Oxidative Stability of Conjugated Linoleic Acid Isomers

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Conjugated linoleic acids (CLAs) have been shown to be a strong anticarcinogen in a number of animal models. Our previous study demonstrated that CLA as a whole was extremely unstable in air. The present study was undertaken further to examine the oxidative stability of individual CLA isomers using the combination of gas−liquid chromatography (GLC) and silver ion high-performance liquid chromatography (Ag-HPLC). It was found that CLA as a whole oxidized rapidly and more than 80% was degraded within 110 h in air at 50 °C. Four *c,c*-CLA isomers were most unstable followed by four *c,t*-CLA isomers. In contrast, four *t,t*-CLA isomers were relatively stable under the same experimental conditions. Both the oxygen consumption and the GLC analysis revealed that 200 ppm jasmine green tea catechins (GTCs) exhibited protection to CLA and were even stronger than 200 ppm butylated hydroxytoluene (BHT) when added to either CLA or canola oil containing 10% CLA. The present study emphasized that oxidative unstability of CLA should not be overlooked although CLA has many biological effects.

Keywords: *Conjugated linoleic acids; oxidation; oxygen consumption; green tea catechins*

INTRODUCTION

Conjugated linoleic acid (CLA) is a generic term used to describe a group of positional and geometric isomers of linoleic acid (LA). In CLA, two double bonds are conjugated, whereas they are methylene-interrupted in LA. CLA is predominantly present in meat and dairy products (Chin et al., 1992; Ha et al., 1989). Low concentrations of CLA also occur in the lipids of human blood, tissue, and milk (Cawood et al., 1983; Harrison et al., 1985; Iversen et al., 1985). CLA can also be prepared enzymatically and chemically in a large quantity (Hughes et al., 1982; Kepler and Tove, 1967; Nichols et al., 1951). In the last two decades, CLA has received considerable attention as a chemopreventive agent since being shown to have inhibitory effects on mammary tumorigenesis, forestomach neoplasia, and skin carcinogenesis (Ha et al., 1987, 1990; Ip et al., 1991, 1994; Schultz et al., 1992). CLA is also hypolipidemic and antiatherosclerotic in several animal models (Lee et al., 1994; Nicolosi et al., 1997). CLA may also enhance immune functions (Cook et al., 1993; Miller et al., 1994) and reduce fat accumulation (Dugan et al., 1997).

The exact mechanism of anticarcinogenic action remains poorly understood. Ha et al. (1990) and Ip et al. (1991) showed that CLA had antioxidative activity and proposed this as a possible explanation for the antiocarcinogenic and antiatherosclerotic effect. However, other investigations have found that CLA was a prooxidant (van den Berg et al., 1995). We have examined the stability of CLA as its chemical form of free fatty acids, methyl esters, and triacylglycerols in various conditions. It was found that CLA as the free fatty acid form was extremely unstable similar to that of docosahexanenoic acid (Chen et al., 1997) and had an oxidation rate considerably greater than that of LA, linolenic acid, and arachidonic acid (Zhang and Chen, 1997). In fact, Yurawecz et al. (1995) reported that CLA was rapidly decomposed to form furan fatty acids when it was oxidized in air.

It is essential to protect CLA from oxidation before it is consumed. Silver ion high-performance liquid chromatographic (Ag-HPLC) analysis has demonstrated that CLA contains at least 12 isomers (Sehat et al., 1998). The present study was designed to examine the stability of these individual CLA isomers in air. It is known that green tea catechins (GTCs) are strong antioxidants. It was also of interest to study the effect of GTC on the stability of CLA.

MATERIALS AND METHODS

Chemicals. CLA as the form of free fatty acids was obtained from either Sigma Chemical Co. (St. Louis, MO) or Natural Lipids Ltd. AS (Hovdebygda, Norway). 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.

Green Tea Catechins Extraction. GTC was extracted as we previously described (Zhang et al., 1997) from jasmine tea (Cheong Hing Tea Co. Ltd., Hong Kong). In brief, 10 g of dry tea leaves was soaked three times with 150 mL of hot distilled water (80 °C). The infusion was cooled to room temperature, filtered, and then extracted with an equal volume of chloroform to remove caffeine and pigment. The GTC in the remaining aqueous layer was then extracted twice with an equal volume of ethyl acetate. After ethyl acetate was removed using a vacuum rotary evaporator, the resulting crude GTC extract was dissolved in 10 mL of distilled water and freeze-dried overnight. The individual catechin derivatives in jasmine tea extract were quantified using a Shimazu LC-10AD HPLC

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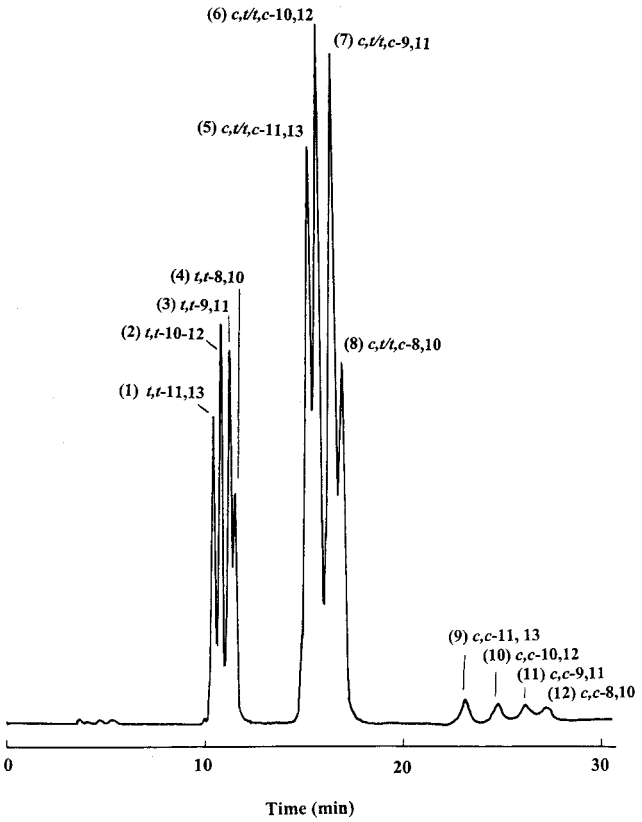

Figure 1. Silver ion high-performance liquid chromatogram of a commercial conjugated linoleic acid methyl esters standard obtained from Sigma, using a silver-impregnated Chrompack analytical column (4.6 mm i.d. × 250 mm), and 0.1% acetonitrile in hexane as a mobile phase at a flow rate of 1 mL min^{-1}.

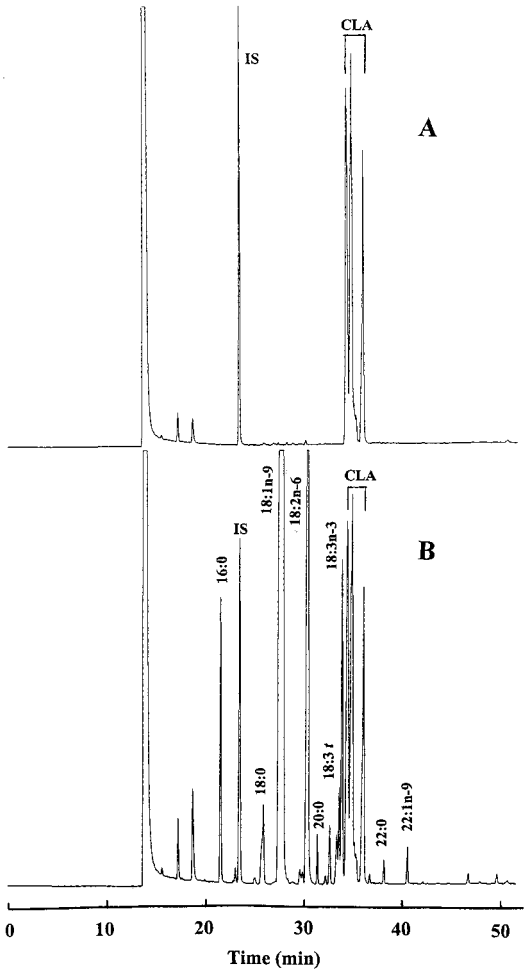

Figure 2. Gas−liquid chromatograms of CLAME obtained from Sigma (A) or in canola oil containing 10% CLA (B). IS = internal standard (heptadecanoic acid methyl ester).

(Tokyo, Japan) equipped with a ternary pump as we previously described (Zhu et al., 1999). The extraction method used in this study yielded 7.5 g of GTC/100 g of jasmine green tea leaves with a purity of 95% in which (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC) accounted for 62.3%, 19.2%, 8.3%, and 4.6%, respectively.

Methylation. Acid-catalyzed methylation was used in the present study (Luddy et al., 1960). In brief, CLA or canola oil samples (10−20 mg) in 1 mL of tetrahydrofuran were transferred into a test tube followed by addition of 2 mL of 5% methanolic hydrogen chloride, which was freshly prepared by adding acetyl chloride (5 mL) slowly to cooled dry methanol (50 mL). The tube was then flushed with nitrogen gas and sealed tightly. The methylation tube was placed in a heat block at 90 °C for 30 min and then cooled to room temperature. Four milliliters of hexane and 3 mL of distilled water were then added and mixed thoroughly. After centrifugation, the top hexane layer containing CLA methyl esters (CLAMEs) was saved and subjected to GLC and Ag-HPLC analysis.

Ag-HPLC Analysis. The individual CLAME isomers were separated using an Alltech model 525 high-performance liquid chromatograph equipped with a ternary pump delivery system as described by Sehat et al. (1998). In brief, 5 μL of CLAME (5 μg/mL) in hexane was injected onto a silver-impregnated column (4.6 mm i.d. × 250 mm stainless steel, 5 μm, Chrompack, Bridgewater, NJ) via a rheodyne valve injector. Hexane containing 0.1% acetonitrile was chosen as a mobile phase at a flow rate of 1.0 mL/min. The separated individual CLAME isomers were monitored at 233 nm using a UV detector (UVIS-205, Alltech, Deerfield, IL). Individual CLA isomers were identified according to the Ag-HPLC eluting pattern (Figure 1) described by Sehat et al. (1998).

Oxygen Consumption Test. The method previously described by Chen et al. (1994) was used to monitor oxygen consumption. In brief, 200 mg of CLA (or LA) was placed in a glass tube (150 × 16 mm o.d.) with or without addition of varying concentrations of butylated hydroxytoluene (BHT) and jasmine GTC in tetrahydrofuran. The components in the test tube were mixed thoroughly. The solvents were removed under a gentle stream of nitrogen at 45 °C. The reaction tube was then flushed with air and sealed tightly with a rubber stopper obtained from an evacuated blood collection tube (100 × 16 mm o.d., Becton-Disckinson, Rutherford, NJ), which usually maintains a vacuum for 2−3 years. The sealed tube was leak-free and was verified by filling the tube with nitrogen gas and monitoring the headspace oxygen concentration by gas chromatography. Oxidation was conducted at 90 ± 2 °C. The headspace oxygen was sampled periodically with a gastight syringe and analyzed in a HP 5890 series II gas−solid chromatograph (Hewlett-Packard, Palo Alto, CA) fitted with a 1/8 in. × 6 stainless steel column packed with molecular sieves 5A (60/80 mesh) and a thermal conductivity detector. The percent oxygen in the headspace was calculated from the ratio of the oxygen to nitrogen.

Fatty Acid Analysis. CLAME was analyzed on a flexible silica capillary column (SP 2560, 100 m × 0.25 mm i.d., Supelco, Inc., Bellefonte, PA) in a HP 5980 series II gas−liquid chromatograph equipped with a flame-ionization detector and an automated injector (Palo Alto, CA) according to the method we previously described (Chen et al., 1997). The column temperature was programmed from 180 to 220 °C at a rate of 1 °C/min and then held for 12 min. The 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 (Figure 2).

Stability of Individual CLA Isomers. CLA (1.5 g) and LA (0.3 g) were mixed in 50 mL beakers (n = 5) and then

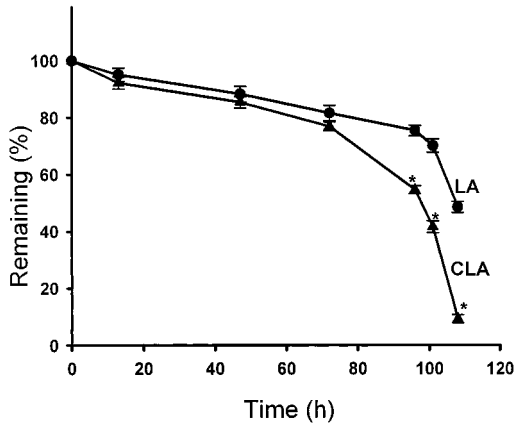

Figure 3. Time course of the remaining free CLA and linoleic acid (LA) heated at 50 °C in air. Data are expressed as means ± SD for $n = 5$ samples. An asterisk represents a significant difference between the CLA and LA samples at the same time point ($p < 0.05$).

flushed with air for 4 min. The reason for choosing this ratio is that the amount of four major *c,t*-CLA isomers would be similar to that of LA and thus the oxidative stability of the former could be compared with the latter. The mixture was then oxidized under continuous stirring in air at 50 ± 3 °C. Five samples (20 mg each) were periodically taken. After heptadecanoic acid (2 mg/mL tetrahydrofuran) was added, the sample was converted to the fatty acid methyl esters as described above and then subjected to GLC and Ag-HPLC analysis.

Stability of CLA in Canola Oil. The objective of this experiment was to test the effect of GTC and BHT on the stability of CLA in canola oil. In brief, 5 g of canola oil containing 10% CLA in the presence of 200 ppm BHT or 100−300 ppm GTC was heated in air at 90 ± 2 °C ($n = 5$). The rationale for choosing these concentrations is that the maximum 200 ppm of a single antioxidant or a mixture of antioxidants is generally permitted in fats and oils in most countries (Botma, 1990). The mixture was sampled periodically and then methylated. The resultant CLAME was analyzed using GLC and quantified using heptadecanoic acid as an internal standard.

Statistics. All the experiments were repeated three times. Data were pooled from each experiment in which five replicates were conducted. Data for the headspace oxygen consumption and fatty acid analysis were subjected to the analysis of variance (ANOVA), and the means were compared among treatments by using Duncan's multiple range test. 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

To study the stability of CLA relative to LA, a mixture of CLA and LA was exposed to air at 50 °C for varying times. The CLA was methylated together with heptadecanoic acid and quantified using GLC according to the amount of heptadecanoic acid added (Figure 2). The stability was expressed as a percentage of its original amount before oxidation. It was confirmed that the oxidation rate of CLA as a mixture of 12 isomers was considerably faster than that of LA under the present experimental conditions (Figure 3). It was found that more than 80% CLA was lost within 110 h in air at 50 °C.

To assess the relative stability of CLA isomers, the decrease in each CLA isomer was monitored using the combination of GLC and Ag-HPLC methods. A typical Ag-HPLC chromatogram of CLAME is shown in Figure

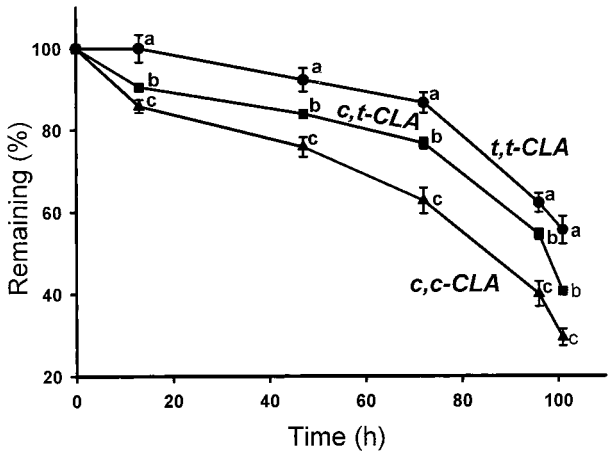

Figure 4. Time course of the remaining total *c,c*-CLA isomers (sum of *c,c*-11,13, *c,c*-10,12, *c,c*-9,11, and *c,c*-8,10), total *c,t*-isomers (sum of *c,t*/*t,c*-11,13, *c,t*/*t,c*-10,12, *c,t*/*t,c*-9,11, and *c.t*/*t,c*-8,10), and total *t,t*-isomers (sum of *t,t*-11,13, *t,t*-10,12, *t,t*-9,11, and *t,t*-8,10), heated at 50 °C in air. Data are expressed as mean ± SD for $n = 5$ samples. Means at the same time point with different superscript letters (a−c) differ significantly ($p < 0.05$).

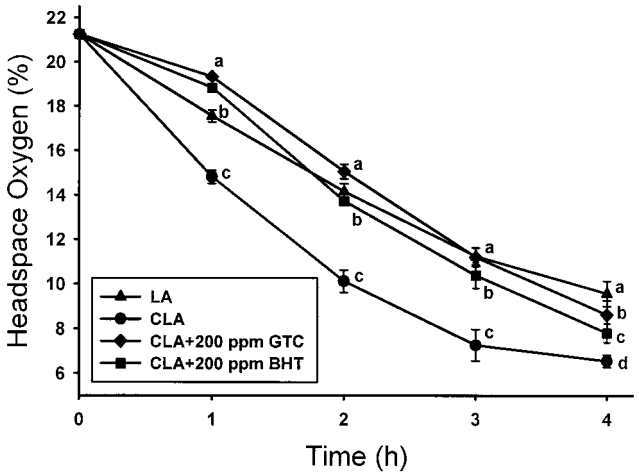

Figure 5. Headspace oxygen consumption profile of LA and CLA with or without addition of 200 ppm GTC or 200 ppm BHT at 90 °C. Data are expressed as mean ± SD for $n = 5$ samples. Means at the same time point with different superscript letters (a−d) differ significantly ($p < 0.05$).

1. Twelve isomers were eluted, generally in three clusters of four isomers each. The identification of these isomers was made according to Sehat et al. (1998). The first cluster of four peaks represents *t,t*-isomers, whereas the second and third clusters represent *c,t*-isomers and *c,c*-isomers, respectively. As shown in Figure 4, three groups of CLA isomers demonstrated varying oxidative stability. Four *t,t*-isomers including *t,t*-11,13, *t,t*-10,12, *t,t*-9,11, and *t,t*-8,10 were most stable, while four *c,c*-isomers, namely, *c,c*-11,13, *c,c*-10,12, *c,c*-9,11, and *c,c*-8,10, were most unstable (Figure 4). Among each group, the oxidation rate of individual isomers was very similar and no statistical significance was observed (data not shown).

The oxygen consumption test showed that the oxygen uptake by the CLA sample was considerably faster than that by LA at 90 °C, indicating that CLA was considerably more unstable than LA (Figure 5). Addition of 200 ppm GTC significantly decreased the oxygen uptake by CLA as compared with the control CLA sample. It was noteworthy that 200 ppm GTC was more effective than

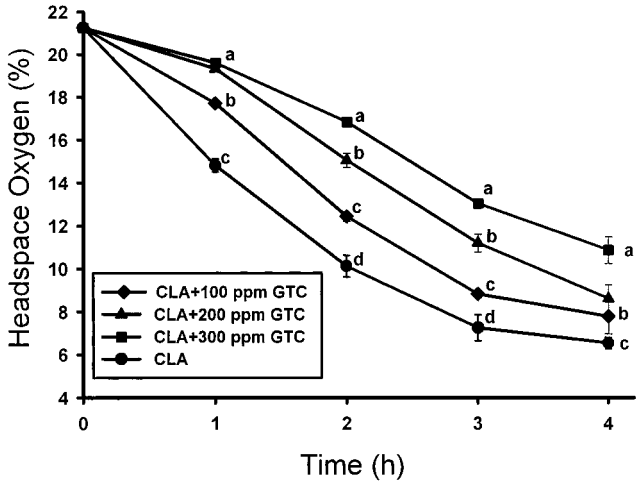

Figure 6. Headspace oxygen consumption profile of CLA with or without addition of 100, 200, and 300 ppm GTC at 90 °C. Data are expressed as mean ± SD for $n = 5$ samples. Means at the same time point with different superscript letters (a−d) differ significantly ($p < 0.05$).

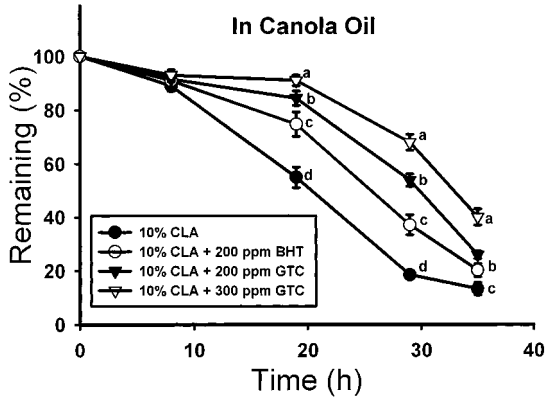

Figure 7. Time course of the remaining CLA in canola oil containing 10% CLA with addition of 200 ppm BHT or 200−300 ppm GTC at 90 °C in air. Data are expressed as means ± SD for $n = 5$ samples. Means at the same time point with different superscript letters (a−d) differ significantly ($p < 0.05$).

200 ppm BHT in protecting CLA from oxidation (Figure 5). The protective effect of GTC on the oxidation of CLA was dose-dependent (Figure 6). The higher the dose of GTC added, the stronger the GTC protected the CLA from oxidation.

The oxidative stability of CLA (10%) in canola oil was also examined at 90 °C. As shown in Figure 7, the CLA present in canola oil was rapidly oxidized and reduced to 14% after 35 h of heating at 90 °C. Addition of BHT and GTC significantly slowed the rate of CLA oxidation in canola oil. At the concentration of 200 ppm, GTC was more protective to CLA than BHT. The protection of GTC in canola oil was again confirmed to be dose-dependent.

DISCUSSION

The present study was the first to examine the relative stability of 12 CLA isomers. The four *t,t*-CLA isomers were most stable followed by the four *c,t*-CLA isomers. In contrast, the four *c,c*-CLA isomers were most susceptible to autoxidation. A *cis*-double bond is chemically more unstable than a *trans*-double bond because the former has a higher free energy level and is more vulnerable to oxygen attack. The present study also demonstrated that the four *c,c*-CLA isomers were oxidized at a similar rate. Similarily, the same oxidative stability was also observed among the four *c,t*-CLA isomers, or among the four *t,t*-CLA isomers. These results indicate that the stability of CLA isomers is at least partially determined by its *cis*- or *trans*-configuration but not by the position of their double bonds.

The present study also showed that CLA was oxidized faster than LA (Figure 3), suggesting a conjugated double bond is more vulnerable to autoxidation than a nonconjugated double bond under the present experimental conditions. This was in agreement with those of previous observations (Chen et al., 1997; van den Berg et al., 1994; Zhang and Chen, 1997). CLA with two conjugated double bonds might be more vulnerable to autoxidation than α-linolenic acid with three methylene-interrupted double bonds (Zhang and Chen, 1997). This is because that CLA can readily donate an electron or a hydrogen to form a CLA free radical intermediate due to resonance delocalization. In fact, these CLA free radicals have been shown to be rapidly decomposed to furan fatty acids (Yurawecz et al., 1995). However, the oxidation systems used have to be taken into consideration when the oxidative stability of CLA is assessed. Sea et al. (1999) demonstrated that CLA was more stable than LA in the aqueous system when 2,2′-azobis(2-amidinopropan) dihydrochloride was used as a free radical initiator. In contrast, the reversal was seen when oxidation was conducted in benzene. In light of this, we are currently examining the stability of CLA under the various conditions including pH values of the medium, temperature, UV light, and interaction with other food components.

CLA has been claimed to have many biological effects although the biochemical mechanisms and the active isomer(s) for these effects still remain unknown. The present results together with our previous reports (Chen et al., 1997; Zhang and Chen, 1997) clearly showed that CLA was very susceptible to autoxidation when it was exposed to air under the present experimental conditions. To our best knowledge, there has been no study to date taking efforts to prevent CLA from oxidative deterioration. Jasmine green tea is an excellent source of natural antioxidants, which are mainly GTC derivatives including EGCG, EGC, EC,G and EC. GTC has been shown to be effective at protecting fats and oil in food from oxidative rancidity (Chen and Chan, 1996; Chen et al., 1996; Lunder, 1992; Tanizawa et al., 1984). It was of interest to test whether GTC was effective in protecting CLA from oxidation as compared with BHT, a most commonly used synthetic antioxidant in food. When GTC was added to either a CLA mixture or canola oil containing 10% CLA, it significantly prevented the oxidation of CLA as monitored using the oxygen consumption test and GLC. The inhibition of 200 ppm GTC on CLA oxidation was even stronger than that of 200 ppm BHT under the same conditions. It is known that canola oil contains α-tocopherol (Yuki and Iskikawa, 1976). It is also possible that GTC and α-tocopherol have a synergistic effect on the oxidation of CLA when added in canola oil.

In conclusion, CLA was extremely unstable in air. Ag-HPLC analysis revealed that there were at least 12 isomers present in the CLA preparation. The oxidative stability test showed that four *c,c*-CLA isomers were most susceptible to oxidative degradation while the four

t,t-CLA isomers were most stable in air under the same conditions. Jasmine GTC (200 ppm) was more effective as an antioxidant than BHT (200 ppm) in protecting CLA from oxidation. Although CLA might have many beneficial biological effects, the prevention of oxidative deterioration must be taken into consideration when CLA is prepared, stored, transported, and supplemented in the diet.

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