Characterization and Oxidative Stability of Enzymatically Produced Fish and Canola Oil-Based Structured Lipids

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ABSTRACT: Two-kilogram quantities of structured lipids (SL) of menhaden fish and canola oils containing caprylic acids (8:0) were produced in a laboratory-scale packed-bed bioreactor by acidolysis catalyzed by an immobilized lipase, Lipozyme IM, from *Rhizomucor miehei*. SL were characterized and their oxidative stabilities investigated. The SL contained 29.5% 8:0 for fish oil and 40.1% for canola oil. Polyunsaturated fatty acids (PUFA) of fish oil remained unchanged after the modification while PUFA of canola oil were reduced from 29.6 to 21.2%. Monoenes, especially 18:1n-9, were completely replaced by 8:0 in fish oil and reduced from 61.9 to 34.7% in canola oil. Downstream processing of enzymatically produced SL led to loss in natural total tocopherol contents of the fish and canola oils. The effects of antioxidants such as α-tocopherol (TOC), *tert*-butylhydroxyquinone (TBHQ), and combinations thereof on the oxidative stability of SL were investigated. SL were analyzed for oxidative stability index, peroxide value, conjugated diene content, free fatty acid content, iodine value, saponification number, and thiobarbituric acid value. Iodine value of unmodified fish oil (154.71) was reduced to 144.10 and that of canola oil (114.49) to 97.27 after modification. The SN increased from 183.72 to 242.63 for fish oil and from 172.50 to 227.90 for canola oil. TBHQ exhibited better antioxidant effects than TOC. A combination of TBHQ/TOC also proved to be an effective antioxidant for SL. We suggest the addition of antioxidants to enzymatically produced and purified SL.

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KEY WORDS: Antioxidants, canola, conjugated diene, menhaden, monoenes, oxidative stability, polyunsaturated fatty acids, structured lipids, thiobarbituric acid.

With increasing awareness by consumers and the public of the negative health effects associated with excessive intake of fats and oils, much research attention on lipids has been focused on the development of a new generation of healthful fats and oils known as structured lipids (SL) (1). SL, "fats of the future," are functional lipids that can be considered as nutraceuticals, i.e., lipids with potential for the treatment and/or prevention of disease (2,3). SL are triacylglycerols containing mixtures of short-chain or medium-chain fatty acids, or both, and long-chain fatty acids, preferably in the same triacyglycerol molecule in order to exhibit their maximal potency (4). For SL to be beneficial, a minimum of long-chain fatty acid is needed to meet the essential fatty acids requirements.

The realization of the possible health benefits of n-3 polyunsaturated fatty acids (PUFA) has created a market for a new generation of fats and oils rich in PUFA such as fish, soybean, and canola oils. Further, advances made in the field of interesterifcation have created new possibilities for the industry to add value to existing fats and oils (5).

Fish and canola oils, with their high PUFA contents, have the potential to develop undesirable odors and flavors during storage. The primary and secondary oxidation products of PUFA have been implicated as the major cause of unpleasant flavors in stored oil (6). Due to the major findings of possible beneficial health effects of PUFA, such as 20:5n-3, 22:6n-3 in fish oil and 18:3n-3 in canola, these oils are being used in a variety of products for human consumption, animal feeds, cosmetics, and other applications to provide n-3 fatty acids (7–9). Structured lipids containing PUFA at the *sn*-2 and caprylic acids at the *sn*-1,3 positions are expected to supply quick energy to individuals with lipid malabsorption disorder and enhance the absorption of the n-3 PUFA. Efficient absorption, stability, and functionality in food products are desirable characteristics of SL described in this paper. It was reported that SL with 20:5n-3 and 22:6n-3 predominantly at the *sn*-2 position of triacylglycerols were a more readily absorbed source of these fatty acids (10,11).

SL can be produced by chemically catalyzed and lipase-catalyzed transesterifications. The first is inexpensive and easy to scale up, but the reaction lacks specificity and offers little or no control over the positional distribution of fatty acids in SL. Enzymatic interesterification offers a better control over the positional distribution of fatty acids or regiospecificity (12), but there are difficulties associated with process scale-up.

Large-scale enzymatic synthesis of SL presents some downstream processing problems. The lipase-catalyzed interesterification results in a mixture of products that make the purification of SL often difficult when produced in large scale. For example, how are free fatty acids (FFA), fatty acid methyl esters, diacylglycerols, or undesired triacylglycerols that may be formed as a result of the esterification or transesterification process to be removed from the desired product (4)? Conventional means of lipid fractionation such as thin-

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layer chromatography, solvent evaporation, column or flash chromatography, preparative high-performance liquid chromatography (HPLC), supercritical fluid chromatography, and short-path distillation can be used to remove reactants and by-products. In our laboratory, we have been successful in using short-path distillation to purify enzymatically produced SL (13). However, from our previous work we noticed that SL products purified by short-path distillation and without antioxidant addition are not very stable to oxidation.

Because of the relatively high PUFA content of the SL produced and its increased risk of stability, the need to stabilize them before nutritional applications cannot be overemphasized. Therefore, inhibition of oxidation would be a major criterion when SL are to be incorporated into food products.

This paper presents a study of the characterization and the stability with respect to the fatty acid composition, tocopherol content, and the effects of α-tocopherol (TOC) and *tert*-butylhydroxyquinone (TBHQ) alone and in combination on the oxidative stability of SL from fish and canola oils.

MATERIALS AND METHODS

Oils. Refined, bleached, and deodorized canola oil from Hunt-Wesson, Inc. (Fullerton, CA) was purchased from the local market and fish oil (menhaden) was provided by Zapata Protein, Inc. (Reedville, VA). Menhaden oil was supplied already stabilized with 200 ppm TBHQ and 1,000 ppm mixed tocopherols. Caprylic acid (99% pure) was obtained from Sigma Chemical Company (St. Louis, MO). Antioxidants TBHQ, supplied by Eastman Chemical Products, Inc. (Kingsport, TN), and TOC (95%), obtained from Sigma Chemical Company, were both food-grade. Iodine trichloride was obtained from Fisher Scientific (Fair Lawn, NJ). Potassium iodide, sodium thiosulfate, and starch (soluble potato powder) were obtained from J.T. Baker, Inc. (Phillipsburg, NJ). All solvents used were HPLC grade.

Synthesis and purification of SL. SL (2 kg each) from canola and fish oils were synthesized in a packed-bed bioreactor. The setup of the stainless steel bioreactor was similar to the glass bioreactor described by Mu *et al.* (14). SL of fish and canola oils containing caprylic acids (8:0) were produced by acidolysis using immobilized Lipozyme IM from *Rhizomucor miehei* (Novo Nordisk A/S, Bagsvaerd, Denmark). The production of SL were performed under optimal conditions as described by Mu *et al*. (14). SL were purified using UIC's KDL-4 short-path distillation system (UIC Inc., Joliet, IL) with 0.04 m^2 heated evaporation surface, 500 mL 2-neck receivers with valved adapters for semicontinuous operation as previously reported by Moussata and Akoh (13). SL were passed through short-path distillation twice to obtain FFA (as oleic acid) concentration less than 1%. SL were further purified at 185°C for canola oil and 170°C for fish oil. FFA were removed from SL under a vacuum of 0.2 mTorr. The flow rate was 500 mL/h. SL samples were cooled at room temperature and placed in amber bottles, then flushed with nitrogen and stored at –96°C for further analysis.

Characterization of SL. FFA, saponification number (SN), iodine value (IV) (Wijs method), and peroxide value (PV) were determined by the American Oil Chemists' Society (AOCS) Official Methods Ca 5a-40, T1 1a-64, and Cd 1b-87, and Cd 8-53 (15), respectively. Thiobarbituric acid (TBA) values were measured by using the method of Kikuzaki and Nakatario as modified by Nakayama *et al.* (16). The clear pink solution obtained was used to estimate TBA values at 532 nm. The Oxidative Stability Index (OSI) of SL treated with antioxidants were determined with an Oxidative Stability Instrument manufactured by Omnion (Rockland, MA) using the AOCS method Cd12b-92 (15) at 110°C for canola oil and 80°C for fish oil SL. Additional treatments included controls (SL) of canola and fish oils containing no antioxidants. Samples of unmodified fish and canola oils were characterized or analyzed with the same methods for comparison purposes. All tests were performed in duplicate and average values reported.

Fatty acid and tocopherol analysis. The fatty acid and tocopherol composition of the SL were determined according to Moussata and Akoh (13) and Chase *et al.* (17) by means of gas–liquid chromatography and HPLC procedures, respectively.

Oxidation experiments. SL samples (5 g) treated with antioxidants were weighed into screw-capped 25-mL Erlenmeyer flasks and oxidized uncovered for 72 h at 60°C in the dark in a shaking water bath (New Brunswick Scientific Co., Edison, NJ). The oils were sampled every 24 h for PV, conjugated diene (CD) (15), and TBA measurements. SL without antioxidant (control) and fresh oils (unmodified) were also oxidized under the same conditions.

Statistical analysis. Analysis of variance (ANOVA) was performed using the ANOVA program of the SAS package (18). Significant differences among and by treatments were determined.

RESULTS AND DISCUSSION

Fatty acid composition. The fatty acid composition of oils before and after lipase-catalyzed acidolysis is given in Table 1. This table shows the effects of transesterification on the contents of PUFA, saturates, and monoenes of SL. SL contained 29.5 and 40.1% 8:0 for fish and canola oils, respectively. The incorporation of 8:0 did not change the total PUFA (20:5n-3 and 22:6n-3) content of fish oil. However, 22:6n-3 significantly increased from 12.9 to 18.3% in the SL indicating that fish oil probably contained more of this fatty acid at the *sn*-2 position than 20:5n-3. Caprylic acids were mainly incorporated at the *sn*-1,3 positions where they replaced most of the n-3 PUFA at those positions. This suggests that most of the 20:5n-3 and 22:6n-3 were located at the *sn*-2 position and conserved during Lipozyme IM catalyzed acidolysis. In canola oil, however, changes were observed mainly in 18:2n-6 and 18:3n-3; 18:2n-6 was reduced from 22.0 to 15.8% and 18:3n-3 from 7.6 to 5.4%, i.e., a reduction of about 29%. Incorporation of 8:0 resulted in significant changes in the con-

TABLE 1
Fatty Acid Composition of Purified Fish and Canola Oil-Based Structured Lipids (SL)

	Fatty acid composition (mol %)			
	Fish oil		Canola oil	
	Unmodified	SL	Unmodified	SL
8:0	ND[a]	29.5		40.1
14:0	8.5	6.5	—	—
16:0	19.4	14.9	5.0	2.0
16:1n-7	10.1	7.5	—	—
17:0	3.9	2.4	—	—
18:0	5.4	2.1	2.5	1.3
18:1n-9	15.0	ND	61.9	34.7
18:2n-6	3.5	1.4	22.0	15.8
18:3n-3	1.7	ND	7.6	5.4
18:4n-3	3.6*	3.7	—	—
20:1	1.4	ND	—	—
20:5n-3	11.9*	11.1*	—	—
22:5n-3	2.5*	2.6*	—	—
22:6n-3	12.9	18.3	—	—
Others	0.3	ND	1.0*	0.7*
Saturates	37.2	55.4	7.5	43.4
Monoenes	26.7	7.5	61.9	34.7
PUFA	36.1*	37.1*	29.6	21.2

[a]ND, not determined. PUFA, polyunsaturated fatty acids. *Means of duplicates not significantly different ($P < 0.05$) between unmodified and SL for the same oil. SL were purified by short-path distillation.

tent of saturates and monoenes. The monoenes 18:1n-9 and 16:1n-7 were remarkably reduced in fish oil by acidolysis. Indeed, 18:1n-9 was completely replaced by 8:0 in fish oil whereas it was reduced to 34.7% in canola oil. This suggests that canola oil contains a high level of 18:1n-9 at the *sn*-2 position. From previous work, we have demonstrated that a good balance of monoenes, especially 18:1n-9, and essential fatty acid is required for melon seed oil stability (19).

Although the unmodified or fresh fish and canola oils used in this study were of good initial quality with low PV, FFA, and TBA values (Table 2), important physicochemical changes occurred after acidolysis and downstream processing of the SL. There was a significant difference between unmodified oils and SL in the characteristics measured. The high IV of fresh oils (20), 154.71 (fish) and 114.49 (canola), agree with the high unsaturation of their fatty acids, making them susceptible to oxidation. Lipase-catalyzed transesterification reduced the IV of fish oil to 144.10 and canola oil to 97.27. The low IV of SL can be explained by the incorporation of 8:0. Also, the molecular weights and the SN of SL were significantly different from that of the fresh oils (21). Generally, oils containing a relatively high amount of short-chain, low molecular weight acylglycerols have relatively higher SN. SL exhibited a general increase in the SN. Lee and Akoh (22) reported the same trend previously.

Oxidative stability index. The OSI values of SL are reported in Table 3. Unmodified samples were more stable than controls (SL with no antioxidant addition). For example, the unmodified canola oil had an OSI value of 9.65 h at 110°C against 3.5 h for SL. Modified fish oil had a much lower OSI

TABLE 2
Physicochemical Quality Characteristics of SL[a]

	Canola oil		Fish oil	
Characteristics	Unmodified	SL	Unmodified	SL
FFA (%)	0.04	0.38	0.09	0.70
IV	114.49 (110–126)[b]	97.27	154.71 (150–170)[b]	144.10
SN	172.50 (168–181)[c]	227.90	183.72 (192–199)[c]	242.63
PV	0.67	1.27	0.61	1.50
TBA	0.17*	0.16*	1.00	1.50

[a]FFA, free fatty acid; IV, iodine value; SN, saponification number; PV, peroxide value; TBA, thiobarbituric acid. SL were purified by short-path distillation. *Means of duplicates not significantly different ($P < 0.05$) between unmodified and SL for the same oil.
[b]From Reference 20.
[c]From Reference 21. See Table 1 for other abbreviation.

value at 80°C compared to the unmodified oil. In addition to the fatty acid composition of triacylglycerols, other factors such as the loss of non-triacylglycerol components (tocopherols and phospholipids) during short-path distillation may have contributed to the low stability of SL (23). The effect of purification by short-path distillation on the tocopherol content of SL is given in Table 4. There were significant differences and loss in tocopherol contents for all homologs between unmodified samples and SL. Total tocopherol loss in fish and canola oil SL were approximately 1270 and 450 ppm, respectively, compared to the unmodified oils. More TOC was lost (170 ppm) in canola than in fish oil (75 ppm). When we modified Pronova fish oil (Pronova Biocare, Sandefjord, Norway) to form SL, approximately 82% loss in total tocopherol content was observed (24), suggesting possible loss during acidolysis reaction and processing. Exposure to light and heat during these processes may have contributed to the loss. Substantial decreases in α-, β-, and γ-tocopherols were observed (24). This SL product was not purified by short-path distillation. Tocopherols and tocotrienols are retained for the most part throughout the oil refining process, although there is some loss during the deodorization step. Short-path distil-

TABLE 3
Effect of Antioxidants on the OSI Values (h) of SL[a]

Sample	Canola oil (110°C)	Fish oil (80°C)
Unmodified	9.65	14.33
Control SL (no antioxidant)[b]	3.50	1.99*
SL1 (100 ppm TBHQ)[b]	4.25	1.99*
SL2 (200 ppm TBHQ)	5.52	8.36
SL3 (100 ppm TOC)*	3.72	1.99*
SL4 (200 ppm TOC)	3.90	3.98
SL5 (50 ppm TBHQ + 50 ppm TOC)	4.10	2.31
SL6 (100 ppm TBHQ + 100 ppm TOC)	4.57	3.58

[a]OSI, oxidative stability index; TBHQ, tert-butylhydroxyquinone; TOC, α-tocopherol. *Means of duplicate not significantly different ($P < 0.05$) between unmodified and SL for the same oil. SL were purified by short-path distillation.
[b]SL1, SL2, SL3, SL4, SL5, and SL6 all originated from the SL. See Table 1 for other abbreviation.

TABLE 4
Tocopherol Content (mg/100 g) of SL Purified by Short-Path Distillation After Acidolysis Reaction[a]

Sample	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total
Fish oil:					
Unmodified	20.36	1.16	95.73	47.57	164.82
SL	12.87	—	24.49	0.87	38.23
Canola oil:					
Unmodified	17.59	—	26.86	0.89	45.34
SL	0.59	—	0.18	—	0.77

[a]See Table 1 for abbreviation.

lation process is used for the same purpose as a deodorizer with application of heat. α-TOC and α-tocotrienols are the most effective antioxidants, but are less stable and have less antioxidant activity. Therefore, heat-sensitive antioxidants are expected to change in concentration. Our current paper suggests that SL purified by short-path distillation after enzymatic synthesis need to be supplemented with appropriate antioxidants before storage and food applications. The final product yield after short-path distillation ranged from 84–87% for both fish and canola oil SL. Table 3 also shows the effect of TOC and TBHQ, and their combinations (TBHQ/TOC) on the OSI time of SL. TBHQ alone at 100 and 200 ppm increased the induction period of SL of canola oil from 3.50 (SL) to 4.25 (SL1) and 5.52 h (SL2), respectively; whereas OSI values of TOC at 100 and 200 ppm were 3.72 and 3.90 h. However, for fish oil, neither TBHQ nor TOC at 100 ppm concentration improved the oxidative stability. This could be due to the fact that these concentrations were too low to compensate for the loss caused by the distillation (approximately 1,270 ppm). Mixtures of 50% TBHQ and 50% TOC, at concentrations of 100 and 200 ppm, proved to be effective against autoxidation of SL. In both cases, the addition of antioxidants significantly improved the OSI values of SL when compared to the control SL (no antioxidant addition) with TBHQ at 200 ppm being the most effective.

PV, CD, and TBA. PV results (Fig. 1) of the oxidation at 60°C show that the PV of unmodified fish oil (FO) and canola oil (CO) samples, Figures 1A and 1B, respectively, increased with oxidation time similar to that of SL before 48 h for fish oil and 24 h for canola oil. The rate of increase was greatly different between the samples for the rest of the time of oxidation. It should be noted that accelerated oxidation at 60°C can lead to the development and breakdown of primary oxidation products, which are not comparable with storage experiments at room temperature. Examples will be the formation of volatile decomposition products and ketodiene compounds at 60°C. Incorporation of caprylic acids helped stabilize the fish oil SL to some extent. Again, the addition of TBHQ at 200 ppm was more effective in stabilizing the SL than TOC and their combinations. Changes in CD content (Fig. 2) did not exceed 0.8 in canola oil (Fig. 2B) whereas in fish oil (Fig. 2A), only with the addition of 100 and 200 ppm of TOC were above 2. The PV decreased, but the CD increased after addition of 100 or 200 ppm TOC in both fish and canola oil SL, contrary to the trend observed after addition of TBHQ. This may be due to the breakdown of the initial hydroperoxides to ketodienes or hydroxydienes (25). The mechanism of action of TOC in slowing lipid oxidation is by donating hydrogen to a peroxyl radical, thus preventing them from participating in propagation reactions. Therefore, the differences between the addition of 100 and 200 ppm of

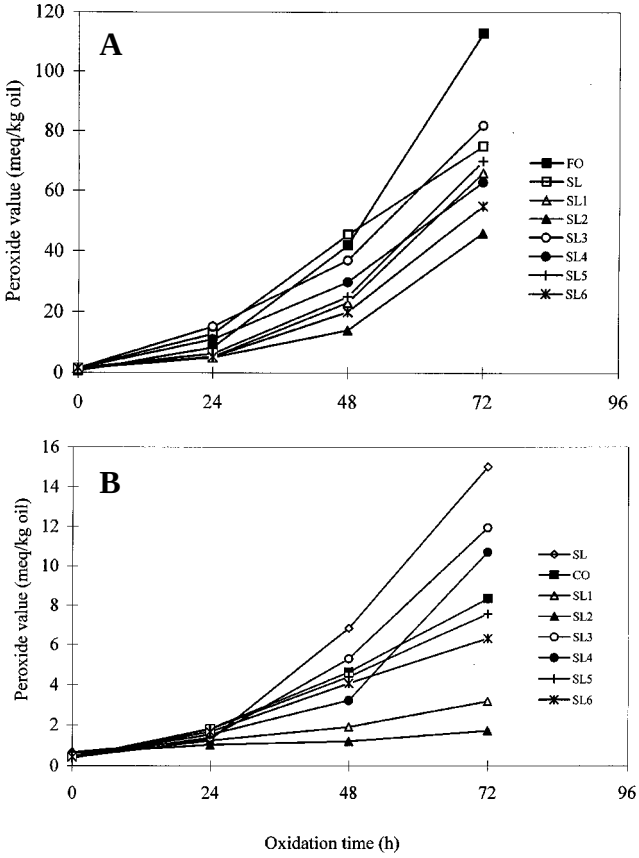

FIG.1. Effect of antioxidants on peroxide value (PV) of fish oil structured lipids (SL) (A) and canola oil structured lipids (B). SL, structured lipids without antioxidants; SL1, structured lipids with 100 ppm TBHQ; SL2, structured lipids with 200 ppm TBHQ; SL3, structured lipids with 100 ppm TOC; SL4, structured lipids with 200 ppm TOC; SL5, structured lipids with 50 ppm TBHQ/50 ppm TOC; SL6, structured lipids with 100 ppmTBHQ/100 ppm TOC; FO, unmodified fish oil; and CO, unmodified canola oil.

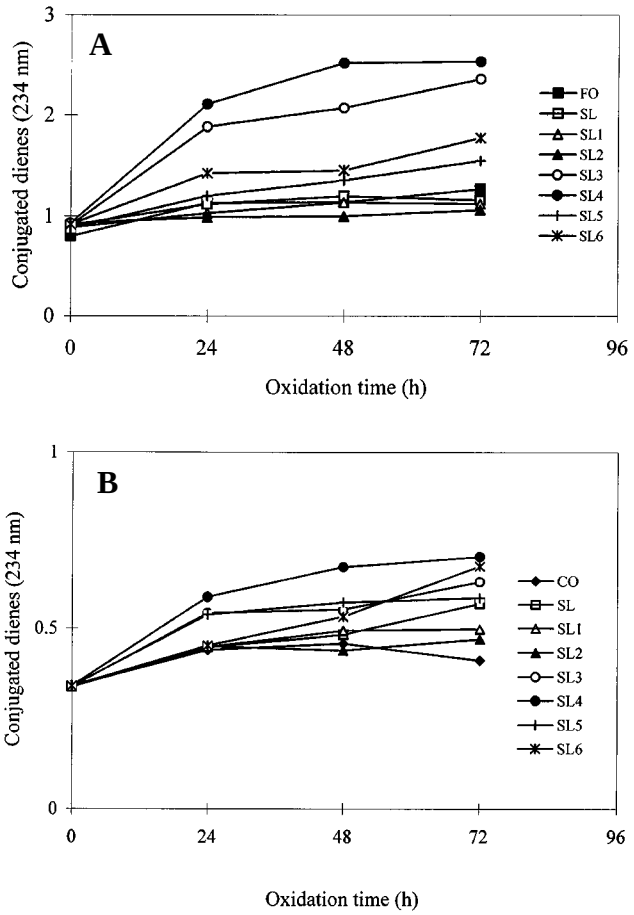

FIG. 2. Effect of antioxidants on conjugated dienes content of fish oil structured lipids (A) and canola oil structured lipids (B). See Figure 1 for abbreviations.

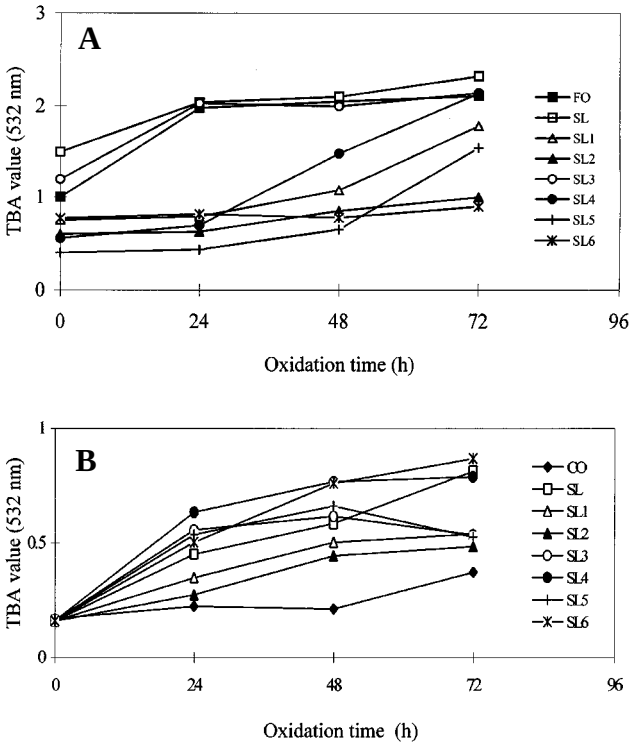

FIG. 3. Effect of antioxidants on TBA values of fish oil structured lipids (A) and canola oil structured lipids (B). See Figure 1 for abbreviations.

TBHQ on one hand and 100 and 200 ppm of TOC on the other may well reside on the ability of TBHQ and tocopherols to serve as hydrogen donor. Various mechanisms of antioxidant actions have been reported by Hopia *et al.* (25). Figure 3 shows that there were significant differences between mean TBA values among samples. In fish oil, TBA values of FO, SL without antioxidant, and that with 100 ppm TOC were higher than others (Fig. 3A). Addition of 200 ppm TBHQ and combined TBHQ/TOC at 100 ppm each, greatly reduced the TBA values of SL. There was a continuous increase in TBA values of canola oil during oxidation (Fig. 3B). CO had the lowest TBA value, whereas SL without antioxidant had a high TBA value. Again, 200 ppm TBHQ was effective in reducing the TBA value. Fish oil contained high levels of n-3 PUFA and therefore was more susceptible to oxidation than canola oil, which contained high levels of monoenes (18:1n-9). This may explain in part the higher TBA values for fish oil-based SL compared to canola oil-based SL. Loss of natural protective antioxidants and addition of inadequate levels of appropriate antioxidants may lead to oxidation of SL and high TBA values. Our study supports the fact that a combination of chemical and instrumental tests is required to assess the oxidative stability of SL. Measurement of PV, CD, and TBA values alone may not fully explain the oxidative changes that occur in SL as well as in regular TAG. Other factors such as heat, storage and assay conditions, presence, amount or absence of tocopherols and phospholipids, fatty acid types, and downstream processing may contribute to the stability of SL. More studies are needed to fully understand lipid oxidation of different types of SL.

This study characterized two different SL and demonstrated the need to protect SL containing highly unsaturated fatty acids. While it was not possible to restore the stability of SL to pre-modification values with the levels of antioxidants used in this study, we suggest that some of the protective antioxidants must be added back to the SL after downstream processing to protect them from further deterioration. Compared to the synthesized SL control, addition of antioxidants did indeed improve the oxidative stability of the enzymatically produced SL.

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