Reassessment of the Antioxidant Activity of Conjugated Linoleic Acids

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ABSTRACT: The anticarcinogenic effect of conjugated linoleic acids (CLA) has been attributed to their antioxidant activity. Strong evidence is lacking, however, to substantiate that CLA is an antioxidant. The objective of this study was, therefore, to test whether CLA is an antioxidant or a prooxidant in canola oil. The oxidation was conducted at 90°C by monitoring oxygen uptake and changes in linoleic acid and α-linolenic acid. Free CLA and CLA methyl ester (CLAME) accelerated lipid oxidation in canola oil. The prooxidant activity of CLA and CLAME was dose-dependent at concentrations ranging from 0.1 to 1.0% in canola oil heated at 90°C. CLA-containing triacylglycerol, however, was neither an antioxidant nor a prooxidant. Under the present experimental conditions, CLA was not an antioxidant in fats and oil.
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KEY WORDS: Conjugated linoleic acids, oxidation, oxygen consumption, prooxidant.

There is increasing interest in dietary conjugated linoleic acids (CLA) as a protective agent against the formation of free radicals *in vivo* and cancer. CLA is a term used to describe a group of positional and geometric isomers of linoleic acid (LA) in which the two double bonds are conjugated instead of being methylene-interrupted. CLA is predominantly found in meat and dairy products (1,2). Low concentrations of CLA also occur in the lipids of human blood, tissue, and milk (3–5).

Whether CLA is an antioxidant remains controversial. CLA is known to be a strong anticarcinogen in a number of animal models (6–12). The exact mechanism of anticarcinogenic action remains poorly understood, but it is believed to be caused by the antioxidant action of CLA (7,8). There were, however, only three studies to date that examined the antioxidant properties of CLA. Two of them showed that CLA acted as an antioxidant (7,8), whereas one demonstrated that CLA did not act as an antioxidant and might be a prooxidant instead (13). It thus appears that there is insufficient evidence to substantiate CLA as an antioxidant. The present study was, therefore, undertaken to study further the antioxidant activity of CLA in three forms (free fatty acids, methyl esters, and triacylglycerols) in canola oil.

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MATERIALS AND METHODS

Chemicals. CLA and conjugated linoleic acid methyl esters (CLAME) were obtained from Sigma Chemical Company (St. Louis, MO). CLA and CLAME were analyzed on a flexible silica capillary column (SP 2560, 100 m × 0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA) in an HP 5890 Series II gas–liquid chromatograph, equipped with a flame-ionization detector (Hewlett-Packard, Palo Alto, CA). CLA and CLAME consisted of the following isomers: *c*-9,*t*-11/*t*-9, *c*-11, 40.3%; *t*-10,*c*-12/*c*-10,*t*-12, 43.9%; *c*-10,*c*-12, 12.4%; *c*-9,*c*-11, 1.8%; *c*-9,*c*-12, 0.3%; others, 1.0%. Lipozyme IM20 (25 units/mg, corresponding to 25 μmol of palmitic acid incorporated into triolein per min) was a gift from Novo Nordisk A.S. (Hong Kong). Canola oil without addition of any synthetic antioxidants was obtained from Lam Soon Marketing Service Ltd. (Kowloon, Hong Kong). Canola oil was chosen because it contained an undetectable amount of CLA.

Purification of CLA and CLAME. Thin-layer chromatography (TLC) was employed to purify CLA and CLAME. In brief, 20 mg CLA or CLAME was applied to a TLC plate (20 × 20 cm, precoated with 250 μm silica gel 60Å; Macherey-Nagel, Duren, Germany) and developed by using a solvent system of hexane–diethyl ether–acetic acid (80:20:1; vol/vol/vol). A CLA or CLAME band was recovered from the TLC plates and eluted from the silica gel with 60 mL hexane–diethyl ether (9:1, vol/vol). After evaporation of the solvent under a stream of nitrogen gas, total CLA or CLAME was redissolved in hexane (1 mg/mL), flushed with nitrogen, and saved until used.

Lipase-catalyzed interesterification (acyl exchange) between CLA and canola oil triacylglycerols. Incorporation of CLA into canola oil triacylglycerols was accomplished by lipase-catalyzed interesterification (14). In brief, a mixture of 100 mg free CLA and 200 mg canola oil, 40 mg Lipozyme, and 4 mL hexane was stirred at 60°C for 6 h. The mixture was then centrifuged, and the supernatant hexane was separated and evaporated under a gentle stream of nitrogen. An aliquot of the mixture was then subjected to TLC separation (20 mg/plate) by using a solvent system of hexane–diethyl ether–acetic acid (80:20:1, vol/vol/vol). The CLA-containing triacylglycerol (CLATG) band was scraped off the plate and eluted with 60 mL of hexane–diethyl ether (9:1, vol/vol). After evaporation of hexane–diethyl ether under nitrogen,

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total CLATG was redissolved in hexane (5 mg/mL). To confirm and quantitate the incorporation of CLA into canola oil triacylglycerols, 5 mg CLATG was converted to the corresponding fatty acid methyl esters with a mixture of 14% BF_3 in methanol (Sigma Chemical Co.) under nitrogen at 90°C for 45 min. The gas–liquid chromatography analysis showed that the lipase-catalyzed acyl exchange between free CLA and canola oil triacylglycerols was efficient in hexane (Fig. 1). CLATG contained 24.5% CLA.

Oxygen consumption test. The method previously described by Chen *et al.* (15) was used to monitor oxygen consumption. In brief, 1 mL of hexane that contained 200 mg of canola oil was placed in a glass tube (150 × 16 mm, o.d.) with the addition of varying amounts of CLA, CLAME, and CLATG. For CLA and CLAME, 1.0, 0.5 and 0.1% were added into canola oil, respectively. For CLATG, 4.0 and 2.0% were added into canola oil to correspond to 1.0 and 0.5% CLA, respectively. The components were mixed thoroughly. The hexane was removed under a gentle stream of nitrogen at 45°C. These concentrations were chosen because an average of 0.5–1.0% CLA is found in fats and oils (1). The reaction tube was then flushed with air and sealed tightly with a rubber stopper from an evacuated blood collection tube (100 × 16 mm, o.d.; Becton-Dickinson, Rutherford, NJ), which usually maintains a vacuum for 2–3 yr. The sealed tube was leak-free as verified by filling the tube with nitrogen gas and monitoring headspace oxygen concentration by gas chromatography. Oxidation was conducted at 90°C ± 2°C. The headspace oxygen was sampled periodically with a gas-tight syringe and analyzed in an HP 5890 series II gas-solid chromatograph (Hewlett-Packard), fitted with a 1/8" × 6′ stainless-steel column packed with Molecular Sieve 5Å (60:80 mesh) and a thermal conductivity detector. The percentage oxygen in the headspace was calculated from the ratio of oxygen to nitrogen. After headspace oxygen analysis, the canola oil was extracted with 10 mL chloroform, and an aliquot containing 20 mg canola oil was taken for fatty acid analysis.

Fatty acid analysis. Fatty acids of heated canola oil with or without addition of CLA, CLAME, and CLATG were converted to the corresponding fatty acid methyl esters with a mixture of 14% BF_3 in methanol and toluene (1:1, vol/vol) under nitrogen at 90°C for 45 min (15). Fatty acid methyl esters were analyzed on a flexible silica capillary column (SP 2560; 100 m × 0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA) in an HP 5980 Series II gas-liquid chromatograph, equipped with a flame-ionization detector and an automated injector (Hewlett-Packard). Column temperature was programmed from 180 to 220°C at a rate of 1°C/min and then held for 10 min. Injector and detector temperatures were set at 250 and 300°C, respectively. Hydrogen was used as the carrier gas at a head pressure of 15 psi.

Thermal stability of CLA relative to LA. One mL of hexane that contained 5 mg free CLA, 5 mg free LA and 5 mg heptadecanoic acid was delivered into each Pyrex tube (13 × 100 mm; Corning, NY). The hexane was removed under a gentle stream of nitrogen. The tube was then flushed with air and heated at 90°C. After heating, the mixture was cooled to room temperature and converted to the corresponding fatty acid methyl esters as described above. After the gas–liquid chromatographic analysis, the remaining free CLA and LA were

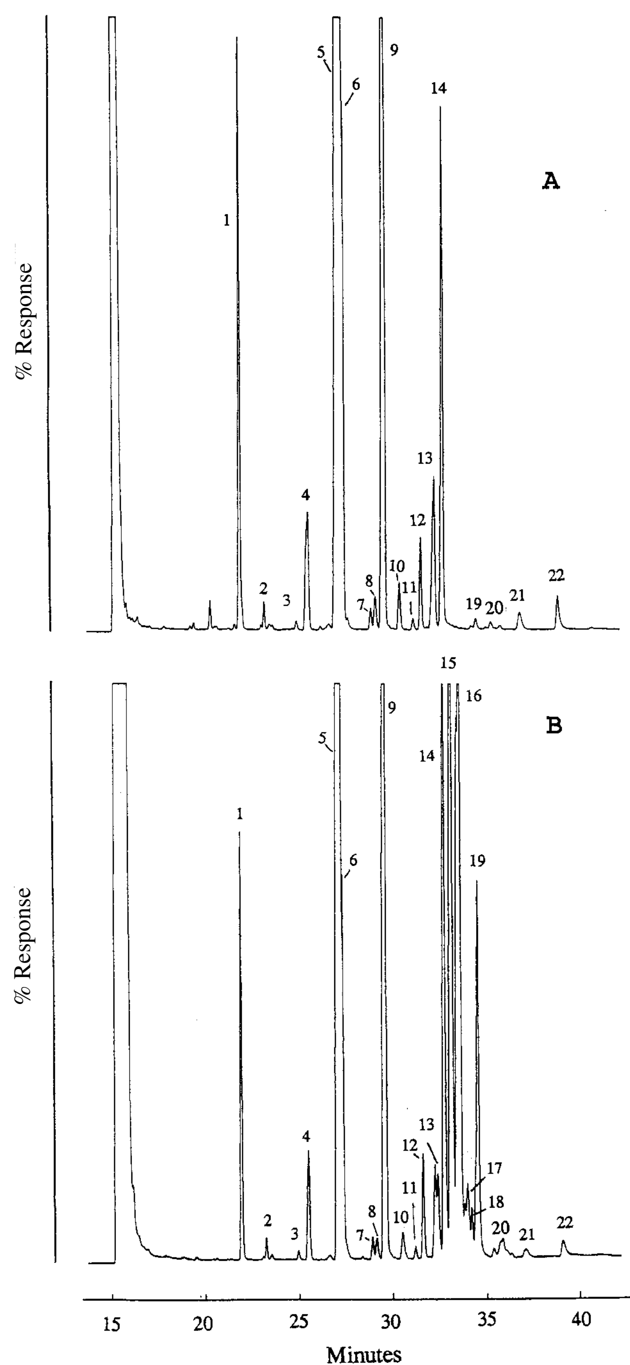

FIG. 1. Gas–liquid chromatograms of the fatty acid methyl esters from canola oil (A) and conjugated linoleic acid-containing canola oil triacylglycerols (B). Peaks were identified as 1, 16:0; 2, 16:1Δ7*c*; 3, unknown; 4, 18:0; 5, 18:1Δ9*c*; 6, 18:1Δ11*c*; 7, 18:2Δ9*c*,12*t*; 8, 18:2Δ9*t*,12*c*; 9, 18:2Δ9*c*,12*c*; 10, 20:0; 11-13, 18:3*t*; 14, 18:3Δ9*c*,12*c*,15*c*; 15, 18;2Δ9*c*,11*t*/18:2Δ9*t*,11*c*; 16, 18:2Δ10*t*,12*c*/18:2Δ10*c*,12*t*; 17, 18:2Δ9*c*, 11*c*; 18, unknown; 19, 18:2Δ10*c*,12*c*; 20, unknown; 21, 22:0; 22, 22:1Δ15*c*.

quantitated by using heptadecanoic acid as an internal standard.

Statistics. All experiments were repeated two to three times. Data were pooled from each experiment, which had three to four replicates (total 8–12 reaction tubes/time point). Data for the headspace oxygen consumption and fatty acid analysis were subjected to analysis of variance, and the means were compared among treatments by using Duncan's multiple range test (16). This was done by running data on the PC ANOVA software (PC ANOVA for the IBM Personal Computer, Version 1.1, 1985; IBM; Armonk, NY).

RESULTS AND DISCUSSION

Results of the headspace oxygen consumption test for CLA and CLAME at 90°C are shown in Figures 2 and 3. The following features were observed: (i) both CLA and CLAME possessed prooxidant activities in canola oil; (ii) the prooxidant effect of CLA and CLAME was dose-dependent within the range of 0.1–1.0%; and (iii) under the same conditions, CLA as a prooxidant appeared to be more effective than CLAME.

The results from the fatty acid analysis were generally in agreement with those from the oxygen consumption test (Tables 1 and 2). LA and α-linolenic acid were lowered in canola oil, a change that was positively associated with the decrease in the headspace oxygen concentration. Addition of CLA and CLAME significantly decreased relative proportions of LA and α-linolenic acid while it increased proportions of oleic acid and total saturated fatty acids in canola oil heated at 90°C. It seemed that the loss of LA and α-linolenic acid in canola oil heated at 90°C was dose-dependent on the amount of CLA and CLAME added except that there was no difference between samples with addition of 0.5% CLAME and 1.0% CLAME for 32 h at 90°C (Table 2).

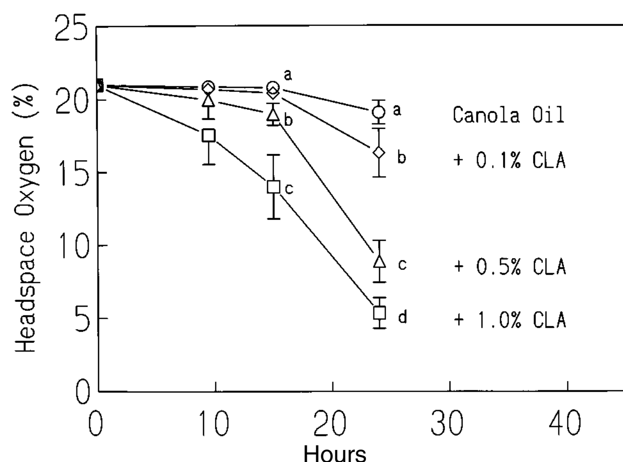

FIG. 2. Effect of free conjugated linoleic acid (CLA) on oxidation of canola oil at 90°C. Data are expressed as mean ± SD; n = 8–12 samples; ○, canola oil; ◇, canola oil + 0.1% CLA; △, canola oil + 0.5% CLA; □, canola oil + 1.0% CLA. Means at the same time point with different superscript letters differ significantly ($P < 0.05$).

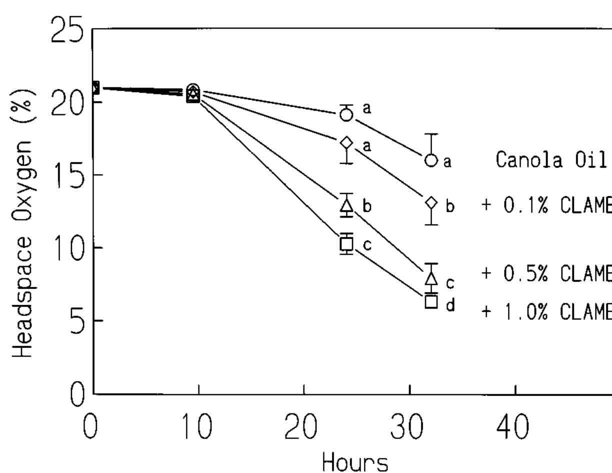

FIG. 3. Effect of free conjugated linoleic acid methyl esters (CLAME) on oxidation of canola oil at 90°C. Data are expressed as mean ± SD; n = 8–12 samples, ○, canola oil; ◇, canola oil + 0.1% CLAME; △, canola oil + 0.5% CLAME; □, canola oil + 1.0% CLAME. Means at the same time point with different superscript letters differ significantly ($P < 0.05$).

The effect of CLA, esterified in triacylglycerols, on oxidation of canola oil was also examined under the same conditions. As shown in Figure 4, the prooxidant activity of CLA in the form of triacylglycerols was diminished. Fatty acid analysis showed that there was no difference in composition of LA and α-linolenic acid between the samples with and without addition of CLATG (data not shown).

The present observation is in agreement with the report of van den Berg *et al.* (13), but it is in contrast to that of Ha *et al.* (2,7). We have no explanation for this discrepancy. Probably, CLA may behave differently, either as an antioxidant or a prooxidant, in various systems. In the study by Ha *et al.* (2), a phosphate buffer/ethanol mixture (60:40, vol/vol) was used as medium in which the free CLA acted as an antioxidant more effectively than did α-tocopherol. As noted by van den Berg *et al.* (13), the phosphate buffer/ethanol system used by Ha *et al.* (2) was dissimilar to biological systems. Another study, conducted by Ip *et al.* (8), examined the antioxidant activity of CLA by using the thiobarbituric acid-reactive substance assay, which is known to measure only the secondary oxidation products and may not necessarily reflect the course and degree of lipid oxidation. Based on these considerations, van den Berg *et al.* (13) reinvestigated the effect of CLA on lipid oxidation in a membrane composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine. Their results did not support CLA as an antioxidant. In fact, they noted that CLA may be a prooxidant instead (13). To our knowledge, the present study is the first report that demonstrates that CLA, under these experimental conditions, is not an antioxidant in fats and oils. Contrary to our expectation, free CLA and CLAME functioned as prooxidants, whereas CLATG had no influence on lipid oxidation of canola oil.

The mechanism by which free CLA and CLAME accelerates lipid oxidation in canola oil remains unknown. It is possible that free CLA and CLAME as reductants are suscepti-

TABLE 1
Effect of Conjugated Linoleic Acids (CLA) on Change in Unsaturated Fatty Acids of Canola Oil (wt% total fatty acids[a]) heated at 90°C for 24 h

Fatty acids	Unheated canola oil	Heated			
		Canola oil	+ 0.1% CLA	+ 0.5% CLA	+ 1.0% CLA
Linoleic acid	21.9 ± 0.1^b	21.7 ± 0.1^c	21.2 ± 0.2^d	19.6 ± 0.2^e	19.0 ± 0.2^f
α-Linolenic acid	8.0 ± 0.1^b	7.6 ± 0.1^c	7.3 ± 0.2^d	6.0 ± 0.2^e	5.5 ± 0.1^f
Oleic acid	55.5 ± 0.1^b	56.1 ± 0.1^c	56.9 ± 0.3^d	59.3 ± 0.3^e	60.2 ± 0.3^f
Total polyunsaturated[b]	29.9 ± 0.1^b	29.3 ± 0.1^c	28.4 ± 0.3^d	25.6 ± 0.3^e	24.5 ± 0.3^f
Total monounsaturated[c]	58.4 ± 0.1^b	59.2 ± 0.1^c	59.9 ± 0.3^d	62.4 ± 0.3^e	63.4 ± 0.3^f
Total saturated[d]	6.9 ± 0.1^b	7.0 ± 0.1^b	7.1 ± 0.1^c	7.6 ± 0.1^d	7.9 ± 0.1^e
Others[e]	4.7 ± 0.1^b	4.6 ± 0.1^c	4.5 ± 0.1^c	4.3 ± 0.1^d	4.2 ± 0.1^d

[a]Excluding CLA. Means at the same row with different superscripts (b–f) differ significantly ($P < 0.05$). Data are expressed as mean ± SD of n = 8–9 samples.
[b]Polyunsaturated = linoleic acid + α-linolenic acid.
[c]Monounsaturated = oleic acid (18:1n-9) + 18:1n-7.
[d]Saturated = myristic acid + palmitic acid + stearic acid + arachic acid.
[e]Others = *trans* isomers of linoleic acid and α-linolenic acid.

TABLE 2
Effect of Conjugated Linoleic Acid Methyl Esters (CLAME) on Change in Unsaturated Fatty Acids of Canola Oil (wt% total fatty acids[a]) heated at 90°C for 32 h

Fatty acids	Unheated canola oil	Heated			
		Canola oil	+ 0.1% CLA	+ 0.5% CLA	+ 1.0% CLA
Linoleic acid	21.9 ± 0.1^b	21.0 ± 0.6^b	20.0 ± 0.5^c	18.8 ± 0.1^d	18.9 ± 0.1^d
α-Linolenic acid	8.0 ± 0.1^b	7.0 ± 0.5^c	6.2 ± 0.4^d	5.3 ± 0.1^e	5.3 ± 0.1^e
Oleic acid	55.5 ± 0.1^b	57.1 ± 0.8^c	58.6 ± 0.8^d	60.4 ± 0.1^e	60.6 ± 0.5^e
Total polyunsaturated[b]	29.9 ± 0.1^b	28.1 ± 1.0^b	26.2 ± 0.9^c	24.1 ± 0.2^d	24.1 ± 0.1^d
Total monounsaturated[c]	58.4 ± 0.1^b	60.4 ± 0.9^c	62.0 ± 0.8^d	63.9 ± 0.2^e	63.8 ± 0.3^e
Total saturated[d]	6.9 ± 0.1^b	7.2 ± 0.3^c	7.6 ± 0.2^d	8.0 ± 0.1^e	8.0 ± 0.1^e
Others[e]	4.7 ± 0.1^b	4.3 ± 0.2^c	4.2 ± 0.1^{cd}	4.1 ± 0.1^d	4.1 ± 0.2^d

[a]Excluding CLA. Means at the same row with different superscripts (b–f) differ significantly ($P < 0.05$). Data are expressed as mean ± SD of n = 8–9 samples.
[b]Polyunsaturated = linoleic acid + α-linolenic acid.
[c]Monounsaturated = oleic acid (18:1n-9) + 18:1n-7.
[d]Saturated = myristic acid + palmitic acid + stearic acid + arachic acid.
[e]Others = *trans* isomers of linoleic acid and α-linolenic acid.

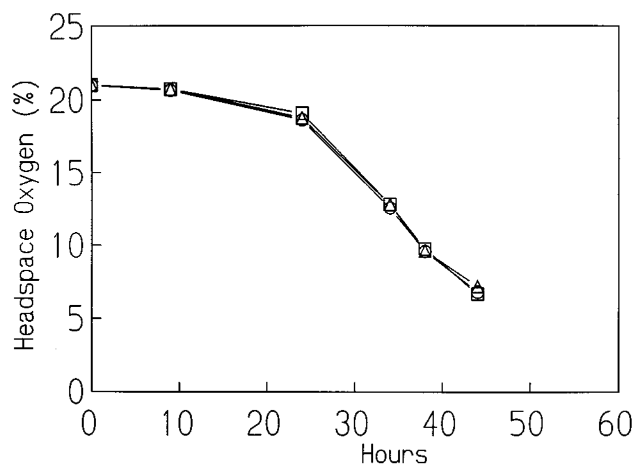

FIG. 4. Effect of conjugated linoleic acid-containing triacylglycerols (CLATG) on oxidation of canola oil at 90°C. Data are expressed as mean ± SD; n = 8 samples; ○, canola oil; △, canola oil + 2.0% CLATG; □, canola oil + 4.0% CLATG.

ble to oxidation. When heated together with canola oil, the free CLA and CLAME were preferentially oxidized and then triggered the oxidation of canola oil triacylglycerols. To prove this, we demonstrated that free CLA is remarkably less stable than LA in air (Fig. 5). This observation is in agreement with that of van den Berg *et al.* (13), who showed that CLA oxidized considerably faster than LA when incubated either separately or together with LA. In fact, conjugated double bonds in a fatty acid have been shown to be more susceptible to free-radical attack than nonconjugated double bond systems (17).

The effectiveness of an antioxidant is determined by many factors, including activation energy, rate constant, oxidation reduction potential, ease of antioxidant loss or destruction, and solubility properties (18). In general, an antioxidant should be an excellent donor of electrons or hydrogens, and the resulting antioxidant free-radical intermediate should be relatively stable and should not initiate new free radicals or not be subject to rapid oxidation by a chain reaction. In this regard, like phenolic antioxidants, CLA can readily donate an

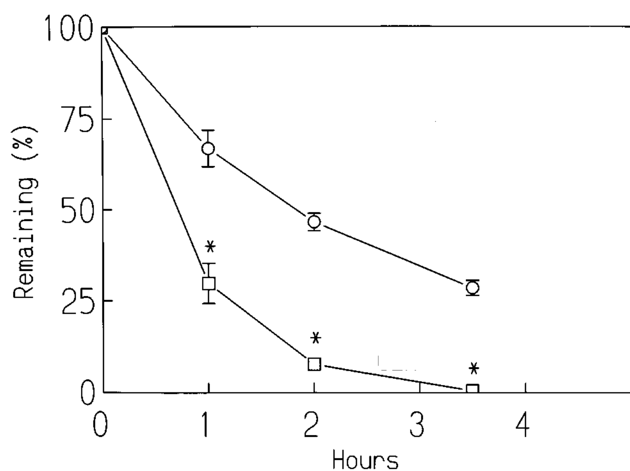

FIG. 5. Time course of the remaining conjugated linoleic acids (CLA) and linoleic acid (LA) heated at 90°C. Data are expressed as mean ± SD; n = 8 samples; ○, LA; ❑, CLA. *Represents a significant difference between CLA and LA.

electron or a hydrogen and form a CLA free-radical intermediate due to resonance delocalization. However, unlike phenolic antioxidants, the CLA free-radical intermediate may be unstable and subject to further oxidative degradation. In fact, it has been shown that CLA is rapidly decomposed to form furan fatty acids (19).

The mechanism by which the CLA present in triacylglycerols exhibited no prooxidant activity remains unclear. It is possible that the CLA esterified in triacylglycerol is less mobile or that the CLA free radical in triacylglycerols is more stable than that in the form of free CLA and CLAME. It will be of interest to study oxidative features of CLA in different chemical forms and identify their corresponding oxidation products.

The present study clearly indicates that CLA does not serve as an antioxidant in fats and oils. Contrary to current thought, CLA in the form of free fatty acid and methyl ester functions as a prooxidant instead. Together with findings of van den Berg *et al.* (13), the current results indicate that CLA as an antioxidant cannot be warranted.

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