979).
5. Baranowski, A., C.W.M. Adams, O.B. Baylliss High and D.B. Bowyer, Atherosclerosis 41:255 (1982).
6. Peng, S.K., B.C. Taylor, E.H. Mosbach, W.Y. Huang, J. Hill and B. Mikkelsson, *Ibid. 41*:395 (1982).
7. Kummerow, F.A., *Ann. NY. Acad. Sci. 4*:29 (1983).
8. Imai, H., N.T. Werthessen, C.B. Taylor and K.T. Lee, *Arch. Pathol. Lab. Med. 100*:565 (1976).
9. Peng, S.K., C.B. Taylor, J.C. Hill and R.J. Morin, *Atherosclerosis 54*:121 (1985).
10. Jacobson, M.S., M.G. Price, A.E. Shamoo and F.P. Heald, *Ibid. 57*:209 (1985).
11. Matthias, D., C.H. Becker, W. Gödicke, R. Schmidt and K. Ponsold, *Ibid. 63*:115 (1987).
12. Kandutsch, A., and H.W. Chen, *J. Biol. Chem. 249*:6057 (1974).
13. Peng, S.K., R.J. Morin, P. Tham and C.B. Taylor, *Artery 13*:144 (1985).
14. Parish, E.J., V.B.B. Nanduri, H.H. Kohl and F.R. Taylor, *Lipids 21*:27 (1986).
15. Bischoff, F., *Adv. Lipid Res. 7*:165 (1969).
16. Sevanian, A., and A.R. Peterson, *Proc. Natl. Acad. Sci. USA 81*:4198 (1984).
17. Sevanian, A., and A.R. Peterson, *Food Chem. Toxic. 24*:1103 (1986).
18. Raaphorst, G.P., E.I. Azzam, R. Langlois and J.E. Van Lier, *Biochem. Pharmac. 36*:2369 (1987).
19. Csiky, I., *J. Chromatogr. 241*:381 (1982).
20. Peng, S.K., and C.B. Taylor, in *Dietary Fats and Health*, edited by E.G. Perkins and W.J. Visek, American Oil Chemists' Society, Champaign, IL, 1983, pp. 919–933.
21. Finocchiaro, E.T., K. Lee and T. Richardson, *J. Am. Oil Chem. Soc. 61*:877 (1984).
22. Fischer, K.-H., G. Laskawy and W. Grosch, *Z. Lebensm. Unters. Forsch. 181*:14 (1985).
23. Gray, J.I., B.R. Harte, J.M. Luby, T.C. Ryan and S.L. Cuppett, in *Euro. Food Pack.*, edited by E. Bojkow, S. Bruin, S. Czedik-Eysenberg, F. Paine and W. Pfannhause, Vienna, Austria, 1985, pp. 275–279.
24. Luby, J.M., J.I. Gray, B.R. Harte and T.C. Ryan, *J. Food Sci. 51*:904 (1986).
25. Luby, J.M., J.I. Gray and B.R. Harte, *Ibid. 51*:908 (1986).
26. Nourooz-Zadeh, J., and L.-Å. Appelqvist, *Ibid. 51*:908 (1986).
27. Nourooz-Zadeh, J., and L.-Å. Appelqvist, *Ibid. 53*:74 (1988).

[Received December 28, 1987; accepted April 6, 1988]