Comparative Study of the Autoxidation of TAG Containing Conjugated and Nonconjugated C_{18} PUFA

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ABSTRACT: Four TAG containing linoleate (soybean-TAG), α-linoleate (perilla-TAG), conjugated linoleate (CLA-TAG), and conjugated linolenate (bitter gourd-TAG) were oxidized in the bulk phase at 50°C. The effects of α-tocopherol and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) on the oxidation of these TAG were also studied. Progress of oxidation was determined by measuring the oxygen consumption, peroxide formation, and polymer formation. The rates of oxygen consumption and polymer formation of bitter gourd-TAG and CLA-TAG were faster than those of perilla-TAG and soybean-TAG, respectively. The present results revealed that the main oxidation products of bitter gourd-TAG and CLA-TAG were dimers and polymers, whereas hydroperoxides were the main products in the oxidation of perilla-TAG and soybean-TAG. These differences in the oxidation of TAG were characterized mainly by oxidation of the main PUFA contained in each TAG, namely, linoleate (56.1%) for soybean-TAG, α-linolenate (54.5%) for perilla-TAG, conjugated linoleate (69.5%) for CLA-TAG, and conjugated linolenate (61.6%) for bitter gourd-TAG, respectively. α-Tocopherol and Trolox inhibited the oxidation of TAG. The inhibitory effect of these antioxidants was more effective against the oxidation of CLA-TAG and bitter gourd-TAG than that of soybean-TAG and perilla-TAG, respectively. This was probably due to the high rate at which the antioxidants inhibited the dimerization and polymerization of CLA-TAG and bitter gourd-TAG.

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KEY WORDS: CLA, CLN, polymer formation, tocopherol, Trolox.

Conjugated PUFA are unique FA that contain conjugated double bonds in their molecules. Among these conjugated FA, CLA are well known, and many papers have been published on the biological activities of CLA (1–3). Conjugated linolenic acids (CLN) also reportedly show cytotoxic effects on mouse and human tumor cells (4); and the anticarcinogenic effect of CLN has been confirmed in an *in vivo* system (5). Seed oils from some kinds of edible plants contain a very high level (30–70%) of CLN (6). Therefore, these seed oils may be used for functional foods and as nutraceuticals.

With the increasing interest in CLA and CLN, more information on the oxidative stability of these conjugated PUFA is required. Oxidation of PUFA not only produces rancid flavors in foods but also can decrease their nutritional quality and safety. Therefore, it is important to establish effective control methods against the oxidation of conjugated PUFA. Early studies (7) revealed differences between the oxidation of conjugated PUFA and their corresponding nonconjugated PUFA. Allen *et al*. (8) reported that the autoxidation of CLA formed less peroxide than the autoxidation of LA. In addition, Holman (7) found that polymers form with the oxidation of conjugated PUFA. Recently, Yurawecz *et al*. (9) reviewed the pathway of CLA oxidation, including hydroperoxide formation, and Hämäläinen *et al*. (10) showed the presence of monohydroperoxides in oxidized CLA. However, the oxidation of CLA and CLN is still not well understood, and less is known about the effects of antioxidants on the oxidation of these conjugated PUFA.

In the present study, we evaluated the oxidative stability of TAG containing CLA, CLN, LA, and LN by measuring their oxygen consumption, peroxide formation, and polymer formation. The effect of antioxidants on the oxidation of TAG containing conjugated and nonconjugated PUFA was also compared. The measurement of oxygen consumption is a good method for comparing the oxidative stabilities of oils producing different oxidation products. The analyses of peroxides and polymers can give useful information on differences in the oxidation of TAG containing conjugated and nonconjugated C_{18} PUFA.

MATERIALS AND METHODS

Preparation of TAG. Soybean oil was obtained from Nacalai Tesque (Kyoto, Japan). Perilla oil and CLA-containing oil were kindly donated by NFO Co. (Oji, Japan) and Kaneka Co. (Takasago, Japan), respectively. Bitter gourd (*Momordica charantia*) seeds were kindly donated by Tohoku Seed Co. (Tochigi, Japan). The seeds were ground to a powder with an electric mill and extracted twice with *n*-hexane at room temperature. After the seeds had been allowed to stand with occasional shaking for about 6 h, the *n*-hexane was evaporated below 30°C by using a rotary evaporator and a vacuum pump. These oils (30 g) were passed through a column (50 cm × 4 cm i.d.) packed with a 1:1 *n*-hexane slurry mixture (w/w) of activated carbon and Celite 545 to remove the tocopherols and pigments by eluting them with *n*-hexane (1500 mL). Column chromatographic separations were performed more than twice, confirming that the oil contained no tocopherol. The

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tocopherol content was measured by a high-performance liquid chromatograph equipped with a fluorescence detector (11). The recovered oil (*ca.* 30 g) was refined on a silicic acid column (50 cm × 4 cm i.d.) (Silica gel 60; Merck, Darmstadt, Germany) by eluting with *n*-hexane (200 mL) and a solution of diethyl ether/*n*-hexane (vol/vol) (5:95, 200 mL; 10:90, 1200 mL; or 20:80, 200 mL). TAG fractions were eluted with diethyl ether/*n*-hexane (10:90; vol/vol) and were then used for the present study as soybean-TAG, perilla-TAG, CLA-TAG, and bitter gourd-TAG, respectively. Each purified oil sample gave only a single spot corresponding to TAG on the thin-layer chromatogram with normal-phase silica plates (Merck), developed with diethyl ether/*n*-hexane/acetic acid (40:60:1, by vol). The detection of the spot on TLC was done by spraying the plate with 50% aqueous H_2SO_4 and heating it on a hot plate to clear the organic material. Triolein (Merck) was used as the TAG standard. The PV of each sample was less than 1.0 as determined by AOCS Official Method Cd 8-53 (12).

Preparation of methyl esters (ME) from TAG. Mixed ME were obtained from each TAG by transesterification using sodium methoxide as the catalyst. After placing the TAG (10 g) into a 300-mL flask, 100 mL of methanol containing 0.5 M sodium methoxide was added, and transesterification was completed by heating the mixture at 60°C for 1 h. The ME were then extracted with *n*-hexane, and the hexane solution was washed with water. The solution was dried with sodium sulfate and concentrated below 30°C by using a rotary evaporator and a vacuum pump. The recovered ME (*ca.* 8 g) were refined on a silicic acid column (22 cm × 2.1 cm i.d., Silica gel 60; Merck) by eluting them with *n*-hexane (100 mL) and a solution of diethyl ether/*n*-hexane vol/vol (5:95, 600 mL; 10:90, 200 mL). The ME fractions eluted with diethyl ether/*n*-hexane (5:95 vol/vol) were used for the present study as soybean-ME, perilla-ME, CLA-ME, and bitter gourd-ME, respectively. Each purified ester gave only a single spot by TLC with normal-phase silica plates developed with diethyl ether/*n*-hexane/acetic acid (30:70:1, by vol). The detection of the spot by TLC was done by spraying the plate with 50% aqueous H_2SO_4 and heating it on a hot plate to clear the organic material. Methyl oleate (Merck) was used as the ME standard. The PV of each sample was less than 1.0 as determined by AOCS Official Method Cd 8-53 (12).

Analysis of the FA composition of ME. FA compositions of the ME were determined by GLC. GLC analyses were performed on a Shimadzu GC-14B chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an FID and a capillary column (Omegawax 320, 30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA) at a column temperature of 200°C. The injector and detector temperatures were held at 250 and 260°C, respectively. Helium was used as carrier gas, with a flow rate of 50 kPa. The FA compositions are shown in Table 1. Component peaks were identified by comparison with standard FAME (6).

Oxidation and analysis. TAG and ME (100 mg) were placed in a 10-mL sealed aluminum vial with a butyl-gum septum (GL Science, Tokyo, Japan) and then incubated at 50°C in the dark. Before incubation, the level of oxygen in the headspace gas of the vial was estimated by GLC (model GC-14B; Shimadzu) (13). The chromatograph was equipped with a thermal conductivity detector and a stainless steel column (3 m × 3.0 mm i.d.) packed with molecular sieve 5A (GL Science). The temperatures of the injection port, detector port, and column oven were 100, 100, and 50°C, respectively. The helium flow was 50 kPa. A small portion (50 μL) of the headspace gas was withdrawn by a microsyringe through the butyl-gum septum at selected oxidation intervals and reduction in the oxygen level of the headspace gas in the vial was estimated by GLC. The reduction (%) in oxygen was calculated from changes in the ratio of oxygen to nitrogen as compared with that before incubation. In this case, one vial of each sample was sampled with a syringe fewer than 15 times. Samples from each of three separate vials were subjected to oxidation. For each determination, there was little difference in the oxidation rate, and the order of oxidative stability of the different samples was unchanged. Another sample (10 g) was also placed in a 30-mL flat-bottomed glass tube and incubated at 50°C in the dark. Aliquots were withdrawn from the oxidized sample at selected time intervals for determinations of PV and polymer formation. PV were determined by AOCS Official Method Cd 8-53 (12). Polymer formation was analyzed by size-exclusion HPLC on an HPLC system consisting of an LC-10A pump (Shimadzu), a Shimadzu RID-6A differential refractometer, and a Shimadzu C-R4AX integrator. HPLC analyses were performed on a pair of TSK gel G2000HR (30 cm × 7.8 mm i.d.) and TSK gel 1000HR (30 cm × 7.8 mm i.d.) columns in series with a TSK HHR-L guard column (4 cm × 6.0 mm i.d.; Toso Co., Yamaguchi, Japan). The sample concentration was 50 mg/mL in chloroform; 0.5 mg was injected into the column. THF (100%) was used as the eluent at a flow rate of 0.4 mL/min. When oxidized ME and TAG were subjected to size-exclusion HPLC, one peak was detected before the peak of unoxidized esters on the chromatogram in the early stage of oxidation of the bitter gourd-ME and bitter gourd-TAG. This peak was identified as the dimer of each bitter gourd-ME. Dimeric triolein was used as a standard (14,15). Triolein was obtained from Nu-Chek-Prep (Elysian, MN).

Analysis of the relationship between oxygen consumption and polymer formation. The same amount (100 mg) of each TAG was placed in 10 sealed aluminum vials (10 mL) with a butyl-gum septum (GL Science), and all vials were then incubated at

TABLE 1
FA Profile of Each Lipid

FA (wt%)	Soybean	Perilla	CLA	Bitter gourd
16:0	10.3	6.4	6.9	1.4
18:0	3.2	1.7	2.1	25.7
18:1n-9	24.7	22.6	16.6	6.1
18:2n-6	56.1	14.4	1.2	4.4
c9,t11-18:2 (CLA)	—	—	34.3	—
t10,c12-18:2 (CLA)	—	—	35.2	—
18:3n-3	5.1	54.5	—	—
*c*9,*t*11,*t*13-18:3 (CLN)[a]	—	—	—	61.6

[a]CLN, conjugated linolenic acid.

50°C in the dark. Before incubation, the level of oxygen in the headspace gas of each vial was estimated by GLC as just described. The ratio of oxygen to nitrogen in the headspace gas of all vials was the same (*ca.* 0.261). At selected intervals of oxidation, one of the 10 vials was taken from the incubator, and a small portion (50 μL) of the headspace gas was withdrawn by a microsyringe. The reduction in the level of oxygen in the headspace gas of the vial was estimated by GLC as just described. The entire TAG sample in the vial was then dissolved in *n*-hexane, and part of the solution was injected into a chromatograph to determine polymer formation. The analysis was duplicated, and little difference was found in the oxygen consumption and polymer formation rates for each determination.

Antioxidant activity of tocopherol and Trolox. D-α-Tocopherol and its water-soluble derivative, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), were obtained from Tokyo Kasei Co. (Tokyo, Japan) and ICN Biomedicals Inc. (Aurora, OH), respectively. Both antioxidants were mixed with TAG in chloroform. The solvent was then removed by gently sweeping with nitrogen. Each TAG (100 mg) with or without 0.5% of each antioxidant was placed in a 10-mL sealed aluminum vial with a butyl-gum septum (GL Science) and then incubated at 50°C in the dark. Before incubation, the level of oxygen in the headspace gas of the vial was estimated by GLC. At selected intervals of oxidation a small portion (50 μL) of the headspace gas was withdrawn, and the decrease in the level of oxygen (%) was estimated by GLC as just described. The analysis was duplicated, and little difference was found in the oxygen consumption rate for each determination. In addition to the above samples, CLA-TAG (10 g) with or without tocopherol was incubated at 50°C in the dark. At selected times, an aliquot of the sample was weighed and the PV was measured by the colorimetric iodine method (16). Another aliquot of the sample was dissolved in *n*-hexane and injected into a high-performance liquid chromatograph to determine of polymer formation and tocopherol content. HPLC analyses were performed as just described.

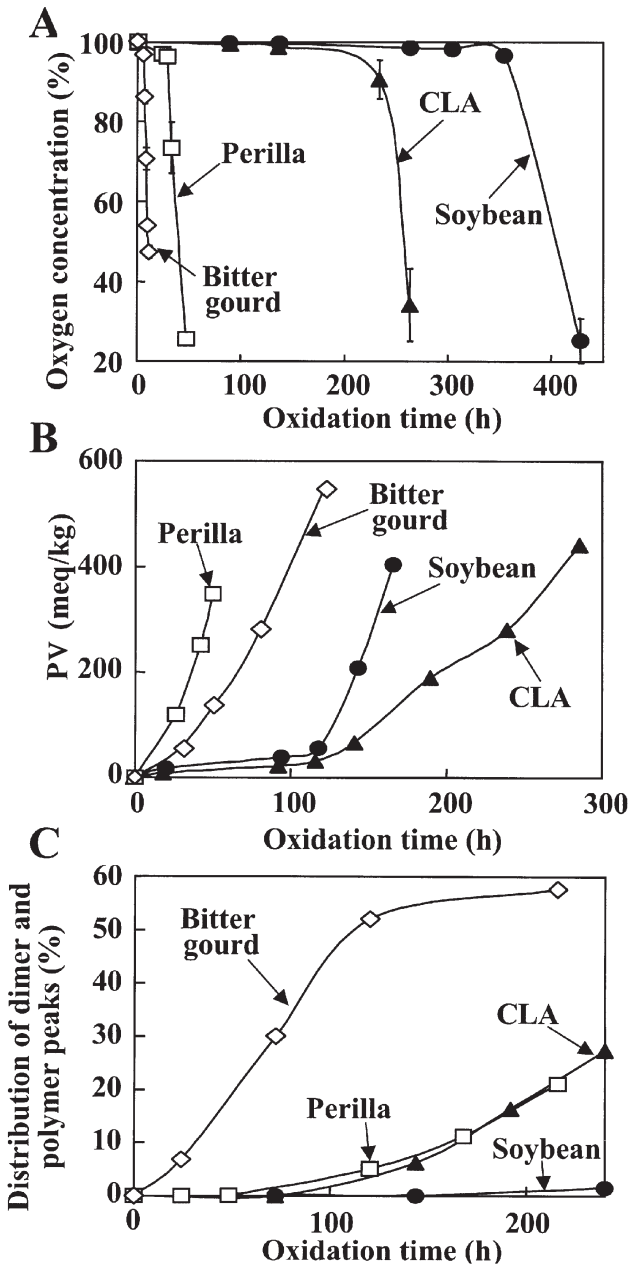

FIG. 1. Oxidative stability of methyl esters (ME) prepared from four kinds of TAG. Each ME (100 mg) was put in a 10-mL sealed aluminum vial and then incubated at 50°C in the dark. The reduction (%) in oxygen concentration (A) was estimated by GLC from changes in the ratio of oxygen to nitrogen in the headspace gas. In addition to the above samples, another sample of ME (10 g) was oxidized at 50°C in the dark. Aliquots were taken from the oxidized sample at selected time intervals to determine PV (B) and for HPLC analysis of dimer and polymer formation (C). Data are expressed as mean ± SD (n = 3).

RESULTS AND DISCUSSION

When ME from the four kinds of TAG were oxidized in the dark at 50°C, the rate of oxygen consumption of CLA-ME was faster than that of soybean-ME, and bitter gourd-ME showed a higher oxygen consumption rate than perilla-ME (Fig. 1A). On the other hand, a reverse relationship was obtained between CLA-ME and soybean-ME and between bitter gourd-ME and perilla-ME in the analysis of PV (Fig. 1B). The same result was also observed in the oxidation of TAG (Figs. 2A and 2B). The main PUFA of soybean-, CLA-, perilla-, and bitter gourd-ME esters were LA (56.1%), CLA (69.5%), LN (54.5%), and CLN (61.6%), respectively (Table 1). Therefore, the oxidative stability and oxidation products of each ester could be characterized by the oxidation of each conjugated or nonconjugated PUFA. Ha *et al*. (17) reported that CLA was more oxidatively stable than LA at room temperature. In that study, oxidative stability was followed by measuring the formation of hydroperoxides, and their results agree with those of the present study (Figs. 1B and 2 B).

On the contrary, van den Berg *et al*. (18) and Zhang and Chen (19) reported that CLA was oxidized more rapidly than LA. They evaluated the oxidative stability of CLA by measuring the

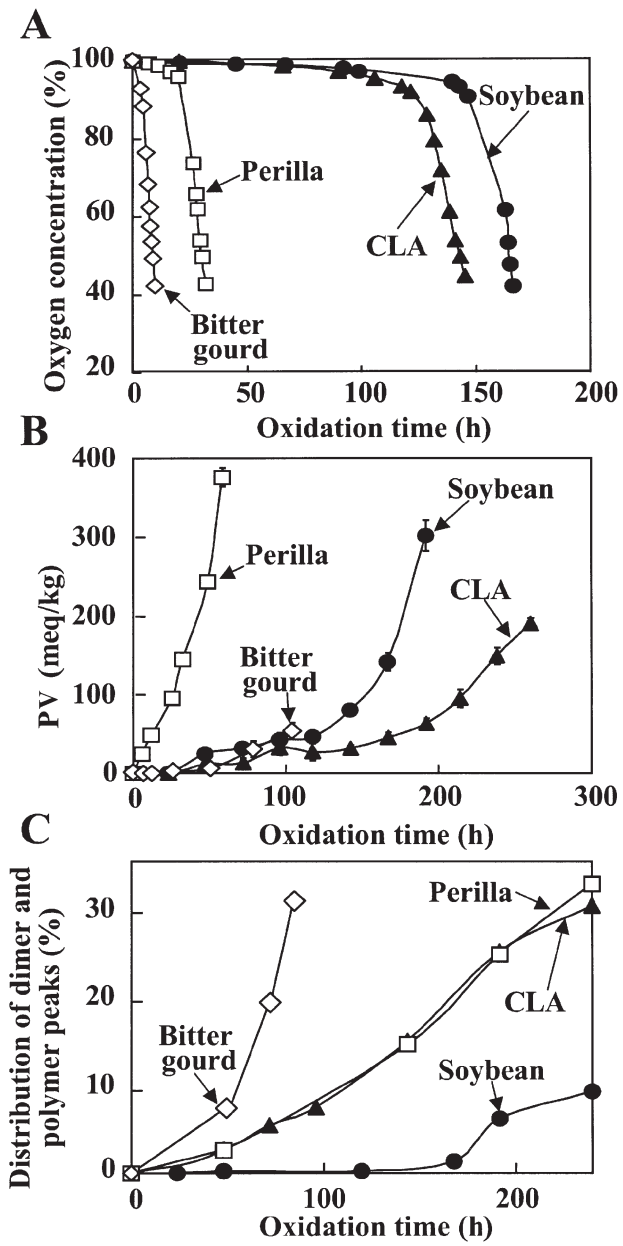

FIG. 2. Oxidative stability of four kinds of TAG. Oxidation was followed by the reduction in oxygen concentration (A), PV (B), and polymer formation (C). Experimental procedures were the same as those for ME (see Fig. 1). Data are expressed as mean ± SD (n = 3).

remaining unoxidized substrate. This relationship agrees with the present results in the analysis of oxygen consumption (Figs. 1A and 2A). It has been pointed out that the oxidation mechanism of conjugated PUFA is different from that of the corresponding nonconjugated PUFA, and that the oxidation products are also different (7,9). Holman (7) compared the oxidation of LA and CLA and found that little peroxide accumulated in the first stage of oxidation in CLA, whereas most of the oxygen absorbed was found as peroxide in the case of LA. Hence, analysis for a particular oxidation product would give a misleading picture when the comparing the oxidative stabilities of conjugated and nonconjugated PUFA. Measuring oxygen consumption or the remaining substrate may be necessary when comparing the oxidation of lipids that produce different oxidation products. However, the lipid oxidation products are responsible for the deterioration of lipid-containing foods. Therefore, clarifying the difference in the oxidation products between nonconjugated and conjugated C_{18} PUFA is also important.

The oxidation of nonconjugated PUFA such as LA and LN has been studied extensively, and many of the oxidative mechanisms have been demonstrated in great detail (20). In the oxidation of nonconjugated PUFA, monohydroperoxides are the main oxidation products, ones that are easily decomposed to produce scission products such as aldehydes, alcohols, and FFA. These volatile compounds cause food to deteriorate.

On the other hand, Yurawecz *et al*. (9) showed that CLA undergoes 1,2- and 1,4-cycloadditions with oxygen to form cyclic endoperoxides. These peroxides are easily decomposed to produce scission products such as aldehydes and furan FA. They also demonstrated that hydroperoxides are formed from CLA by 1,3-addition of oxygen and that radical-induced oligomerization occurs. Hämäläinen *et al*. (21) confirmed the formation of positional and geometrical isomers of hydroperoxides in the autoxidation of CLA, whereas Holman (7) suggested that dimers composed of two molecules of CLA and one molecule of absorbed oxygen are formed as the main products of CLA oxidation. In such a case, the loss of two CLA molecules could occur as a result of the absorption of one oxygen molecule. Holman (7) also pointed out that dimerization and polymerization are likely occurring simultaneously with the addition of oxygen in the oxidation of conjugated trienes such as CLN. The higher formation rate of dimers and polymers may therefore cause different results for oxidative stabilities obtained by different methods, namely, measurements of peroxides, oxygen consumption, and remaining substrate in the oxidation of conjugated PUFA.

With the progress of oxidation of ME and TAG, a new peak corresponding to a dimer was found before the peak of the unoxidized ester on size-exclusion HPLC. The dimer peak area increased rapidly, and another minor new peak was detected before the dimer peak. This new peak could be identified as polymers. Dimer and polymer formation was also observed in the oxidation of perilla esters (Figs. 1C and 2C). The total peak area of these dimeric and polymeric products increased with a decrease in the corresponding unoxidized ME or TAG peak. However, the rate of polymer formation of perilla-ME was much lower than that of bitter gourd-ME. The oxidation of CLA-ME also produced dimers and polymers, but there was little formation of dimers and polymers in the oxidation of soybean-ME (Figs. 1C and 2C). These results suggest that esters containing conjugated C_{18} PUFA such as CLA and CLN are more easily polymerized than those containing the corresponding nonconjugated C_{18} PUFA.

This comparative study of oxygen consumption, peroxide formation, and polymer formation (Figs. 1 and 2) clearly showed a difference in the oxidation of esters containing conjugated and nonconjugated C_{18} PUFA. The main oxidation

products of bitter gourd-ME were dimers, but less peroxide was accumulated during oxidation. In the oxidation of CLA esters, dimers were also detected as the main oxidation product at an early stage of oxidation. On the other hand, hydroperoxides were the main products in the oxidations of perilla-ME and soybean-ME. As shown in Figure 3, dimer and polymer distribution on the HPLC chromatogram quickly increased with increasing oxygen consumption in the oxidation of bitter gourd-TAG and CLA-TAG. However, in the case of soybean-TAG and perilla-TAG, fewer polymers were formed with oxygen loss. Most of the oxygen could have been consumed during hydroperoxide formation in these cases.

Figure 4 shows the effects of α-tocopherol and Trolox on the oxygen consumption rate in the oxidation of soybean-TAG and CLA-TAG. A comparison of this result with that in Figure 2A indicates that oxygen consumption was inhibited by the addition of both antioxidants. This antioxidant activity was more effective with Trolox than with tocopherol. As shown in Figure 2A, the oxygen consumption rate of CLA-TAG was higher than that of soybean-TAG. However, a reverse order in the consumption rate was obtained in the presence of antioxidants (Fig. 4). Figures 5A and 5B show the effect of tocopherol on PV and polymer formation, respectively. Polymer formation was effectively inhibited by the addition of tocopherol. This inhibition pattern was almost the same as the oxygen consumption rate (Fig. 4A). Figure 5C also shows a decrease in the tocopherol concentration during oxidation. Polymer formation was suppressed as long as tocopherol was present in the oxidation system. On the other hand, the addition of tocopherol had little effect on peroxide formation in the early stages of oxidation (Fig. 5A). The PV increased prior to the loss of tocopherol. The higher inhibitory effect of tocopherol on oxygen consumption found in the oxidation of CLA-TAG could be due mainly to the inhibition of dimerization and polymerization of CLA-TAG by the antioxidant.

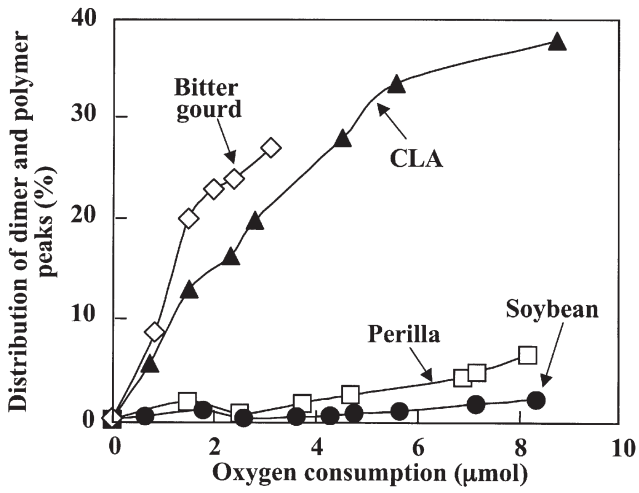

FIG. 3. The relationship between polymer formation and oxygen consumption in the oxidation of TAG. The same amount (100 mg) of TAG was placed in 10 sealed vials, and all vials were then incubated at 50°C in the dark. At selected intervals of oxidation, one of the 10 vials was taken, and the reduction of oxygen was estimated by GLC. The entire sample in the vial was then dissolved in chloroform and analyzed by HPLC for dimer and polymer formation.

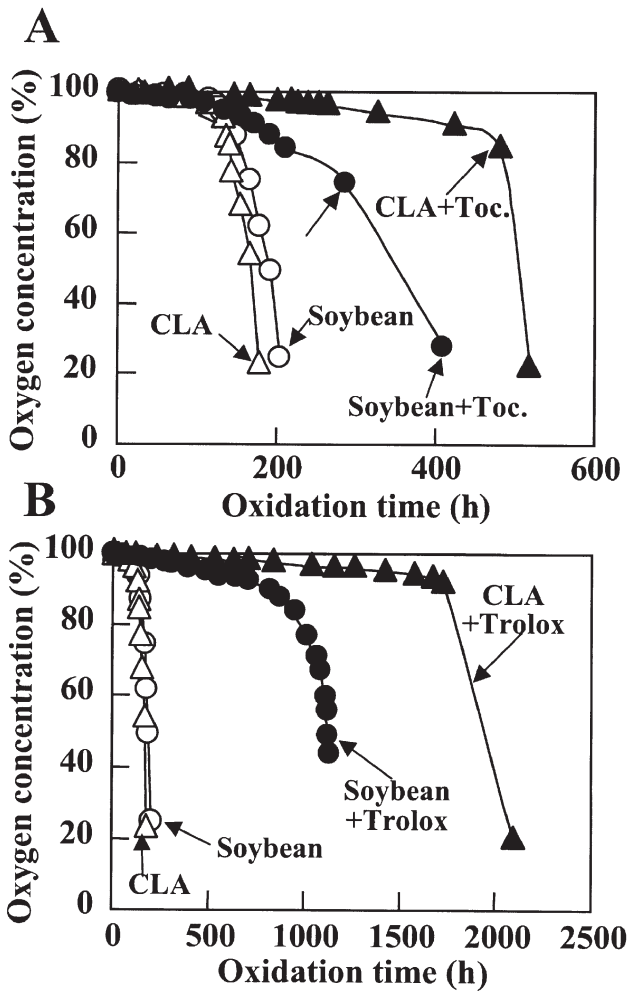

FIG. 4. Effect of tocopherol (Toc) (A) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (B) on the oxidation of soybean-TAG and CLA-TAG. The same amount (100 mg) of TAG with or without antioxidant was placed into 20 sealed vials and all vials were then incubated at 50°C in the dark. At selected oxidation intervals, one of the 20 vials was withdrawn and reduction in oxygen was estimated by GLC.

The rate of oxygen consumption of perilla-TAG and bitter gourd-TAG was also effectively inhibited by the addition of tocopherol and Trolox (Fig. 6). The hydrophilic antioxidant Trolox was more effective than tocopherol in the case of CLA-TAG as well (Fig. 4). This difference in the efficiency of the two types of antioxidants has been explained by their affinities toward the air–oil interface in the bulk oil (22). Thus, in a bulk oil system, the hydrophilic antioxidant Trolox is believed to be located at the oil–air interface and may be more protective against oxidation. On the other hand, the lipophilic antioxidant tocopherol is apparently less protective by remaining in solution in the oil.

In the early stages of oxidation, the oxygen consumption of bitter gourd-TAG was inhibited more effectively than that of perilla-TAG by the addition of both antioxidants. As shown in Figure 2, the main oxidation product of bitter gourd-TAG

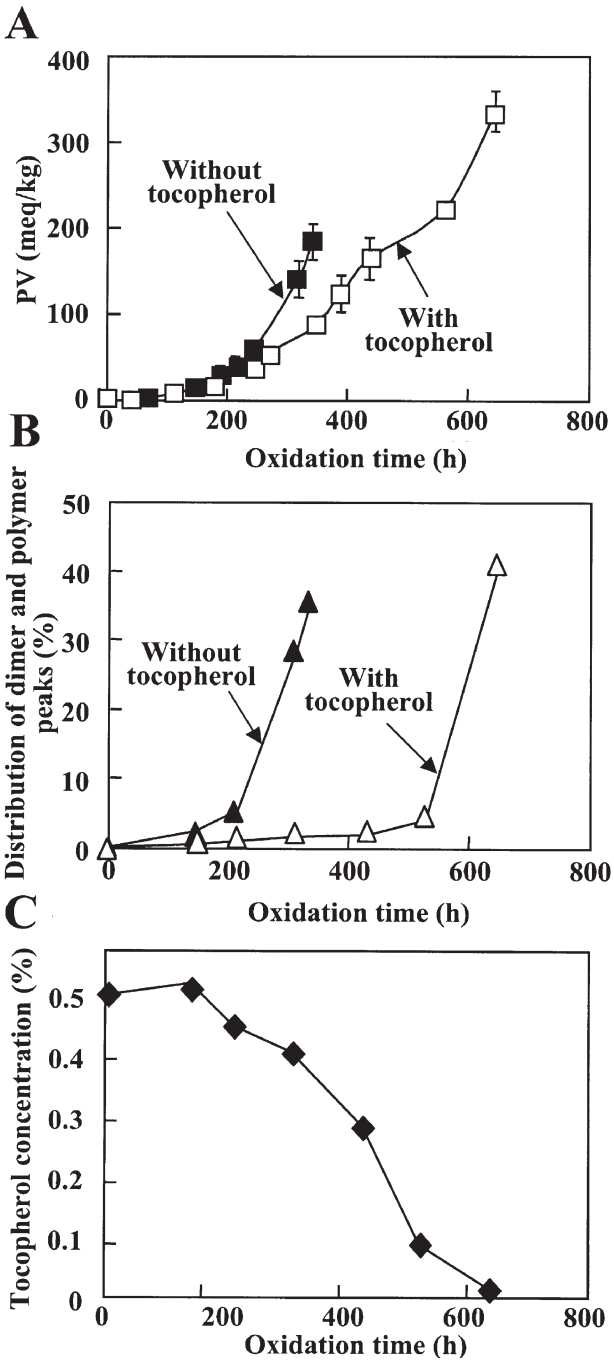

FIG. 5. Peroxide formation (A), polymer formation (B), and reduction in tocopherol (C) in the oxidation of CLA-TAG with or without tocopherol. CLA-TAG (10 g) with or without tocopherol was incubated at 50°C in the dark. At selected intervals, an aliquot of the sample was taken and the PV was measured by the colorimetric iodine method. Another aliquot of the sample was dissolved in *n*-hexane and injected into a high-performance liquid chromatograph for the determination of polymer formation and tocopherol content. Data are expressed as mean ± SD (n = 3).

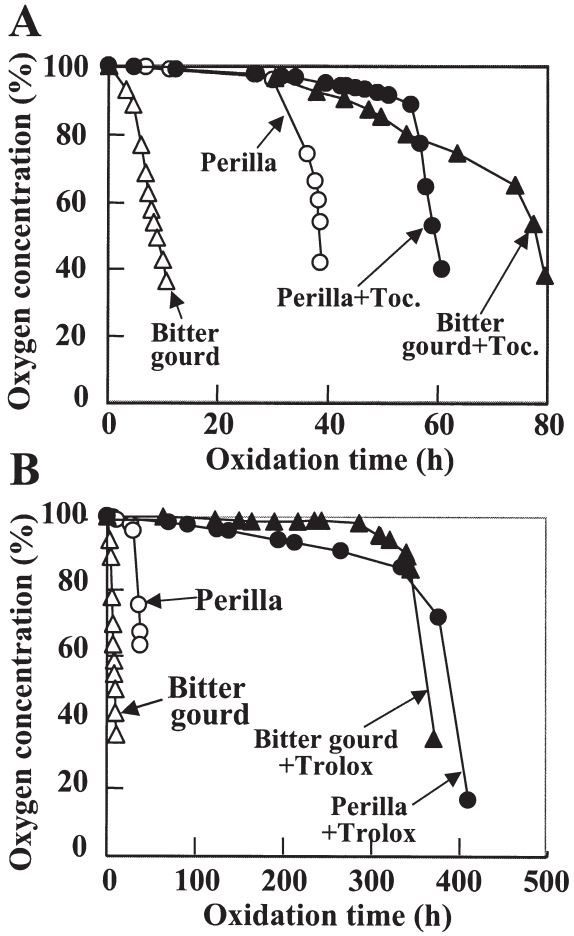

FIG. 6. Effect of tocopherol (TOC) (A) and Trolox (B) on the oxidation of perilla-TAG and bitter gourd-TAG. Experimental procedures were the same as those described in Figure 4. For other abbreviation see Figure 4.

was polymers, and the formation of peroxide was relatively low, although most of the oxygen was consumed. Furthermore, Figure 3 shows that most of the oxygen was consumed for polymer formation in the oxidation of bitter gourd-TAG. Therefore, the effects of antioxidants on the oxidation of bitter gourd-TAG could be due mainly to the inhibition of dimerization and polymerization of bitter gourd-TAG. Although quick oxygen consumption and a high rate of polymer formation were observed in the oxidation of bitter gourd-TAG (Figs. 2A and 2C), the addition of antioxidants, especially Trolox, effectively inhibited their oxidative deterioration. These results suggest that the choice of antioxidant is very important in applications using oils containing conjugated C_{18} PUFA, such as CLA and CLN, in functional foods and nutraceuticals.

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