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Identification and Quantification of Cholesterol Oxides in Grated Cheese and Bleached Butteroil

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ABSTRACT

Butteroil samples bleached with benzoyl peroxide (BP) and 17 commercial cheeses were screened for oxidized sterols by thin layer chromatography (TLC). Ungrated cheeses made from bleached milk and freshly bleached butteroil contained no detectable oxidized sterols. Oxidized sterols were detected in stored, bleached butteroils and in grated cheeses. Four major oxidation products were the isomeric 5,6-epoxycholesterols and the epimeric 7-hydroxycholesterols identified by TLC, high performance liquid chromatography (HPLC) and mass spectrometry (MS). Additional sterol oxides (tentatively identified and not quantified) present in these samples included low levels of 7-ketocholesterol and cholesta-3,5-dien-7-one. The epimeric 7-hydroxycholesterols were detected in bleached butteroils stored in air (BP-A) and nitrogen (BP-N) for 22 days at 15 C. Butteroil, after 90 days of storage at 15 C, had 30 (BP-N) and 60 (BP-A) μg total oxides/g of bleached oil and, after 1-year at −20 C, had 70 (BP-N) and 180 (BP-A) μg/g butteroil. A grated, unbleached cheese packaged in clear glass contained the most oxidized sterols (44 μg/g). Sterol oxides were not detected in bleached cream using a simulated industrial process.

INTRODUCTION

The toxicity of dietary oxidized cholesterol has been studied using both in vivo (1,2) and in vitro (3-6) systems. The 25-hydroxy cholesterol and the isomeric 5,6-epoxides are common autoxidation products of cholesterol that have received considerable attention in recent years (7). The epoxides as well as other cholesterol oxides are formed by attack of several oxygen species (7), some of which occur in food systems (8). Although little is known about the biological effects of the β-epoxide (1,9,7,10), many studies have linked the α-epoxide to the development of atherosclerosis and cancer (1-4,7,11,12).

Smith's comprehensive review (7) on the autoxidation of cholesterol and biological effects of cholesterol oxides illustrates the need to characterize and quantify sterol oxides in processed foods. Recent reviews (7,13) have documented the probable occurrence of common sterol autoxidation products in food. Two studies that have clearly identified cholesterol epoxide(s) (14,15) involved dried egg products. Some studies have used cholesterol-rich foods subjected to high oxidative stress, which would rarely occur in foods. For example, Chicoye et al. (14) identified the β-epoxide and other sterol oxides in spray-dried egg yolks exposed to direct sunlight for 5 hours. Tsai et al. (15) found 1-33 μg/g α-epoxide in commercial dried-egg products (7).

Although autoxidation of sterols in most foods will occur in time, little is known of the rate of oxidation, the quantities and distribution of the oxidation products and the factors that accelerate sterol oxidation in foods. Attention should be given to foods rich in cholesterol that have been processed with prooxidants, which could generate sterol oxides. Benzoyl peroxide (BP), a widely used free-radical generating agent (16), is allowed in the US to bleach flour and in milk (at 20 μg/g) to make Blue, Swiss and Italian cheeses (17). Although BP itself or flour treated with it, and bread baked from BP-treated flour, carry no significant carcinogenic hazard (18), recent studies using mice have shown BP to be a promoter of skin tumors, possibly from the generation of free radicals (16). Thus, residual BP in foods should be of concern to the food industry. This research detected, and quantified, oxidized sterols in butteroil and in cream treated with BP and in commercial cheese samples purchased from local sources.

MATERIALS AND METHODS

Bleaching of Butteroil and Cream

Anhydrous butteroil was prepared from fresh, unsalted butter (University of Wisconsin Dairy Laboratory) by melting it at 60 C, separating it by centrifugation, washing it once with distilled water and drying it over anhydrous sodium sulfate. Butteroil samples were bleached (in duplicate) with BP (Aldrich Chemical Co., Milwaukee, WI, 99.2% peroxide by analysis (19)). Sufficient BP dissolved in 50.0 mL ethyl acetate was added to 1,440 g of oil to give a final concentration of 500 μg/g. After being stirred (in darkness) for 2 hr at 60 C, 40 mL portions of bleached oil were stored (in darkness) in test tubes sealed with Teflon-lined screw caps (Scientific Products, Inc., McGraw Park, IL). Samples were stored either under N_2 (BP-N) or air (BP-A) at 15 C for 90 days, then an additional yr at −20 C. Controls were treated only with solvent and stored as

above. Portions (2.00-5.00 g) were assayed for peroxide values (PV) during storage. Duplicate (3.0 g) portions of oil were saponified immediately after each peroxide assay and examined for oxidized sterols. Antioxidants (5 mg butylated hydroxytoluene (BHT, Sigma Chemical Co., St. Louis, MO) and 30 mg dilaurylthiodiproprionate (TDP, Evans Chemetics, Inc., Darien, CT)) were added before saponification.

Fresh raw cream (University of Wisconsin Dairy Laboratory, 38.2% fat (w/w) by analysis (20)) was bleached with 154 μg BP/g cream by adding 25.0 mL of an ethyl acetate solution of BP to 3 kg cream and shaking (in darkness) at 80 oscillations per min (opm) for 2 hr at 60 C. Portions of cream were withdrawn at timed intervals and milk fat was isolated using a modified method of Stine and Patton (21). Samples (150 mL) were added to 200 mL centrifuge tubes containing 7.5 mL Tergitol-7 (Sigma Chemical Co., St. Louis, MO), and centrifuged (3,000 g, Sorvall centrifuge, DuPont Co., Newtown, CT) for 10 min to isolate milk fat. Recovered milk fat (10.0 g samples in triplicate) was used for peroxide determination during bleaching. Controls were treated only with solvent. Duplicate (3.0 g) samples of isolated milk fat were examined for oxidized sterols immediately after the 2 hr bleaching treatment. Antioxidants (5 mg BHT and 30 mg TDP) were added before saponification.

The scheme for the extraction, purification and analysis of oxidized sterols in cheese is shown in Figure 1.

Isolation of Cheese Lipids

The method of Bligh and Dyer (22) was used to extract lipids from commercial cheeses. Twenty-five grams of shredded cheese (in duplicate) were blended for 1 min each with 200 mL methanol/chloroform (2:1 v/v) containing 10 mg BHT and 10 mg TDP. Water (53 mL) was added to give a methanol/chloroform/water ratio of 2:1:0.8 (v/v/v) before blending the mixture for 1 min. Solids were removed by filtration through a triple layer of cheesecloth before lipids were partitioned into the chloroform phase by adding appropriate volumes of chloroform and water to the filtrate (22). The chloroform phase was dried with anhydrous sodium sulfate followed by centrifugation (3,000 g) for 15 min. Solids on top of the chloroform were removed by aspiration before decanting. Portions of the chloroform solution were dried to constant weight to determine yield.

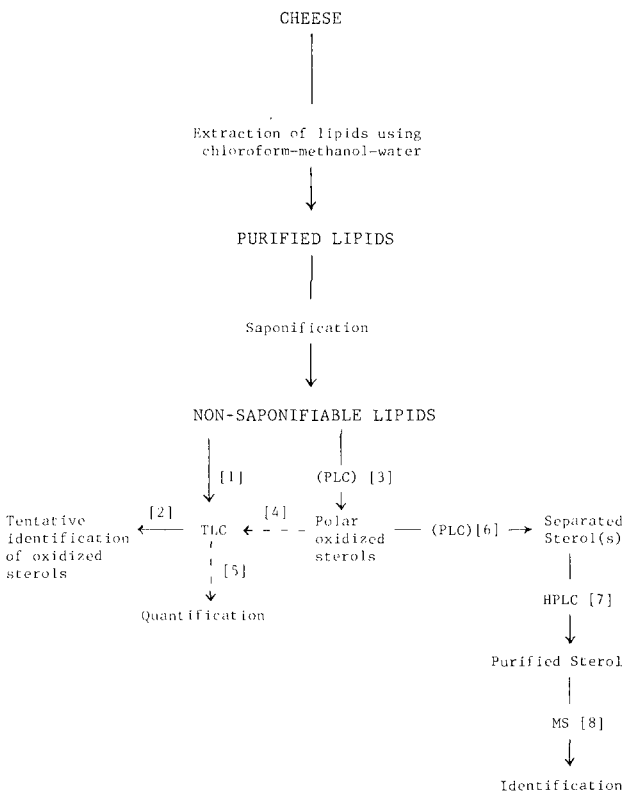

FIG. 1. Scheme for the extraction, purification and analyses of oxidized sterols in commercial cheese samples. Preparative layer chromatography, PLC; thin layer chromatography, TLC; high performance liquid chromatography, HPLC; mass spectrometry, MS.

Most sterol standards (i.e., cholesterol, the epimeric 7-hydroxycholesterols, 7-ketocholesterol and cholestane-3β,5α,6β-triol) were obtained commercially (Steraloids, Inc., Wilton, NH) and purified where necessary. The 5α,6α-epoxide was synthesized from purified cholesterol (23) and recrystallized from 20% aqueous acetone. Identities of standard sterols were confirmed using chromatographic, spectral and chemical analyses.

Saponification and TLC

Cholesterol and oxidation products were concentrated as nonsaponifiable lipids before TLC analyses. Purified samples were saponified (protected from light) for 2 hr in 30 mL 95% ethanolic 1.5 N KOH. After dilution with 100 mL water, nonsaponifiable lipids were extracted with ethyl ether (3 × 100 mL), washed with 0.5 N KOH (4 × 100 mL) and distilled water (4 × 100 mL). The lipid residue was concentrated in vacuo at 35 C to 500 μL. One hundred microliters of each ether solution, along with oxidized cholesterol standards, was applied to heat-activated (120 C, 1 hr) silica gel TLC plates (Polygram Sil G (0.25 mm), Brinkman Instruments, Inc., Westburgy, NJ). Plates were developed in ethyl ether (solvent A) (Fig. 1 [1]), dried, sprayed with 25% aqueous para-toluene sulfonic acid (p-TSA, Sigma Chemical Co.) and heated at 110 C for 5 min for color development (Fig. 1 [2]) (24). The 5,6-epoxides and cholestane-triol exhibited fluorescence under 254 nm light after p-TSA treatment. This TLC system (Fig. 1 [1] [2]) was used as a preliminary test for oxidized sterols. On tentative identification of cholesterol oxidation products, additional chromatograms were run 3 times successively in ethyl acetate/heptane (1:1 by vol, solvent B) (Fig. 1 [1]) or twice (successively) in acetone/heptane (1:1 by vol, solvent C) (Fig. 1 [1]) and developed with p-TSA as before (Fig. 1 [2]). Relative migration values (R_c), defined as the ratio of distance to the center of the sample spot from origin to that of the cholesterol spot, were computed for each solvent.

Preparative Layer Chromatography (PLC)

Silica gel UV_{254}-PLC plates (1 mm thick) (25) enabled isolation of total oxidized sterols (Fig. 1 [3]) before quantitative TLC (Fig. 1 [4]) or HPLC. Nonsaponifiable lipids from 3 g of duplicate oil samples were loaded on the center of the plate and sterol standards were applied to each edge, chromatographed with solvent B (2 successive developments) (Fig. 1 [3]) and illuminated with 254 nm light to detect fluorescence-quenching compounds (including unsaturated ketosteroids) (7). Sterol zones were located by spraying with a 2% chloramine (Sigma Chemical Co.) solution in H_2SO_4 (26). The center lane, containing compounds more polar than cholesterol, was scraped from the plate and extracted with chloroform. Each PLC extract containing mixtures of polar cholesterol oxidation products was concentrated in vacuo to 50 μL before quantitative TLC.

Quantitative TLC

Sterols were quantified after chromatographic separation

(Fig. 1 [4]) using the spot area method (Fig. 1 [5]) (27). Twenty-five μL of oxidized sterol isolates in chloroform were applied to TLC plates, which were developed 3 times in solvent B (Fig. 1 [4]). For the quantification of unoxidized cholesterol (before the above PLC isolations), 3.0 μL of the original nonsaponifiable lipids (in 500 μL ether) were applied to the plates for development as before (Fig. 1 [1] [5]). Separated sterols were seen as colored spots after treating with p-TSA. Spot area was measured at least 3 times with a digital planimeter (Planix 2, Tamaya and Co., Tokyo, Japan). Sterol concentrations were derived from a standard curve prepared using indicated sterol standards. The epimeric 7-diols were quantified from the epoxide standard curve. The correlation coefficient (r) for each standard curve was determined from linear regression analysis (cholesterol r = 0.992, 5,6-epoxide r = 0.979, triol r = 0.937).

High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS)

Individual sterols isolated from a cheese nonsaponifiable sample were purified and analyzed by PLC, HPLC and MS to confirm the tentative TLC identifications. Before HPLC, polar sterols extracted as a single isolate from PLC developed twice in solvent A (Fig. 1 [3]) were rechromatographed on PLC plates (Fig. 1 [6]) employing triple development using solvent B. Each purified sample and standard was filtered through a 0.5 μm filter (Millipore Corp., Bedford, MA) and subjected to HPLC (Fig. 1 [7]) with a μ-Bondapak C_{18} reversed-phase column equipped with a C_{18} precolumn (Waters Associates) with acetonitrile/H_2O (9:1, v/v) (28) as the mobile phase. Column effluent was monitored with a Waters Associates differential refractometer (Model 401) for the detection of the epoxides and triol, whereas a variable UV detector (Schoeffel Spectroflow 770) at 212 nm detected the 7-diols. Purified sterols were further characterized using MS (Fig. 1 [8]) as detailed by Daly et al. (25) for oxidized β-sitosterol derivatives.

RESULTS AND DISCUSSION

Peroxide Analyses

Mean peroxide values (PV) for butteroils treated with BP during storage at 15 C for 90 days are reported in Figure 2. Peroxide values for untreated, control samples were zero throughout. Freshly prepared butteroils were bleached with 500 μg BP/g butteroil, which represents the highest level expected in the fat phase if all the BP legally allowed for bleaching milk (20 μg/g) partitions into the fat phase. Storage for 90 days at 15 C under air resulted in a 97% increase in PV compared with a 16% increase when samples were kept under nitrogen. Furthermore, frozen storage (−20 C) of these fats for an additional year (data not shown) resulted in a 240% increase in peroxides (PV = 36 ± 1.8, n = 6) for BP-A, whereas BP-N exhibited a 26% decrease in peroxides (PV = 4.6 ± 0.6, n = 6). This large increase in PV noted for the BP-A sample during −20 C storage might be partially caused by the increased solubility of oxygen in the liquid (uncrystallized) portion of milk fat at lower temperatures (29). Although O_2 is effectively excluded from the crystalline milk fat (29), the enhanced concentration of O_2 in the more unsaturated liquid-fat phase should sustain continued peroxidation of the lipids.

These data demonstrate the development of lipid hydroperoxides in aged, bleached butteroil samples, but most bleached cream used in commercial cheese has about 154 μg BP/g cream (30). This level of BP was therefore used in other studies for bleaching 38.2% cream. Peroxide values during the 2 hr bleaching process are shown in Figure 3. An

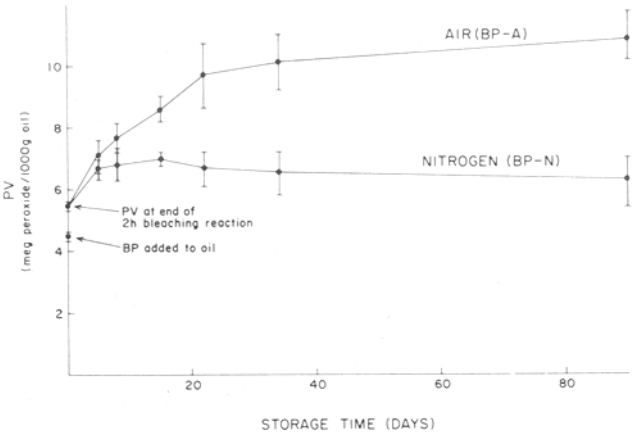

FIG. 2. Peroxide values (PV) of butteroils bleached with benzoyl peroxide (BP) stored under air (BP-A) or nitrogen (BP-N) at 15 C (mean ± SD of duplicate samples, each assayed in triplicate [n = 6]).

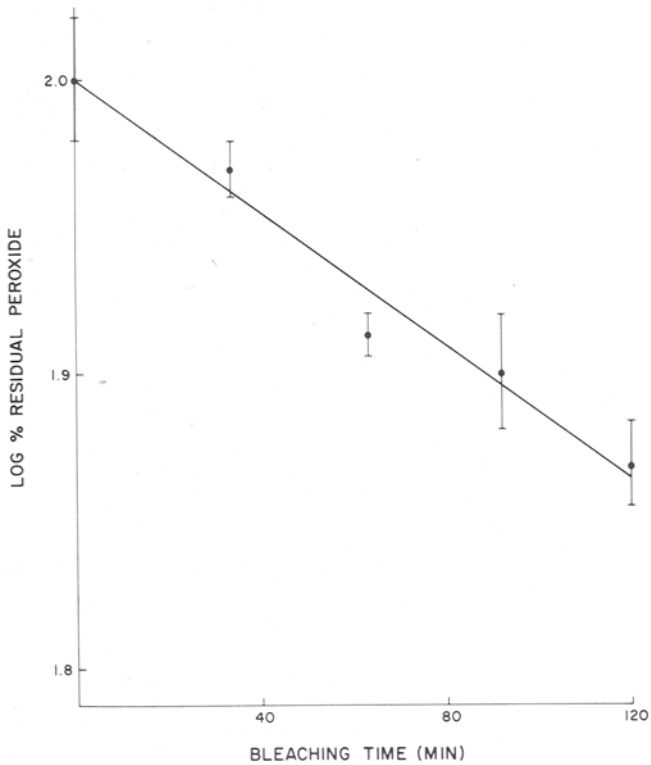

FIG. 3. Residual peroxide detected in cream during 2 hr bleaching with benzoyl peroxide at 60 C (means ± SD, n = 3). Slope determined by linear regression analysis (r = −0.939).

insufficient number of half-life values were available for the decomposition of BP to adequately define the kinetics, but an apparent first-order rate of peroxide decrease was observed. This was analogous to that determined during the bleaching of whey (31). Moreover, 73% of the original peroxide remained in the cream after the 2 hr bleaching indicating the possibility for residual BP in commercially bleached cream.

Sterol Analyses

A problem in analyzing cholesterol-containing foods is the formation of artifacts (7). Control experiments indicated that the analytical and preparative procedures used in these studies did not create artifactual oxidized sterols. Freshly recrystallized cholesterol (30 mg) was added to each duplicate 25 g portion of a commercially available Parmesan cheese packaged in a clear glass bottle. The procedures used

for sterol quantification resulted in no significant increase in endogenous oxidized sterols when compared with control samples (Students *t*-test at $p > .01$).

Butteroil containing purified sterol standards was used to determine yields. Butteroil containing added cholesterol (4 mg/g) subjected to the above isolation procedures had an average sterol yield of 96 ± 7% (n = 12, not shown). No differences were found in sterol yields (one-way analysis of variance at P = .01) when butteroil was subjected to saponification, chloroform-water purification and saponification or chloroform-water purification, saponification and PLC isolation of cholesterol. Studies with purified α-epoxide and cholesterol subjected to the above isolation techniques indicated an average yield of 72 ± 6% (n = 12) and 95 ± 7% (n = 12), respectively. Again, no significant difference in epoxide or cholesterol yield (P = .01) was noted using the above 3 isolation techniques. The antioxidants added to the saponification mixture had no significant effect on epoxide recovery. Recovered cholestane-triol (expected from the hydrolysis of the epoxide) accounted for only 4% of the original epoxide. Experiments with purified 7-ketocholesterol indicated the 7-ketone was completely destroyed during saponification with possible formation of its thermal degradation product (cholesta-3,5-dien-7-one) (7) indicated by TLC (not shown).

Cholesterol oxidation products identified and quantified in bleached butteroils during storage at 15 C and at −20 C are listed in Table I. The epimeric 7-hydroxysterols were detected in both BP-N and BP-A samples as early as 22 days of storage at 15 C. The 5,6-epoxide was detected in BP-A after 6 mo at −20 C. Peroxide values per se cannot be correlated to the amount and occurrence of cholesterol oxides in purified lipids. This is illustrated by comparing the PV of the 15-day BP-A sample that had no detectable oxidized sterols against the lower PV of the 22-day BP-N sample which contained oxidized sterols (Fig. 2). Moreover, a BP-A sample stored at ambient temperature ca. 10 ft below ceiling fluorescent lamps contained 7-hydroxysterols (when examined by TLC) after 2 days of storage. Photocatalyzed oxidative changes are indicated in view of the 22 days required for development of the same oxidized sterols from BP-A stored in the dark at 15 C.

Commercial cheese samples surveyed for cholesterol oxidation products are listed in Table II. Federal regulations require the labeling of products that contain milk bleached with BP (17). The 4 bleached cheeses examined (Table II) contained no detectable sterol autoxidation products, indicating commercial bleaching does not oxidize cholesterol.

The data in Table II indicate 4 of 8 commercial grated cheeses were the only samples that contained oxidized sterols. A comparison of the oxidized sterol data (Table I) illustrates the problems of using model systems for extrapolation to complex food products such as aged, grated cheese. Bleached butteroils contained greater amounts of the epimeric 7-hydroxysterols compared with the epoxide (Table I). However, the opposite trend was observed with the cheese samples (Table I). These differences indicate different oxidative mechanisms in the 2 systems. Trace levels of certain metals in milk (32) catalyze the epoxidation of cholesterol (7) in cheese fat associated with protein-metal complexes. Our data also indicate, in some cases,

TABLE I

Sterol Contents of Bleached Butteroils (as a Function of Storage) and Aged Italian Cheese Samples

	μg/g Oil[a,b] (μg/g cheese)[c]				
	Cholesterol	5,6-Epoxycholesterol	7β-Hydroxycholesterol	7α-Hydroxycholesterol	Cholestane-triol
Bleached b butteroils					
Nitrogen (BP-N)					
90 days at 15 C	3400 ± 350	ND[d]	20 ± 3	10 ± 3	ND[d]
One year at −20 C (after 90 days at 15 C)	3300 ± 190	20 ± 3	30 ± 6	20 ± 6	NQ[e]
Air (BP-A)					
90 days at 15 C	3400 ± 250	ND[d]	30 ± 2	30 ± 2	ND[d]
One year at −20 C (after 90 days at 15 C)	3300 ± 300	30 ± 5	90 ± 5	60 ± 4	NQ[e]
Cheese samples[f]					
Parmesan					
Brand A in clear glass bottle	4100 ± 510	110 ± 10 (32 ± 2.9)	20 ± 2 (6 ± 0.6)	20 ± 2 (6 ± 0.6)	4 ± 2 (2 ± 0.6)
Brand A in cardboard shaker box	4100 ± 340	30 ± 4 (9 ± 1.2)	10 ± 4 (3 ± 1.2)	10 ± 4 (3 ± 1.2)	NQ[e]
Brand D in cardboard shaker box	4400 ± 440	20 ± 4 (6 ± 1.2)	NQ[e]	NQ[e]	NQ[e]
Romano					
Brand A in clearn glass bottle	4300 ± 570	50 ± 5 (16 ± 1.6)	10 ± 2 (3 ± 0.6)	10 ± 2 (3 ± 0.6)	NQ[e]

[a]Means ± SD of 2 replicates, each in duplicate (n = 4).

[b]Sterol content corrected for the following recoveries of standards: cholesterol and 7-hydroxysterols (based on cholesterol)−95%; 5,6-epoxides −72%.

[c]Based on the following fat (w/w) content of the cheeses as determined by the Babcock modified fat test (20): Parmesan samples−29.2%; Romano samples−32.3%.

[d]Not detected. Detection limit for sterols is approximately 3 μg/g oil.

[e]Not quantifiable.

[f]Commercial cheese purchased as grated products (see Table 2).

TABLE II

Commercial Cheese Samples Assayed for Oxidized Sterols

Type	Brand	Purchased form
Parmesan	A[a]	Grated[b]
	A[a]	Grated[c]
	D[a]	Grated[b]
	E	Grated[d]
Romano	A	Grated[b]
	A[a]	Grated
	D	Wedge
	E	Grated[d]
Provolone	B[e]	Block
	D	Sliced
	E	Grated[d]
Swiss	A	Sliced
	F	Block
	G	Sliced
Blue	B[e]	Wedge
	B[e]	Crumbled
	C[e]	Block

[a]Oxidized sterols detected and quantified in these samples (see Table I). Remaining products did not contain detectable levels of sterol oxides (less than 3 μg of each sterol/g oil).

[b]Purchased in cardboard shaker box.

[c]Purchased in clear glass shaker bottle.

[d]Grated at time of purchase.

eLabeled "milk bleached with benzoyl peroxide."

greater amounts of the 7β-epimer compared with that of the α-epimer (Table I). These data are consistent with published observations and apparently result from greater stability of the equitorial (7β-epimer) over the axial 7α-epimer (7).

Although the data in Table I are from a single random sampling of commercial products, the same oxidation products were found in 2 other grated Parmesan samples (both brand A) packaged in glass containers. One of the samples contained 80 μg/g oil of the isomeric epoxides (n = 2) whereas the other was used only for qualitative analysis of sterols employing HPLC-MS.

The latter 2 grated cheese products (Parmesan and Romano, brand A) packaged in clear glass bottles contained the highest amounts of oxidized sterols. Preparations packaged in cardboard contained lower amounts of oxidized sterols. A grated Romano (brand A) marketed in cardboard did not contain any detectable oxidized sterols. These results suggest photooxidation is partially responsible for the greater amounts of oxidized sterols found in the cheese packaged in clear glass. Moreover, the grated Parmesan (brand A) contains more oxidized sterols relative to the grated Romano (brand A), regardless of container (Table I). The higher level of oxidized sterols in the Parmesan cheese might reflect longer storage times because it was aged 10 months compared with 5 or more months for the Romano (as indicated by manufacturer's labels).

Only 4 major sterol oxidation products were detected in cheese and butteroil using preliminary TLC (Table III). The sterol oxides cochromatographed with sterol standards when the TLC plates were developed in each of 3 solvent systems. Moreover, R_c values of these sterol oxides are within 86-95% of published values (13,44) obtained using the same solvent systems. Other probable sterol oxides were evident on quantitative TLC analyses of the concentrated extracts (Table IV). Some of these products, however, may be artifacts. Spot 5 (Table IV) could be the 7-alkyl ether of 7-hydroxycholesterol resulting from its

TABLE III

Relative Migration of Cholesterol Oxidation Products Isolated from Cheese Samples and Bleached Butteroils on Thin-Layer Chromatography

Identity	R_c I[b]	R_c II[c]
5,6-Epoxycholesterols	0.68	0.80
7β-Hydroxycholesterol	0.49	0.62
7α-Hydroxycholesterol	0.40	0.62
Cholestane-triol	0.18	0.48

[a]R_c-mobility values relative to cholesterol on single development of silica gel TLC plates.

[b]TLC solvent system—ethyl ether at room temperature.

[c]TLC solvent system—acetone/heptane (1:1 by vol) at room temperature.

TABLE IV

Some Characteristics of Oxidized Sterols and Unidentified, Suspected Sterol Oxidation Products after Thin Layer Chromatography of PLC Isolates[a,b,c]

Spot no.	Identity	R_c	Color[e]
1	Unidentified[f]	0.80	Yellow tan
2	5,6-Epoxycholesterols	0.72	Golden yellow[g]
3	Unidentified[h]	0.66	Pinkish yellow
4	7-Ketocholesterol[i]	0.64	—[j]
5	Unidentified[f]	0.61	Blue
6	7β-Hydroxycholesterol	0.50	Blue
7	7α-Hydroxycholesterol	0.44	Blue
8	Unidentified[g]	0.38	Pinkish tan
9	Cholestane-triol	0.15	Golden yellow[g]

[a]Preparative layer chromatography (PLC) isolates containing compounds more polar than cholesterol (see [3] and [4] in Fig. 1).

[b]From cheese samples listed in Table 2.

[c]From bleached butteroils (stored under air or nitrogen) kept for 1 year at −20 C after storage at 15 C for 90 days.

[d]R_c-mobility value for cholesterol on triple development of silica gel plates using ethyl acetate-heptane (1:1 by vol).

[e]After being sprayed with 25% p-toluene sulfonic acid (p-TSA).

[f]Detected only in bleached butteroil samples and in brand A grated Parmesan cheese packaged in glass containers.

[g]Fluorescence on irradiation with UV (254 nm) light after color development with p-TSA reagent.

[h]Detected in isolates from cheese only.

[i]A standard 7-ketocholesterol that was not detected in isolates from cheese or butteroil samples.

[j]Detected before p-TSA treatment by quenching fluorescence on irradiation of silica gel UV_{254} plates with 254 nm light.

etherification during extraction and saponification (7). The saponified α-epoxide standard contained a small golden yellow spot (when sprayed with p-TSA) with the same R_c as spot 3 (Table IV), suggesting that a portion of this spot may also be an artifact of saponification. The triol quantified in brand A grated Parmesan (Table I) is 4% of the original epoxide. Our studies on the saponification of pure epoxide indicated a triol content equivalent to 4% of the original added epoxide. This observation suggests all of the quantified triol in the Parmesan sample (Table I) was an artifact of saponification.

Because 7-ketocholesterol is often formed simultaneously with the oxidized sterols observed in the present study (7), this ketone was probably present in the original cheese and bleached butteroil but was destroyed during saponification (7). No fluorescence-quenching zones (indicative of the 7-ketone) were present after separation of nonsaponifiable lipids. Although a fluorescence-quenching zone corresponding to the cholesta-3, 5-dien-7-one was

observed, overlapping nonsaponifiable and antioxidant materials precluded verifying the presence of this sterol. Identification of the major oxidized sterols in the cheese required isolation of individual sterol zones from PLC plates, purification by HPLC and MS analysis. All standards were purified by HPLC before MS analysis. A mixture of cheese sterols isolated using PLC and purified standards were cochromatographed on the HPLC column. Results (Table V) indicated the epoxide isolated from cheese (spot 2, Table IV) contained 2 major compounds (R_T = 0.52 and 0.49, Table V) that were probably the isomeric epoxycholesterols with an β/α-ratio of ca. 2:1 (not shown) indicated by peak areas. These epoxide isomers were not resolved using the TLC methods. An additional compound, which exhibited an R_T of 0.37 (Table V), was also noted. Although the above quantitative TLC technique resolved these 2 compounds (i.e., spots 2 and 3, Table IV), the PLC isolation technique did not adequately separate them. Each 7-hydroxysterol band (spots 6 and 7, Table IV) isolated from the PLC plate contained the expected major peak when subjected to HPLC (R_T = 0.25 and R_T = 0.22, respectively, Table V). The triol (spot 9, Table IV), when subjected to HPLC (R_T = 0.18, Table V) also contained smaller unidentified peaks (not shown). Each purified sterol isolated using PLC then HPLC (i.e., the epoxide isomers, epimeric 7-diols and triol) was subjected to MS analysis to confirm identifications. Fragmentation patterns observed for each sterol were consistent with those of the appropriate standards (Table VI). All expected major MS fragments were observed and agree favorably with published data (7).

TABLE V

Relative Retention Times (R_T) of Major Cholesterol Oxidation Products Isolated from a Grated Parmesan Cheese[a] and Separated Using High Performance Liquid Chromatography (HPLC)[b]

Sterol	R_T[c]
5α,6α-Epoxycholesterol	0.52
5β,6β-Epoxycholesterol	0.49
Unidentified compound[d]	0.37
7-Ketocholesterol	0.34
7β-Hydroxycholesterol	0.25
7α-Hydroxycholesterol	0.22
Cholestane-3β,5,6β-triol	0.18

[a]Brand A cheese sample packaged in a clear glass container. 7-Ketocholesterol not detected in cheese sample.

[b]HPLC system using a Bondapak C_{18} column and an acetonitrile-water (9:1 by vol) mobile phase as described in the Materials and Methods section.

[c]Retention times relative to cholesterol.

[d]Possible sterol oxidation product.

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TABLE VI

Fragmentation Patterns From Mass Spectra of Some Oxidation Products of Cholesterol[a]

Sterol	M/e (fragments)[b]	Relative intensities			
		Standard		Cheese isolate	
		α-Isomer		α-Isomer	β-Isomer
5,6-Epoxycholesterols	402 (M^+)[c]	100		100	100
	387 ($-CH_3$)	4		3	5
	384 ($-H_2O$)	50		49	25
	369 ($-[H_2O+CH_3]$)	11		10	8
	366 ($-2H_2O$)	9		5	5
	351 ($-[2H_2O+CH_3]$)	8		7	5
	271 ($-[H_2O$+side chain])	10		8	9
	247 (−[42+side chain])	12		11	13
	229 ($-[H_2O$+42+side chain])	7		4	6
		α-Epimer	β-Epimer	α-Epimer	β-Epimer
7-Hydroxycholesterols	402 (M^+)[c]	5	8	28	5
	384 ($-H_2O$)	83	100	100	100
	369 ($-[H_2O+CH_3]$)	3	4	8	5
	366 ($-2H_2O$)	42	19	4	2
	351 ($-[2H_2O+CH_3]$)	6	5	5	3
	271 ($-[H_2O$+side chain])	2	2	4	3
	247 (−[42+side chain])	15	8	8	5
	229 ($-[H_2O$+42+side chain])	3	2	6	3
Cholestane-triol	420 (M^+)[c]		3		3
	402 ($-H_2O$)		100		100
	387 ($-[H_2O+CH_3]$)		5		4
	384 ($-2H_2O$)		83		82
	369 ($-[2H_2O+CH_3]$)		24		22
	348 (−A ring)		4		5
	289 ($-[H_2O$+side chain])		3		3
	247 ($-[H_2O$+42+side chain])		43		41
	229 ($-[2H_2O$+42+side chain])		40		34

[a]Cheese isolates and appropriate standards purified using high performance liquid chromatography before mass spectral analysis as described in Materials and Methods section.

[b]Characteristic fragments were verified from published data (7).

[c]Parent ion.

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Specific Heats of the Solid-State Phases of Trimargarin and Tristearin

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ABSTRACT

Differential scanning calorimetry is used to obtain specific heats of the α, β_2', β_1' and β phases of trimargarin and tristearin in the temperature range from 190-350 K. Unequal specific heats are observed for β' phases of the 2 lipids in contrast to nearly coincident values for their respective α and β phases. These results are discussed on the basis of odd vs even chain length triglycerides.

INTRODUCTION

Previous investigations of trimargarin and tristearin have established the existence of 2 intermediate β' phases (1,2). They were conducted to gain information on the contrasting physical properties of even vs odd chain-length monoacid saturated triglycerides. Raman and infrared (IR) spectroscopy, X-ray diffraction and differential scanning calorimetry provide insight into the causes, but do not give total understanding of the even-odd anomaly.

In this study, differential scanning calorimetry (DSC) was used to obtain the specific heats of the 4 phases of trimargarin and tristearin. The behavior of saturated triglyceride solid-state phases are compared.

MATERIALS AND METHODS

Tristearin and trimargarin were purchased as white crystalline powders from Nu-Chek-Prep, Inc., Elysian, MN, and were used without further purification. Purity was better than 99% as determined by gas liquid chromatography (GLC).

Specific heat measurements were obtained with a Perkin-Elmer Model DSC-2. The methods followed were fundamentally the same as those described by O'Neill (3) and McNaughton and Mortimer (4). A synthetic sapphire chip was used for calibration. Sample weights ranged from 4.58-13.67 mg. Aluminum cups, covers and sapphire chip were heated at 600 K before each study to achieve and maintain a moisture-free system. Range was 5 mcal/sec. Scanning rate was 20°/min. The temperature range of heat capacity observations from 190 through 350 K was measured in 3 intervals: 185-245 K, 235-295 K and 285-355 K.

Specific phases of tristearin and trimargarin were prepared as in earlier work (1,2). In addition, the β_1' form of trimargarin was obtained by treatment in the DSC as follows. The phase was gradually developed by heating the β_2' phase at 10°/min until initial melting or transition of this phase occurred by the beginning of a sharp endotherm. Heating was halted and isothermal conditions were maintained until equilibrium was again established after a brief exothermal reaction. This procedure was repeated until no subsequent exothermal behavior was apparent.

Each phase was examined for a minimum of 5 runs. Each run consisted of an individually distinct sample, but some samples were used to examine more than one phase.

RESULTS AND DISCUSSION

Tables I and II contain specific heat values obtained for the