Effects of the Degree of Unsaturation of Coexisting Triacylglycerols on Cholesterol Oxidation

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In order to evaluate the effects of the degree of unsaturation of triacylglycerols on cholesterol oxidation, mixtures of purified sardine oil triacylglycerols (iodine value, IV = 182.6) and cholesterol; of partially hydrogenated sardine oil triacylglycerols (IV = 174.5) and cholesterol; and of fully hydrogenated sardine oil triacylglycerols (IV = 92.0) and cholesterol were incubated at 25°C in the dark. The oxidative stability of the samples decreased with increasing degree of unsaturation of the triacylglycerols in the sample mixtures; the induction period for peroxide values (PV) of the sardine oil triacylglycerols and cholesterol was shorter than that of the partially hydrogenated sardine oil triacylglycerols and cholesterol. Certain polyunsaturated fatty acids (PUFAs) in the constituent fatty acids of sardine oil triacylglycerols started to decrease after a shorter induction period compared with that of the partially hydrogenated triacylglycerols. The prominent cholesterol oxides accumulated in the samples were 7β-hydroxycholesterol, 7-ketocholesterol, β-epoxide and cholestane triol. The tendency for accumulation of cholesterol oxides in the time course coincided with the changes in PV as well as the decrease in PUFAs. Cholesterol was oxidized in conjunction with autoxidation of coexisting fish oil triacylglycerols. Although lowering the degree of unsaturation of fish oil triacylglycerols was effective in prolonging the induction period of cholesterol oxidation, the rate of cholesterol oxidation in the cholesterol oxides' formation phase after the induction period was not affected by the difference in the proportion of highly unsaturated fatty acids in the natural and partially hydrogenated triacylglycerols of fish oils.

KEY WORDS: Autoxidation, cholesterol, cholesterol oxides, degree of unsaturation, fish oils, hydrogenation, hydroxycholesterols, 7-ketocholesterol, polyunsaturated fatty acid, triacylglycerol.

It is now accepted that ω3 polyunsaturated fatty acids (PUFAs) from fish oils, e.g., 20:5n-3 and 22:6n-3, reduce the tendency of platelet aggregation (1) as well as levels of cholesterol, triacylglycerols and possibly low-density lipoproteins in serum (2). Commercial fish oils are generally highly susceptible to autoxidation and also contain low levels of cholesterol. The accumulation of several cholesterol oxides in various foods has been reviewed (3,4). Cholesterol oxides are powerful atherogenic reagents *in vivo* (5,6). Steady consumption of clarified butter, which included a high level of cholesterol oxides, in the diet of Indian immigrants in the United Kingdom was assumed to explain the higher-than-expected mortality from atherosclerosis in this group (7). We reported earlier (8) that commercially available fish products also contained significant amounts of cholesterol oxides. We also evaluated the mechanism of cholesterol oxidation and found that cholesterol was oxidized in conjunction with autoxidation of coexisting triacylglycerols that contained PUFAs. In the previous study, however, cod liver oil triacylglycerols were coated on the surface of a microcrystalline cellulose support; and, therefore, the mechanism of cholesterol oxidation in this system was expected to be different from liquid-state cholesterol dissolved in fish oils. In the present study, the effects of the degree of unsaturation of coexisting fish oils on the rate of cholesterol oxidation were evaluated for cholesterol in the normal liquid state.

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EXPERIMENTAL PROCEDURES

Materials. Total lipids were extracted from Pacific sardine (*Sardinops melanostictus*) by the Bligh and Dyer procedure (9). Two-thirds of the total lipids were hydrogenated over a catalytic amount of platinum oxide (10). Two types of hydrogenated oils with different degrees of unsaturation were prepared by altering hydrogenation time. A cholesterol standard (>99% purity; Sigma, St. Louis, MO) was further purified by recrystallization from acetone. Authentic cholesterol oxide standards (>99% purity) were obtained from Steraloids, Inc. (Wilton, NH) and used as purchased.

Purification of triacylglycerols. All triacylglycerols were purified by column chromatography on Silica Gel G (E. Merck, Darmstadt, Germany) and subsequently on Florisil (Floridin, Pittsburgh, OH) (11). The triacylglycerol fractions thus obtained showed a single spot by thin-layer chromatography on a Silica Gel G plate and did not contain any detectable amount of α-tocopherol upon high-performance liquid chromatography (12). The recrystallized cholesterol was further purified by column chromatography on Florisil to remove peroxidized products. The purified cholesterol thus obtained did not contain any detectable amount of hydroperoxides by high-performance liquid chromatography with fluorescence analysis (13).

Preparation of model systems. For the study of cholesterol oxidation, three model systems with different degrees of unsaturation of triacylglycerols were prepared—A mixture of 48.0 g purified sardine oil triacylglycerols and 1.0086 g purified cholesterol (test-I sample); a mixture of 24.5 g partially hydrogenated triacylglycerols and 0.5150 g purified cholesterol (test-II sample); a mixture of 39.7 g of fully hydrogenated triacylglycerols and 0.8305 g purified cholesterol (test-III sample). Each lipid mixture was added to chloroform (200 mL) and thoroughly mixed in a 300-mL taper-necked, round-bottomed glass flask. All solvents were then evaporated *in vacuo*. The lipid mixtures were autoxidized by exposure to air in the flask with mild mechanical stirring at 25°C in the dark. Five separated samples were taken from each test sample in appropriate intervals for the lipid analyses described below.

Determination of cholesterol oxides. Cholesterol oxides in the model systems were determined qualitatively and quantitatively by gas–liquid chromatography (GLC) and GLC/mass spectrometry (GLC/MS) as described previously (8). An aliquot (*ca.* 125 μg) of 5α-cholestane and tricosanoic acid methyl ester (>99% purity; Nu-Chek-Prep, Elysian, MN) was added to the lipid mixture (*ca.* 500 mg), taken from the model system, as internal standards of cholesterol oxides and fatty acid methyl esters

(FAMEs), respectively. To avoid formation of artificial secondary oxidation products (14,15), the lipids were saponified with 1 N methanolic KOH at 37°C for 18 h under N_2. The saponified lipid sample was diluted with 10 vol of deionized water and extracted three times with peroxide-free diethyl ether. The ether extracts of unsaponifiable matter were dissolved in dry pyridine (1 mL) and converted to trimethylsilyl ether (TMS) derivatives by means of hexamethyldisilazane and trimethylchlorosilane as described previously (8). The TMS derivatives of cholesterol oxides were dissolved in *n*-hexane and subjected to GLC, with Shimadzu (Kyoto, Japan) GC-12A gas chromatograph equipped with a flame-ionization detector (FID) and a Shimadzu split injector SPL-G9. The outlet of an open tubular fused-silica column with a nonpolar methyl silicone liquid phase (0.25 mm i.d. × 25 m, 0.1 μm in film thickness; Quadrex, New Haven, CT) was connected directly to the FID jet. The oven temperature was programmed from 180 to 250°C at a rate of 3°C/min. The injector temperature was held at 250°C. Helium was used as carrier gas with a column inlet pressure at 2 kg/cm^2 and a split ratio of 1:1.

Mass spectrometric analysis of the TMS derivatives of cholesterol oxides was carried out with a Shimadzu QP 1000 quadrupole mass spectrometer fitted with an electron ionization source as described previously (8).

Determination of fatty acids. An excess amount of 6 N HCl was added to the aqueous layer (saponifiable matter) of the saponified sample lipids to liberate free fatty acids, followed by extraction three times with diethyl ether. FAMEs were prepared from the free fatty acids. Quantitative analysis of FAMEs was carried out by GLC with a Shimadzu GC 15A instrument equipped with a SUPELCOWAX-10 fused-silica open tubular column (0.25 mm i.d. × 30 m, 0.25 μm in film thickness; Supelco Japan, Tokyo, Japan) and an FID (16). The column temperature was programmed from 170 to 230°C at a rate of 1°C/min. The split injector was kept at 250°C/min. Helium was used as carrier gas at a column inlet pressure at 2 kg/cm^2 and a split ratio of 1:50.

Determination of saponification value (SV), iodine value (IV) and PV. The SV (17) and the Wijs IV (18) of the sample lipids were determined in duplicate by standard methods. Determination of PV of the sample lipids was replicated five times by a colorimetric microdetermination with aluminum chloride as a catalyst (19).

Estimation of induction period and rate of cholesterol oxide formation. Induction periods of PV and cholesterol oxides were determined by plotting data and using an intersection of tangents (20). The rate of increase in cholesterol oxides was expressed as a slope of tangent in the cholesterol oxides formation phase after the induction period on the plotted data.

Statistics. All data were statistically analyzed as the means ± standard error. Probability values less than 0.05 were significant. Correlation between PV and cholesterol oxide levels was obtained by a linear regression analysis.

RESULTS

Chemical characteristics and fatty acid compositions of the purified triacylglycerols. Chemical characteristics as well as fatty acid compositions of the purified triacylglycerols and its hydrogenation products are summarized in Table 1. PUFAs with more than two ethylenic bonds of the sardine oil triacylglycerols (test-I) accounted for 38.57% of total fatty acids. The chemical characteristics of the sardine oil triacylglycerols showed low SV and high IV. The PUFAs of the purified triacylglycerols prepared from the partially hydrogenated sardine oil (test-II) amounted to 32.16% of total fatty acids, consisting mostly of unaltered 20:5n-3 and 22:6n-3. After this partial hydrogenation, the level of PUFAs was decreased by 23% with appropriate increases in the levels of saturated and monoenoic fatty acids. This partial hydrogenation of sardine oil triacylglycerols decreased the IV, although the SV remained unchanged. With more hydrogenation, the purified triacylglycerols (test-III) contained few PUFAs and increased levels of saturated fatty acids and monoenoic fatty acids from samples of test-I and test-II. The prominent monoenoic acids were a mixture of geometric isomers of 16:1, 18:1, 20:1 and 22:1. The IV of the triacylglycerols decreases after full hydrogenation but without change in the SV.

Changes in PV. The initial PV (5.70 meq/kg) of the test-I sample increased after incubation and reached the maximum value, 783 meq/kg, after 32 d of storage (Fig. 1). The PV decreased to 524 meg/kg after 39 d of storage. The PV of the test-II sample (initially 5.09 meq/kg) showed an induction period of *ca.* 15 d and increased to 762 meq/kg after 39 d of storage; the PV decreased to 710 meq/kg after 46 d of storage (data not shown). The PV of the test-III sample remained almost unchanged; 0.14 meq/kg initially and 0.56 meq/kg after 39 d of storage. The test-I sample had a shorter induction period (*ca.* 10 d) than the test-II mixture. Lipid peroxidation was neglible in the test-III sample (15 d). The differences in the autoxidative stability of the lipid mixtures were due to differences in the degree of unsaturation of coexisting triacylglycerols (Table 1).

TABLE 1

Chemical Characteristics and Fatty Acid Compositions of Purified Triacylglycerols Prepared from Sardine Oils and from Their Hydrogenated Oils

	Test-I	Test-II	Test-III
Saponification value	193.6	193.6	193.6
Iodine value	182.6	174.5	92.0
Fatty acid (wt%)			
Saturates			
Total	29.81 ± 0.53[a]	33.85 ± 0.23	47.06 ± 0.20
14:0	7.47 ± 0.23	8.85 ± 0.14	11.47 ± 0.08
16:0	17.65 ± 0.31	19.81 ± 0.07	26.40 ± 0.16
Monoenes			
Total	31.62 ± 0.19	33.99 ± 0.42	51.75 ± 0.26
16:1n-7	8.50 ± 0.12	9.49 ± 0.10	14.72 ± 0.66
18:1n-9	10.48 ± 0.04	11.12 ± 0.18	13.62 ± 0.12
20:1n-9	2.06 ± 0.05	2.33 ± 0.07	5.71 ± 0.09
22:1n-11	2.89 ± 0.13	2.86 ± 0.07	3.25 ± 0.06
Polyenes[b]			
Total	38.57 ± 0.51	32.16 ± 0.31	1.19 ± 0.07
20:5n-3	16.33 ± 0.22	12.69 ± 0.16	trace[c]
22:6n-3	9.23 ± 0.12	7.05 ± 0.03	trace

[a]Values are the means ± standard error (n = 5).
[b]Fatty acids with more than two ethylenic bonds.
[c]Below 0.01%.

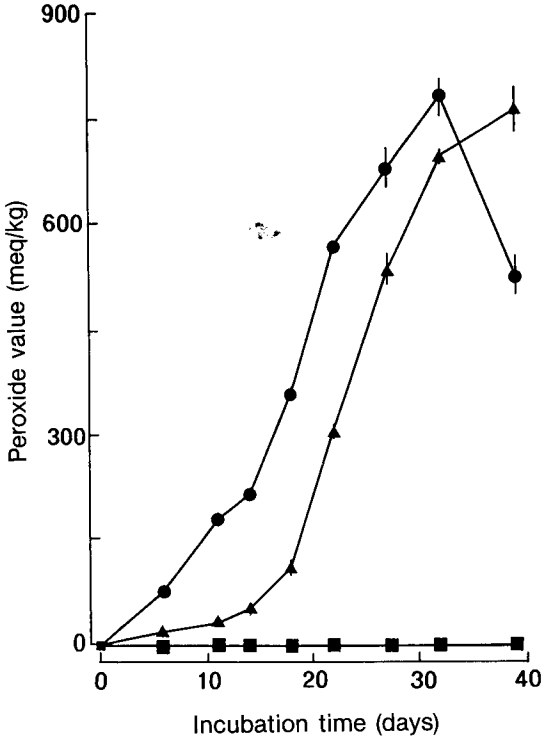

FIG. 1. Peroxide values during incubation at 25°C in the dark of cholesterol plus sardine oil triacylglycerols (test-I), ●; cholesterol plus triacylglycerols purified from partially hydrogenated sardine oils (test-II sample), ▲; cholesterol plus triacylglycerols purified from fully hydrogenated sardine oils (test-III sample), ■. Error bar indicates means ± standard error ($P < 0.05$).

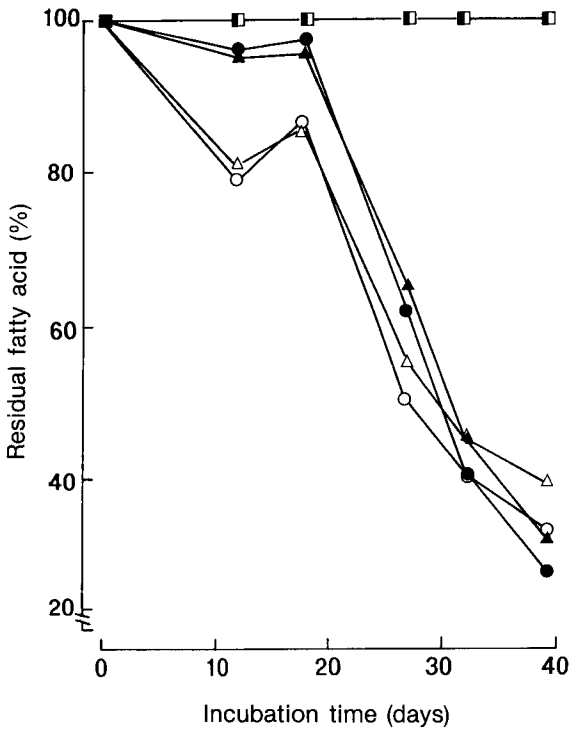

FIG. 2. Changes in the residual amounts of constituent fatty acids of triacylglycerols in the lipid mixtures during incubation. ○, 22:6n-3 of test-I sample; ●, 22:6n-3 of test-II sample; △, 20:5n-3 of test-I sample; ▲, 20:5n-3 of test-II sample; □, 18:1n-9 of test-I sample; ■, 18:1n-9 of test-II sample.

Changes in residual fatty acids of the triacylglycerols. The residual amounts of prominent constituent fatty acids of the triacylglycerols in the lipid mixture were changed as illustrated in Figure 2. The amounts of 20:5n-3 and 22:6n-3 in the test-I sample decreased immediately after incubation began. After 32 d of storage, the residual amounts of 20:5n-3 and 22:6n-3 decreased to 46.2 and 40.7% of the initial levels, respectively. The decrease in the PUFAs was similar in both the partially hydrogenated (test-II) and unhydrogenated (test-I) samples. The level of 18:1n-9 of the test-I sample remained almost unchanged. Amounts of 20:5n-3 and 22:6n-3 in the test-II sample decreased by only 5% after 19 d of storage but to 45.1 and 41.0% of the initial levels, respectively, after 39 d of storage. However, the level of 18:1n-9 in the test-II sample remained unchanged after 39 d of storage. Because the level of PUFA in the test-III sample was low (Table 1), changes in the residual amounts of fatty acids were not plotted. The decrease in the levels of 20:5n-3 and 22:6n-3 (Fig. 2) correlated ($r = -0.974$, $P < 0.05$ in the test-I sample and $r = -0.960$, $P < 0.05$ in the test-II sample) with the changes in PV of the samples (Fig. 1). The induction period in the test-II sample, before the initial decrease in the PUFA (after 19 d), was almost identical with the oxidation induction period of the samples based on PV (*ca.* 15 d). Based on the above data, lipid oxidation, occurring in the test-I sample and in the test-II sample, was mainly due to the oxidative deterioration of PUFA in the triacylglycerols.

Changes in cholesterol oxides contents. Changes in the levels of cholest-5-en-3β,7β-diol (7β-hydroxycholesterol), 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), 5,6β-epoxy-5βcholestan-3β-ol (β-epoxide) and 5α-cholestane-3β,5,6β-triol (cholestane triol) of the samples are summarized in Table 2. In general, identification of cholesterol oxides and their derivatives has been carried out based upon the retention indices in GC. However, cholesterol oxides recovered from complex food systems are subject to interference by coeluting compounds in chromatographic analyses; and therefore, it is necessary to apply other technology for reliable identifications (5). In the present study, cholesterol oxide TMS derivatives were separated in the first instance by GLC and were subsequently identified by electron ion MS as described elsewhere (8).

Cholesterol oxides were not detected initially in any of the samples. The test-I sample accumulated 77.1 μg/g of cholesterol oxides after 6 d of storage. After 39 d of storage, total cholesterol oxides increased to 920 μg/g, composed predominantly of 7β-hydroxycholesterol, β-epoxide and 7-keto cholesterol. Cholestane triol was first detected after 18 d of storage. The cholesterol oxides in the test-II sample first appeared at 11 d of storage, compared to 6

TABLE 2

Cholesterol Oxides (μg/g) in the Lipid Mixtures During Incubation at 25°C

	Incubation time (days)						
	0	6	11	18	27	32	39
Test-I sample							
β-Epoxide	n.d.[a]	12.7 ± 2.70[b]	28.8 ± 4.88	70.3 ± 5.99	171 ± 37.3	183 ± 10.2	235 ± 5.36
7β-Hydroxy[c]	n.d.	27.6 ± 3.16	45.8 ± 3.81	131 ± 10.0	312 ± 11.7	379 ± 11.1	470 ± 12.4
Triol	n.d.	n.d.	n.d.	35.6 ± 2.07	49.3 ± 7.52	52.2 ± 4.74	46.0 ± 6.77
7-Keto	n.d.	36.8 ± 1.11	56.8 ± 5.98	88.2 ± 7.45	136 ± 7.14	158 ± 7.14	169 ± 12.4
Total	n.d.	77.1 ± 5.13	131 ± 11.8	325 ± 23.1	668 ± 43.6	772 ± 25.5	920 ± 14.8
Test-II sample							
β-Epoxide	n.d.	n.d.	8.85 ± 1.04	21.5 ± 1.80	111 ± 8.55	161 ± 12.8	248 ± 10.5
7β-Hydroxy[c]	n.d.	n.d.	22.9 ± 1.45	41.7 ± 4.52	216 ± 12.3	314 ± 14.8	485 ± 14.6
Triol	n.d.	n.d.	n.d.	26.1 ± 2.21	38.7 ± 2.11	41.8 ± 3.13	49.0 ± 5.78
7-Keto	n.d.	n.d.	36.5 ± 3.64	46.9 ± 4.21	106 ± 11.9	153 ± 11.0	169 ± 10.8
Total	n.d.	n.d.	68.3 ± 3.96	136 ± 9.19	472 ± 31.2	670 ± 333.1	951 ± 38.7

[a]Not detected.
[b]Values are the means ± standard error (n = 5).
[c]Including α-epoxide.

d for the test-I sample. The levels of cholesterol oxides increased rapidly after 18 d of storage and reached levels similar to those in the test-I sample after 39 d of storage. The species and composition of the prominent cholesterol oxides accumulated were almost identical to those in the test-I sample. For the test-III sample, no cholesterol oxides were formed under the experimental conditions. The changes in cholesterol oxides described above were correlated with the decrease in the amounts of constituent PUFAs of triacylglycerols ($r = -0.974$, $P < 0.05$ in the test-I sample; $r = -0.960$, $P < 0.05$ in the test-II sample).

DISCUSSION

In our previous study with a model system composed of a mixture of the triacylglycerols purified from cod liver oils and authentic cholesterol coated on the surface of microcrystalline cellulose, we found that cholesterol was oxidized to form several species of oxidation products in conjunction with the autoxidation of PUFAs of the cod liver oil triacylglycerols (8). We also evaluated the levels of cholesterol oxides in commercially available fish products and found that certain products contained relatively high levels, i.e., 138 μg/g of edible parts, of cholesterol oxides. This concentration is equivalent to 240 μg/g cholesterol oxides in the extracted fats. At this stage of oxidation in the model system used in the previous study, the residual amounts of 20:5n-3 and 22:6n-3 were almost 30–40% each, and the residual 18:1n-9 accounted for *ca.* 90% of the fresh sample before storage. In the present model system, however, 14 d of oxidation were needed to accumulate cholesterol oxides to the same level. At this stage of incubation (11–18 d), the levels of residual PUFAs were between 80 and 85%. The level of residual 18:1n-9 remained unchanged for up to 39 d. Based on the above results, it is possible to conclude that a mixture of cholesterol and highly unsaturated triacylglycerols in the liquid state is more susceptible to autoxidation compared to a similar mixture coated in the surface of an inert support. This could be useful in evaluating cholesterol oxidation of heated tallow (14,21,22) and heated lard (23), which must be in the liquid state for oxidation to take place.

The difference in susceptibility of cholesterol oxidation between the test-I sample (sardine oil triacylglycerols and cholesterol) and the test-II sample (partially hydrogenated sardine oil triacylglycerols plus cholesterol) must be due to different degrees of unsaturation of the triacylglycerols. This is supported by the fact that no cholesterol oxides accumulated in the test-III sample, which contained fully hydrogenated triacylglycerols plus cholesterol. These results suggest that susceptibility of cholesterol toward autoxidation would be related to the degree of unsaturation of coexisting triacylglycerols. From this point of view, a correlation between the level of cholesterol oxides and the PV could be evaluated by using the data of both the test-I and the test-II samples. A significant ($r = 0.976$, $P < 0.05$) linear relationship was recognized between these parameters as shown in Figure 3. This evaluation supports the above hypothesis that cholesterol oxidation progresses in conjunction with autoxidation of coexisting highly unsaturated triacylglycerols. In the advanced stage of oxidation (e.g., after 39 d in the test-I sample), when the rate of breakdown of hydroperoxides exceeds the rate of their formation, the level of cholesterol oxides reaches a plateau. It is known that cholesterol itself undergoes autoxidation

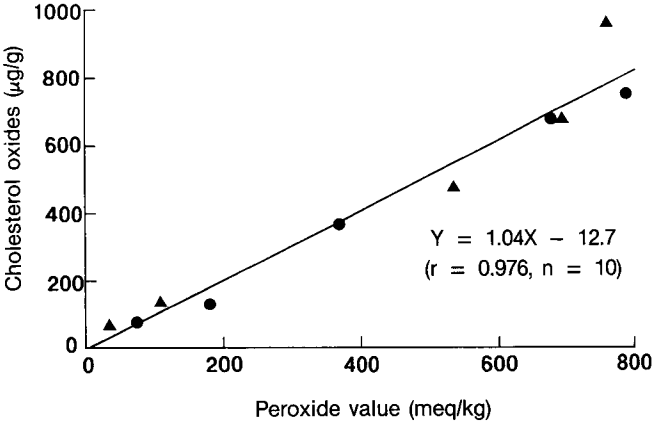

FIG. 3. Relationship between peroxide values of cholesterol oxides of test-I (●) and test-II (▲) samples ($P < 0.05$).

by the accepted chain mechanisms of lipid oxidation (24), although it is relatively stable in the pure state (4). The present results indicate, however, that cholesterol oxidation is strongly accelerated by autoxidation of coexisting highly unsaturated triacylglycerols.

Once the cholesterol oxides began to accumulate after an induction period, there was no difference in the rate of cholesterol accumulation, even though the degree of unsaturation of triacylglycerols was different. The rate of accumulation of cholesterol oxides after 18 d of storage in the test-II sample (37.0 μg/day•g) was almost parallel to that of the test-I sample (37.4 μg/day•g). This would imply that the susceptibility of cholesterol to autoxidation in fish products is similar to that in other foods, even though the induction period in cholesterol oxidation might be different. This indicates that not only fish products but also other foods have the potential to accumulate cholesterol oxides. Indeed, several cholesterol oxides have been detected in foods, such as dried egg products (25–30), milk products (31,32), cheese (33), heated butter (34,35), meat products (15,36–39) and fish products (8,40). Morgan and Armstrong (41) reported the effectiveness of adding 67 ppm butylated hydroxytoluene to spray-dried eggs to prevent formation of cholesterol oxides. Therefore, further studies on the effectiveness of added natural antioxidants, such as vitamins C and E, and synthetic antioxidants, such as butylated hydroxyanisole, should be conducted with cholesterol-containing foods.

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