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Conjugated Linoleic Acid-Rich Soy Oil Triacylglycerol Identification

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Conjugated linoleic acid (CLA)-rich soy oil has been produced by soy oil linoleic acid (LA) photoisomerization, but CLA-rich oil triacylglycerol (TAG) characterization was not described. Therefore, the objectives were to identify and quantify new TAG fractions in CLA-rich oil by nonaqueous reversed-phase high-performance liquid chromatography (NARP-HPLC). Analytical NARP-HPLC with an acetonitrile/dichloromethane (ACN/DCM) gradient and an evaporating light scattering detector/ultraviolet (ELSD/UV) detector was used. New TAG peaks from LA-containing TAGs were observed. The LnLL, LLL, LLO, and LLP (Ln, linolenic; L, linoleic; O, oleic; and P, palmitic) peaks reduced after isomerization with an increase in adjacent peaks that coeluted with LnLnO, LnLO, LnOO, and LnPP. The newly formed peaks were wider than those of the original oil and absorbed at 233 nm, suggesting the possibility of various CLA containing TAGs. The HPLC profile showed five fractions of mixed TAGs, and fatty acid analysis showed that CLA isomers were found predominately in fractions 2 and 3, which originally contained most LA. The CLA isomers were 70−80% *trans,trans* and 20−30% *cis,trans* and *trans,cis*.

KEYWORDS: Conjugated linoleic acid (CLA); triacylglycerols (TAGs); nonaqueous reversed-phase high-performance liquid chromatography (NARP-HPLC); gas chromatography (GC); flame ionization detector (FID); photoisomerization

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term describing certain geometrical and positional isomers of octadecadienoic acid (18:2) naturally found in milk and meat products of ruminant animals. *cis*-9,*trans*-11-Octadecadienoic acid and *trans*-10,*cis*-12-octadecadienoic acid are the most common CLA isomers (*1*), produced predominantly by ruminants as a first intermediate of biohydrogenation of linoleic acid by the anaerobic rumen bacteria *Butyrivibrio fibrisolvens* (*2*). CLA has been reported to have beneficial properties of suppressing carcinogenesis (*3, 4*), reducing atherosclerosis (*5*), enhancing immune system (*6*), reducing body fat (*7*), acting as an antimutagenic agent and antioxidant, and lowering insulin resistance (*8*) in experimental animals. Furthermore, it has been shown that CLA acts as a growth factor, increasing bone (*9*) and muscle mass (*10, 11*). CLA decreased abdominal fat in obese men with metabolic syndrome (*12*). Recently, the *trans*,*trans*-CLA isomers have been shown to have other health benefits. In particular, *trans*-9,*trans*-11-CLA inhibited bovine aortic endothelial cell proliferation by apoptotic pathway and proliferation of human leukemic cell lines (*13*). Also, *trans*, *trans*-CLA isomers decreased the ultraviolet radiation (UVR) induced secretion of interleukin, prostaglandin E2, and carcinogenesis (*14*). CLA can be synthesized by dehydration of hydroxyl fatty acids and enzymatic interesterification of lipids using algal or bacterial enzymes. However, these techniques resulted in a high level of oil oxidation and low CLA yields (*15*). A pilot plant process was developed for photoisomerization of linoleic acid in soybean oil that produced 22% CLA (*16*−*18*). The amount of each CLA isomer in the oil was 1.5% *cis*-9,*trans*-11; 1.5% *trans*-9,*cis*-11/*cis*-10,*trans*-12; 2.0% *trans*-10,*cis*-12; and 17% *trans*-*trans*-8,10/9,11/10,12. However, characterization of the CLA-containing triacylglycerol (TAG) species was not reported. This is the focus of the proposed study.

List et al. (*19*) used nonaqueous reversed-phase high-performance liquid chromatography (NARP-HPLC) to separate soy oil TAG species using an analytical HPLC column (two Inertsil, C_{18}, 250 mm × 4.6 mm i.d. each, 5 μm particle size). This method used a solvent gradient elution system of acetonitrile (ACN)/dichloromethane (DCM), changing over time, and a flow rate of 0.85 mL/min with an atmospheric chemical ionization mass spectrometer (APCI-MS). The TAG species eluted in order of increasing saturation with TAGs containing

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linolenic (Ln) acid (LnLnLn and LnLnL) eluting first followed by TAGs with linoleic (L) rich fatty acid (LLL, LLO, and LLP). Monoenoic-containing TAGs (LOP and LOO) were then eluted followed by TAGs containing saturated fatty acids. Although TAG species could be readily identified, peak resolution was low. However, the data clearly identified the linoleic acid containing TAGs (LnLL, LLL, LLO, and LLP), which potentially could be converted to CLA-containing species during photoisomerization. This NARP-HPLC technique could be useful in identifying new CLA isomer TAG species produced by soy oil linoleic acid photoisomerization. This study will also obtain concentrated CLA fractions or mixed CLA-TAG standards for subsequent research.

The objectives of this research were to (i) determine the HPLC retention times of new CLA-containing TAG species obtained by photoisomerization of pure trilinolein (LLL-TAG), (ii) qualitatively identify new CLA TAG fractions in soy oil obtained by photoirradiation, and (iii) quantitatively determine the fatty acid composition of new CLA TAG fractions formed during soy oil photoisomerization.

MATERIALS AND METHODS

Materials. *Reagents and Samples.* Refined, bleached, and deodorized (RBD) soy oil (Wesson; ConAgra, Irvine, CA) was obtained from a local grocery store (Fayetteville, AR) with 55% linoleic acid and 6% linolenic acid. Glycerol trilinoleate (LLL-TAG, 99.9% pure) standard was obtained (Sigma-Aldrich, St. Louis, MO). HPLC mobile solvents ACN and DCM were of HPLC grade (VWR International, West Chester, PA) and were used without further purification. Nitrogen gas, oxygen-free grade (Scientific Supplies, University of Arkansas, Fayetteville, AR), was used. Commercial heptadecanoic acid methyl ester (HME C17:0; Sigma-Aldrich, St. Louis, MO) was used as a FAME internal standard.

Qualitative TAG Analysis of CLA-Rich Trilinolein and CLA-Rich Soy Oil by NARP-HPLC. *CLA Preparation.* Triplicate 5 g trilinolein (LLL-TAG) and 5 g RBD soy oil samples in 7-mL borosilicate glass vials with 0.35% iodine catalyst were attached to an illuminated laminar flow unit of a photoirradiation unit with photolevel of 3.14 mW as described by Jain and Proctor (*18*). The photoirradiation of the RBD soy oil was done for 12 h, whereas the LLL-TAG was irradiated for 12 and 36 h, respectively. Peroxide value (PV) was then determined in triplicate for each replicate (*20*).

Analytical HPLC of Control Trilinolein and Control Soy Oil before and after Photoisomerization. Triplicate samples of control LLL-TAG, CLA-rich LLL-TAG, control soy oil, and CLA-rich soy oil were dissolved in the HPLC mobile phase of 50:50 ACN/DCM to obtain a concentration of 20 mg/mL for each replicate. The method was adapted from List et al. (*19*). Ten-microliter samples each consisting 0.2 mg of TAG were injected onto the column using the Waters 717 plus autosampler and a Waters model 600 system, equipped with a quaternary pump (Waters Delta 600). Two C_{18} reverse-phase analytical columns (Sunfire, Waters Corp., Milford, MA), 250 mm × 4.6 mm i.d. each, 5-μm particle size, were placed in series, with a 20 mm × 4.6 mm i.d. guard column. The column temperature was kept constant at 30 °C using a Temperature Control Module (Waters Corp.). The solvent gradient elution, pumped at a flow rate of 0.85 mL/min, was as follows: 70% ACN/30% DCM for 40 min; to 65% ACN/35% DCM at 45 min, held until 55 min; to 60% ACN/40% DCM at 60 min, held until 70 min; to 55% ACN/45% DCM at 80 min, held until 100 min; returning to 70% ACN/30% DCM until 120 min. The column effluent was split between an ELSD and a UV-photodiode array (PDA) detector receiving ~720 μL/min (85%) and ~130 μL/min (15%). The ELSD (Waters model 2420) had a drift tube temperature of 48 °C and a gas flow pressure of 25 psi. The photomultiplier gain was 1. High-purity N_2 was used as the nebulizer gas. The UV-PDA (Waters model 2996) was set at 233 nm. The modification of the List et al. method (*19*) was the ELSD and UV detectors. The data output from ELSD was integrated by Waters Empower 2 software. The analysis of each replicate was done in triplicates.

Quantitative Analysis of Fatty Acids in CLA-Rich Trilinolein and New CLA-TAG Fractions in CLA-Rich Soy Oil. *Fatty Acid Composition of Control LLL-TAG and CLA-Rich LLL-TAG.* The control LLL-TAG and 12 and 36 h irradiated LLL-TAGs were obtained after photoisomerization. The TAG samples were then converted to FAMEs (*20*) and were analyzed by GC-FID to evaluate the total fatty acid composition of the LLL-TAGs before and after photoisomerization. This information will be useful in comparing the trends of fatty acids formed in the subsequent study using soy oil.

Semipreparative NARP-HPLC of Control Soy Oil and CLA-Rich Soy Oil. Triplicates of control RBD soy oil and CLA-rich soy oil samples were dissolved in the mobile phase of 50:50 ACN/DCM to obtain a concentration of 250 mg/mL. A semipreparative NARP-HPLC of the CLA-rich soy oil and control soy oil was adapted from the qualitative NARP-HPLC method, described earlier, to obtain large enough TAG fractions for subsequent fatty acid analysis. The method upgrade was done using Water's Preparative Calculator. Three replicates of 100 μL consisting of 25 mg of oil sample were injected using a Waters 717 plus autosampler to obtain enough HPLC TAG fractions for subsequent fatty acid analysis. Two C_{18} reverse-phase semipreparative columns (Sunfire, Waters Corp.), 250 mm × 10 mm i.d. each, 5-μm particle size, were placed in series, with a 20 mm × 10 mm i.d. guard column. The column temperature was kept constant at 30 °C using a Temperature Control Module (Waters Corp.). The solvent gradient elution, pumped at a flow rate of 4.0 mL/min, was used as previously described. A UV-PDA monitored the effluent at 233 nm. The CLA-rich oil TAG fractions (F-1−F-5) obtained by HPLC were then combined individually, as were those from the control soy oil. Each of the five TAG fractions was then evaporated separately using a Rotavapor R-114 (Buchi Corp., New Castle, DE) and converted to FAMEs (*21*). The oil content of each fraction was determined by gravimetric analysis.

Fatty Acid Methyl Ester (FAME) Preparation. The fatty acid composition of LLL-TAG before and after photoisomerization and each control soy oil and CLA-rich soy oil TAG fraction obtained from semipreparative NARP-HPLC was converted to FAMEs by modifying the following micromethod (*21*). Triplicate samples were weighed using a weighing balance (Mettler Toledo Classic AB204-S) in 25-mL centrifuge tubes using a class A pipet. A 1% heptadecanoic acid methyl ester (HME) solution in hexane was prepared. HME equivalent to 5% of the oil weight was added to each centrifuge tube as an internal standard. One milliliter of toluene and 4 mL of 0.5 M sodium methoxide in methanol were added to each centrifuge tube and purged with nitrogen gas. The centrifuge tubes were heated to 50 °C in a water bath for 10−12 min and then cooled for 5 min. To inhibit formation of sodium hydroxide, which could hydrolyze methyl esters to free fatty acids, 0.2 mL of glacial acetic acid was added to the centrifuge tube. Five milliliters of distilled water was added to each centrifuge tube followed by 1 mL of hexane, and the tubes were vortexed for 2 min. The hexane layer was extracted and dried over anhydrous sodium sulfate in a 7-mL glass vial for 15−20 s.

GC-FID Analysis. Fatty acid profiles were then analyzed by measuring the FAMEs by GC in triplicate for each replicate using an SP 2560 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Supelco Inc., Bellefonte, PA) with a flame ionization detector (FID) (model 3800, Varian, Walnut Creek, CA). The samples were injected by an autosampler (Varian). The sensitivity of the GC instrument was manually maximized in Galaxie Chromatography Workstation software (version 1.9.3.2) to 12 to provide sufficient sensitivity. The FID settings were as follows: heater = 250 °C, sensitivity = 12, He gas = 30 mL/min, H_2 = 31 mL/min, air = 296 mL/min, and oven program time = 111 min. Fatty acid concentrations were calculated by the following equation (*21*):

$$\%\ \text{fatty acid concn} = \frac{(\text{int. std concn} \times \text{sample peak} \times \text{relative response factor})}{\text{int std sample peak}}$$

Statistical Data Analysis. Data were processed by analysis of variance (ANOVA) using JMP version 7.1 (SAS Institute, Cary, NC). Least

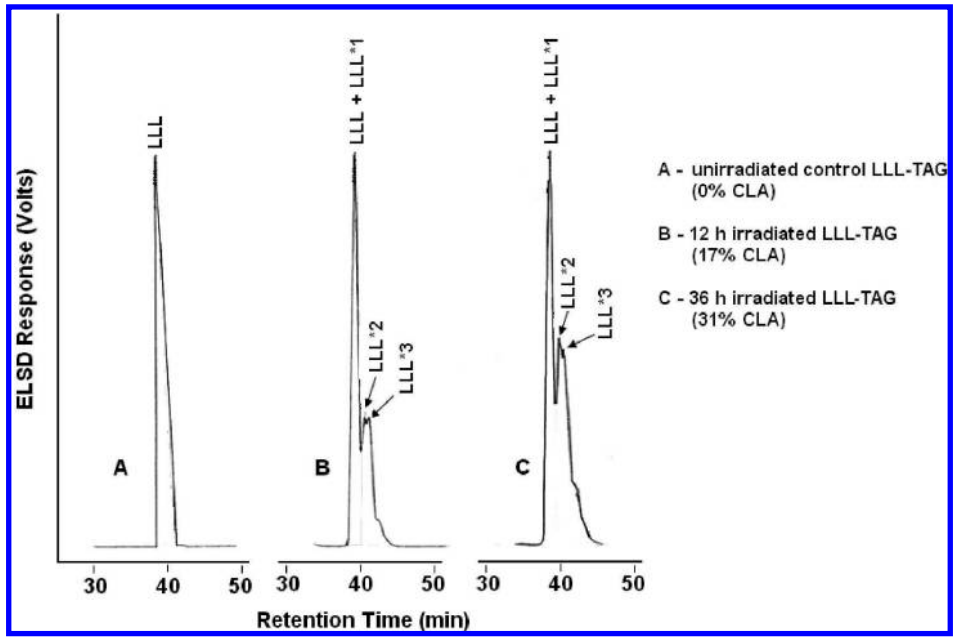

Figure 1. NARP-HPLC fractionation of (**A**) unirradiated control LLL-TAG, (**B**) 12 h irradiated LLL-TAG, and (**C**) 36 h irradiated LLL-TAG detected by ELSD. All samples had zero peroxide value (PV).

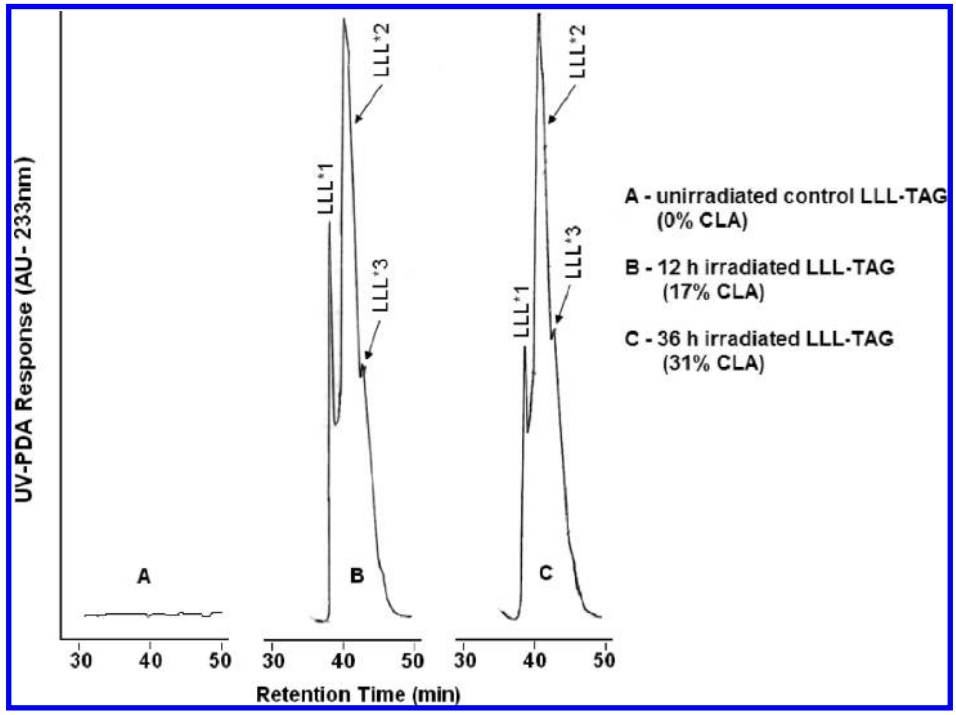

Figure 2. NARP-HPLC fractionation of (**A**) unirradiated control LLL-TAG, (**B**) 12 h irradiated LLL-TAG, and (**C**) 36 h irradiated LLL-TAG detected by UV detector at 233 nm. All samples had zero peroxide value (PV).

significant differences were calculated to compare mean values among replications for significant differences. Comparisons for all concentrations were done using Student's *t* test. Significance was established at $P < 0.05$.

RESULTS AND DISCUSSION

Qualitative TAG Analysis of CLA-Rich Trilinolein and CLA-Rich Soy Oil by NARP-HPLC. A NARP-HPLC chromatogram was obtained by ELSD for (**A**) control LLL-TAG, (**B**) 12 h irradiated LLL-TAG containing 17.0% CLA, and (**C**) 36 h irradiated LLL-TAG with 31% CLA levels as shown in **Figure 1**. The control LLL-TAG chromatogram (**A**) showed a single LLL peak, which identified its retention time for future studies. Chromatogram **B** shows there are new TAG species in CLA-rich LLL-TAG containing 17% CLA levels after 12 h of irradiation. It should be realized that the fatty acid notation (LLL*1) on the irradiated LLL-TAG ELSD chromatogram in **Figure 1B**,**C** describes the original LLL retention time in the control LLL-TAG (**Figure 1A**), where original LLL-TAG coelute together with other new CLA isomers as LLL + LLL*1 after photoirradiation.

After photoisomerization, the LLL peak in control trilinolein decreased relatively to give rise to new LLL*1, LLL*2, and LLL*3 TAG peaks, suggesting linoleic acid (L) isomerization to CLA during photoisomerization. The LLL*2 and LLL*3 peaks are new TAGs formed after photoisomerization, where

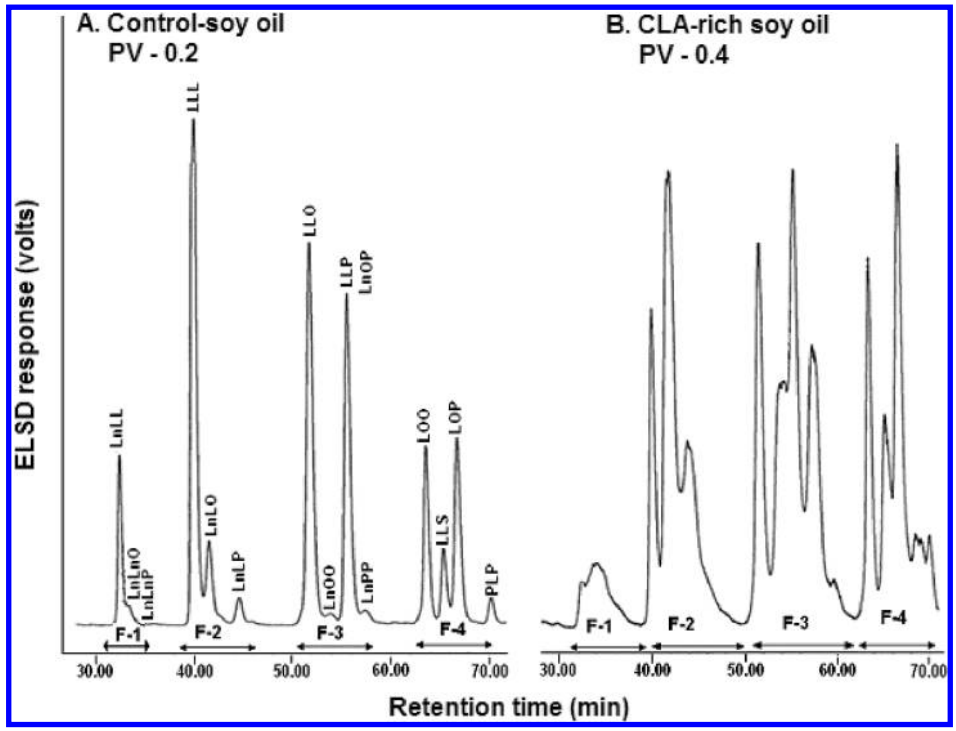

Figure 3. NARP-HPLC fractionation of (**A**) control soy oil and (**B**) 22% CLA-rich soy oil detected by ELSD.

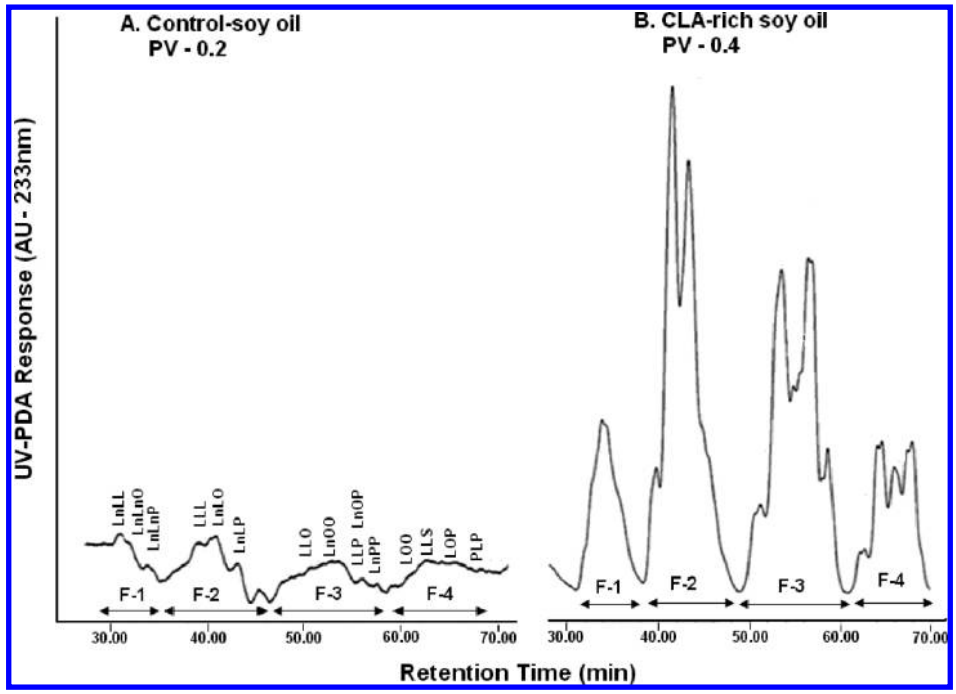

Figure 4. NARP-HPLC fractionation of (**A**) control soy oil and (**B**) 22% CLA-rich soy oil detected by UV-PDA at 233 nm. PV, peroxide value.

as the LLL*1 peak shows retention times similar to that of the control LLL-TAG, suggesting LLL coeluting with CLA isomers as the LLL + LLL*1 peak. The peak widening and relative height reduction suggest the possible conversion of L in LLL to at least two poorly resolved geometrical isomers of CLA resulting in TAG positional isomers. Similarly, when the ELSD chromatogram (**C**) is compared with the control LLL-TAG chromatogram (**A**), there is a relative increase in the LLL*1, LLL*2, and LLL*3 TAG peaks due to increased CLA levels to 31% after 36 h of irradiation. Some more peak widening and broadening (*22*) were observed with increasing CLA levels, suggesting formation of unresolved CLA TAG isomers after photoisomerization. **Figure 2** shows the corresponding UV absorbance at 233 nm of control LLL TAG and CLA-rich LLL-TAGs. Increased UV absorbance at 233 nm indicates conjugated dienes, and the zero PV value before and after photoisomerization suggests diene isomerization rather than oxidation. It should be realized that the fatty acid notation (LLL*1, LLL*2, and LLL*3) on the CLA-rich trilinolein UV chromatogram describes the retention time relative to the ELSD chromatograms of CLA-rich LLL-TAG as shown in **Figure 2B**,**C** chromatograms. The control LLL TAG (**A**) chromatogram in **Figure 2** showed no peaks, suggesting no conjugated dienes or oxidation, which is supported by the zero PV value. In contrast, after irradiation, new TAG peaks LLL*1, LLL*2, and LLL*3 shown in the ELSD chromatogram showed increased UV absorbance

Table 1. Fatty Acid Composition of Control LLL-TAG and 12 and 36 h CLA-Rich LLL-TAGs Analyzed by GC-FID[a]

	% fatty acid composition				
sample	C18:2	CLA1	CLA2	CLA3	CLA4
control LLL-TAG	99.9a				
12 h CLA-rich LLL-TAG	82.5b	1.0a	2.5a	2.3a	11.6a
36 h CLA-rich LLL-TAG	68.5c	1.7b	4.5b	4.1b	21.1b

[a] C18:2, linoleic; CLA1, *cis-9*, *trans-11* CLA; CLA2, *trans-9*, *cis*-11/*cis*-10, *trans*-12 CLA; CLA3, *trans*-10, *cis*-12 CLA; CLA4, *trans-trans* (8, 10/9, 11/10, 12). Values are fatty acid means of triplicate analyses of three replications. Fatty acids in same column with the same letters are not significantly different ($P < 0.05$) as measured by ANOVA using the Student *t* test.

Table 2. Percent of Oil in Each Fraction (F-1−F-5) As Obtained by Semipreparative NARP-HPLC UV-PDA Detector[a]

fraction	control soy oil	CLA-rich soy oil
F-1	11.4 ± 0.12a	10.6 ± 0.11b
F-2	22.1 ± 0.21a	21.8 ± 0.29a
F-3	29.0 ± 0.20a	28.8 ± 0.12a
F-4	21.3 ± 0.09a	21.5 ± 0.07a
F-5	16.2 ± 0.14a	17.4 ± 0.32b

[a] Values are means of triplicate analyses of two replications; ± values denote standard deviation of each fraction collected from two replications. Fractions in the same row with the same letters are not significantly different ($P < 0.05$) as measured by ANOVA using the Student *t* test.

Table 3. Fatty Acid Composition Obtained by GC-FID of the Control Soy Oil and Soy Oil HPLC Fractions (F-1−F-5)[a]

	% fatty acid composition				
	C16:0	C18:0	C18:1	C18:2	C18:3
soy oil	10.7c	4.0b	23.3c	55.7c	6.3b
F-1	1.1a		1.9a	5.7a	2.7a
F-2	1.3a		1.7a	16.8b	2.3a
F-3	3.6b		7.0b	17.1b	1.3a
F-4	3.6b	1.6a	7.4b	8.7a	
F-5	1.1a	2.4a	5.3a	7.4a	

[a] Values are fatty acid means of triplicate analyses of three replications within each fraction. Fatty acids in the same column with the same letters are not significantly different ($P < 0.05$) as measured by ANOVA using the Student *t* test.

Table 4. Fatty Acid Composition Obtained by GC-FID of the CLA-Rich Soy Oil and CLA-Rich Soy Oil HPLC Fractions (F-1−F-5)[a]

	% fatty acid composition								
	C16:0	C18:0	C18:1	C18:2	C18:3	CLA1	CLA2	CLA3	CLA4
CLA-rich oil	10.9c	4.0b	23.0c	21.4c	6.4b	1.4b	5.0b	4.6b	23.4c
F-1	0.2a	0.2a	2.0a	2.0a	2.5a	0.3a	1.4a	1.2a	0.8a
F-2	1.0a		1.5a	4.9b	2.2a	0.4a	1.5a	1.3a	9.0b
F-3	3.6b		6.4a	5.0b	1.7a	0.4a	1.5a	1.4a	8.8b
F-4	3.6b	1.0a	8.2b	4.1b		0.2a	0.4a	0.5a	3.5a
F-5	2.5a	2.8b	4.9a	5.4b		0.1a	0.2a	0.2a	1.3a

[a] C16:0, palmitic; C18:0, stearic; C18:1, oleic; C18:2, linoleic; C18:2, linolenic; CLA1, *cis*-9,*trans*-11 CLA; CLA2, *trans*-9, *cis*-11/*cis*-10, *trans*-12 CLA; CLA3, *trans*-10,*cis*-12 CLA; CLA4, *trans-trans* (8, 10/9, 11/10, 12). Values are fatty acid means of triplicate analyses of three replications within each fraction. Fatty acids in the same column with the same letters are not significantly different ($P < 0.05$) as measured by ANOVA using the Student *t* test.

at 233 nm, suggesting the presence of unresolved CLA isomers with slightly longer retention time. The new peaks shown in **Figure 2** correspond with those in **Figure 1**. The UV absorbance of the peak with retention time of LLL*1 as shown by UV chromatogram decreases over time as later eluting peaks increase with increase in percent CLA. This possibly suggests conversion of earlier formed *cis*−*trans*/*trans*−*cis* isomers of CLA to *trans-trans*, as it is the most stable form of CLA found. **Figures 1** and **2** provide a reference for retention time of LLL and its photoisomerization products. This information was useful in subsequent soy oil studies. A NARP-HPLC fractionation of RBD soy oil and CLA-rich soy oil was obtained by ELSD as shown in **Figure 3**. The control soy oil chromatogram shows a similar pattern of peaks with slightly better resolution than those obtained by List et al. (*19*). The largest peak corresponds to LLL as shown in the previous study and by List et al. (*19*). This enabled the tentative identification of TAG species in the control soy oil chromatogram. Fractions with retention times of >75 min were not collected as they were thought to be TAGs containing monounsaturated and saturated FA. High linoleic acid containing TAG fractions were LnLL, LLL, LLO, LLP, LOO, and LOP. Peaks of unsaturated fatty acid bearing TAGs were not consistently resolved but formed four distinct fractions (F-1, F-2, F-3, and F-4) in the control soy oil (**A**) and CLA-rich soy oil (**B**). To observe TAG changes after photoisomerization, the CLA-rich soy oil chromatogram was compared with that of the control soy oil. Fraction 1 (F-1) of the control oil (**A**) contains mainly Ln and L fatty acids. After processing (**B**), the CLA-rich soy oil the LnLL peak decreased in height with a corresponding increase in the adjacent LnLnO and LnLnP peaks. The peak widening (*22*) and relative height reduction most likely indicate conversion of Ln and L in LnLL to geometrical isomers, resulting in TAG positional isomers that coeluted with LnLnO and LnLnP. The main feature of fraction 2 (F-2) was the large reduction in the LLL peak after photoisomerization, relative to the smaller LnLO and LnLP peaks in the control soy oil. The LLL peak decreased in height with a significant increase in the adjacent LnLO and LnLP peaks in CLA-rich oil, indicating conversion of L in LLL to different CLA isomers that form different CLA containing TAGs with different retention times which coeluted with LnLO and LnLP peaks. In both the LnLO and LnLP peaks, there was evidence of peak broadening (*22*) and at least two poorly resolved isomers, indicating elution of newly formed TAG isomers produced from LLL as shown in the previous study. Similarly, fraction 3 (F-3) showed a large reduction in the LLO and LLP peaks after photoisomerization relative to the smaller LnOO and LnPP peaks in the control soy oil. The LLO and LLP peaks decreased in height with a significant increase in the adjacent LnOO and LnPP peaks, respectively, in CLA-rich oil, possibly indicating L isomerization in both LLO and LLP to different CLA isomers containing TAGs with different retention times which coelute with LnOO and LnPP. Some peak broadening occurs, but the main feature is unresolved peaks at LnOO and LnPP retention times. In fraction 4 (F-4) the relative peak heights of LOO, LLS, and LOP change little after photoisomerization, but with a relative increase in PLP, which consists of unresolved new TAG isomers. This may be due to L isomerization in LOO, LLS, and LOP and coelution of the resulting CLA-containing TAG with PLP.

As a general observation, the new unresolved peaks in CLA-rich soy oil had slightly longer retention time relative to L-containing TAGs in control soy oil. This results in coelution of the new CLA-containing TAGs with adjacent native TAGs in control soy oil, which is the probable cause of the unresolved peaks due to formation of various geometrical fatty acid and positional TAG isomers. **Figure 4** shows the corresponding UV absorbance at 233 nm of control soy oil and CLA-rich soy oil.

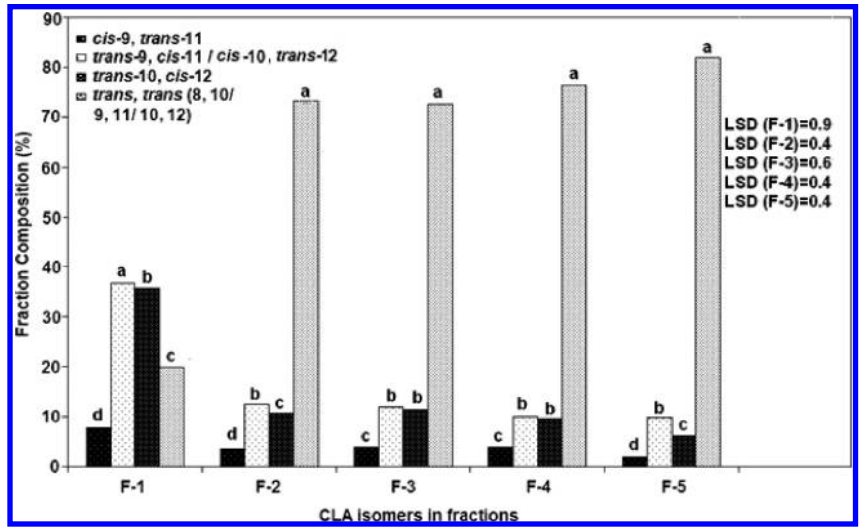

Figure 5. Distribution of percent CLA isomers in 34.2% CLA-rich oil TAG fractions analyzed by FAME GC. Replication (n) = 3. Means with different letters within each fraction differ significantly, $P < 0.05$.

The control soy oil chromatogram is probably noise and suggests little or no conjugated dienes. In contrast, retention times of UV absorption peaks of CLA-rich soy oil corresponded with the new unresolved TAG peaks, detected by ELSD. The L-containing TAG peaks that were reduced relative to adjacent ELSD peaks (LnLL, LLL, LLO, LLP) were also lower than the neighboring peaks in the UV chromatogram. This provides additional evidence that L-containing TAGs were isomerized to form TAG isomers that eluted later than the parent TAG, as unresolved peaks. UV absorbance at 233 nm and the minimal PV value indicated conjugated dienes due to diene isomerization, rather than oxidation.

Quantitative Analysis of Fatty Acids in CLA-Rich Trilinolein and New CLA-TAG Fractions in CLA-Rich Soy Oil. *Fatty Acid Isomers.* The FAME GC analysis of CLA-rich trilinolein and CLA-rich soy oil showed isomerization of L-containing fatty acids to CLA.

Fatty Acid Composition of Control LLL-TAG and CLA-Rich LLL-TAG. The fatty acid composition of control LLL-TAG and 12 and 36 h CLA-rich LLL-TAG analyzed by GC-FID is shown in **Table 1**. It was observed that after 12 h of photoisomerization, the LLL content in control LLL-TAG reduced from 99.9 to 82.5%, suggesting 17.4% of LLL undergoing isomerization to new CLA-containing TAGs, as confirmed by the CLA content of 17%. Similarly, after 36 h of photoisomerization the LLL content in control, LLL-TAG reduced from 99.9 to 68.5%, suggesting 31.4% of LLL undergoing isomerization to new CLA-containing TAGs, as confirmed by the CLA content of 31%. The new TAGs consisted of geometric and positional isomers of CLA and were identified in 12 and 36 h CLA-rich LLL-TAGs as *cis*-9, *trans*-11 (1.0 and 1.7%); *trans*-9,*cis*-11/*cis*-10,*trans*-12 (2.5 and 4.5%); *trans*-10, *cis*-12 (2.3% and 4.1%); and *trans-trans*-8,10/9,11/10,12 (11.6 and 21.2%), respectively, as shown in **Table 1**. One major observation was that LLL isomerization to CLA after photoisomerization of control trilinolein primarily resulted in *trans-trans*-isomers of CLA. This was expected as *trans-trans*-isomers are the stable form of CLA.

Fatty Acid Composition of Control Soy Oil Fractions and CLA-Rich Soy Oil Fractions. Semipreparative NARP-HPLC was used to collect and isolate F-1−F-5 as detected by UV absorbance at 233 nm. The TAG peak fractions collected were similar to those in **Figures 3** and **4**, as described by List et al. (*19*), except that fraction 5 primarily consisting of TAGs with saturated fatty acids was obtained as all peaks eluting after 75 min. The CLA-rich soy oil yielded the same distinct fractions (F-1−F-5) as observed with analytical HPLC. The control soy oil and CLA rich-soy oil each yielded five fractions by semipreparative HPLC. The five fractions were isolated on the basis of their retention times using the UV-PDA detector at 233 nm. The percent of oil recovery in fractions 1−5 is very important to quantify the percent of CLA formation within each fraction. The precision and reproducibility of HPLC method to produce consistent fraction sizes within replicates are shown in **Table 2**. The fractions of control soy oil were collected on the basis of similar retention times obtained from the UV chromatogram of fractions F-1−F-5 of CLA-rich soy oil. The total fatty acid compositions of control soy oil and control soy oil fractions collected from semipreparative NARP-HPLC were measured by GC-FID as shown in **Table 3**. L-containing TAGs primarily were found to be present in F-2 and F-3, consisting of 16.8 and 17.1%. This was because F-2 (LLL, LnLO, and LnLP) and F-3 (LLO, LnOO, LLP, and LnPP) in control soy oil contained high L-containing TAGs. F-1 (LnLL, LnLnO, and LnLnP) consisted of 5.7% of L-containing TAGs, whereas F-4 and F-5 consisted of 8.7% of L (LOO, LLS, LOP, and PLP) and 7.4% (LOS and SLP). The total L% in the control soy oil distributed in the fractions added up to 55.7% of L-containing TAGs, which was similar to the total L% obtained by GC-FID analysis of control soy oil as shown in **Table 3**. The total fatty acid composition of CLA-rich soy oil and CLA-rich soy oil fractions collected from HPLC were measured by GC-FID as shown in **Table 4**. It was observed that after photoisomerization, the percent L content in control soy oil reduced from 55.7 to 21.4%, suggesting 34.3% of L undergoing isomerization to new CLA-containing TAGs, as confirmed by CLA content of 34.4% in CLA-rich soy oil before HPLC fractionation. The new CLA-containing TAGs in the CLA-rich soy oil before HPLC fractionation consisted of geometric and positional isomers of CLA and were identified as *cis*-9,*trans*-11 (1.4%); *trans*-9,*cis*-11/*cis*-10,*trans*-12 (5.0%); *trans*-10,*cis*-12 (4.6%); and *trans-trans*-8,10/9,11/10,12 (23.4%), which were distributed in all five fractions collected as shown in **Table 4**. One major observation was that L isomerization to CLA after

photoisomerization primarily resulted in *trans-trans*-isomers of CLA, which complimented the CLA isomer formation in CLA-rich trilinolein as shown in **Table 1**. This was expected as *trans-trans* is the stable form of CLA.

The fatty acid content of the fractions analyzed by GC showed distribution of total CLA within every fraction. F-1 was found to have 10.8% of the total CLA formed in CLA-rich soy oil after photoisomerization. F-2 (35.7%) and F-3 (35.2%) both constituted about 71% of total CLA formed, which was expected because these were the fractions starting with high L-containing TAGs in control soy oil prior to photoisomerization. F-4 and F-5 were found to form 13.4 and 4.7%, respectively. No statistically significant differences were observed ($P < 0.05$) in the percent CLA levels for each fraction among the three replications analyzed by GC.

Distribution of four major CLA isomers identified as *cis*-9,*trans*-11; *trans*-9,*cis*-11/*cis*-10,*trans*-12; *trans*-10,*cis*-12; and *trans-trans*-8,10/9,11/10,12 in CLA-rich soy oil fractions is shown in **Figure 5**. The distribution of CLA isomers is unusual in F-1 as it is mainly constituted of *trans*-9,*cis*-11/*cis*-10,*trans*-12, *trans*-10,*cis*-12 to a large extent (71%) of the CLA formed in F-1 (10.8%). The reason for this unusual distribution is still unknown and can probably be due to F-1 (LnLL, LnLnO, and LnLnP) containing a higher ratio of Ln to L as compared to other fractions. The L in these TAGs may predominantly get converted to *trans*-9,*cis*-11/*cis*-10,*trans*-12 and *trans*-10, *cis*-12 isomers of CLA. F-2−F-5 showed a different trend from F-1 as every fraction was found to be rich in *trans-trans*-8,10/9,11/10,12 (>80%). This was expected as *trans-trans* is the most stable form of CLA. These three CLA isomers were the sole reason for the poor resolution of TAGs obtained in CLA-rich soy oil by both ELSD and UV chromatograms. This was due to all three CLA isomers having close retention times to each other, causing mixing of TAG isomers during HPLC separation.

In conclusion, linoleic acid in soy oil TAGs isomerizes during photoisomerization to form CLA. Analytical NARP-HPLC provided evidence of new TAG fractions in CLA-rich soy oil, but with poor or no resolution from the original soy oil TAG fractions. Future work includes improving the chromatographic resolution of coeluting TAGs with different positions of double bonds, which can be achieved by optimizing the mobile phase composition and gradient steepness, the column type by using smaller particle size than 5 μm, and column length (*23*) for better precision. A new semipreparative NARP-HPLC method allowed subsequent fatty acid analysis that showed HPLC fractions contained 70−80% *trans*,*trans* and 20−30% *cis*,*trans* and *trans*,*cis* isomers of CLA. This is considerably more than is found in bovine food products such as beef (0.43%) and dairy (0.4−0.55%) (*3*). The resolution of positional and geometrical CLA isomers needs to be improved to determine the fatty acid composition of individual TAGs in CLA-rich soy oil. This study will help to produce concentrated CLA TAGs and may lead to the development of a method to obtain mixed CLA isomer TAG standards in the future.

Abbreviations Used. CLA, conjugated linoleic acid; NARP-HPLC, nonaqueous reversed-phase high-performance liquid chromatography; GC, gas chromatography; FIC, flame ionization detector; UV-PDA, ultraviolet photodiode array detector; ELSD, evaporative light scattering detector; L, linoleic; Ln, linolenic; O, oleic; P, palmitic; S, stearic; LLL-TAG, trilinolein.

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