Determination of *c*9,*t*11-CLA in Major Human Plasma Lipid Classes Using a Combination of Methylating Methodologies

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ABSTRACT: Isomeric CLA exhibit several significant biological activities in animals and humans and are easily isomerized to their corresponding *t,t*-CLA isomers during methylation with various acid-catalyzed reagents. To minimize such isomerization and provide a valid quantification of human plasma CLA content, several methylation methods were tested. Plasma neutral lipid, nonesterified FA (NEFA), and polar lipid classes were separated into the following fractions: (i) cholesteryl ester (CE, 1.2 mg/12 mL, 37.5% lipids), (ii) TAG (0.8 mg/12 mL, 25% lipids), (iii) NEFA (0.2 mg/12 mL, 6.2% lipids), (iv) MAG/DAG/cholesterol (0.3 mg/12 mL, 9.4% lipids), and (v) phospholipid (PL, 0.5 mg/20 mL, 15.6% lipids). Data showed that *c*9,*t*11-CLA found in TAG, MAG/DAG/cholesterol, and PL fractions were converted to methyl esters with sodium methoxide within 2 h at 55°C. However, the *c*9,*t*11-CLA in the CE fraction could not be completely converted to methyl esters by sodium methoxide/acetylchloride in methanol or methanolic KOH; instead, CE was treated with sodium methoxide and methyl acetate in diethyl ether for 1 h. NEFA were converted to methyl esters with trimethylsilyldiazomethane (TMSDAM). All reaction mixtures were monitored by TLC prior to GLC analysis. The highest enrichment of *c*9,*t*11-18:2 (% FA) was in TAG (0.31%), followed by CE (0.14%) and PL (0.13%). The above methylation methods were then applied to a small subset ($n = 10$) of nonfasting plasma lipid fractions to confirm the applicability of these data. Results from this subset of samples also indicated that the greatest enrichment of *c*9,*t*11-CLA was present in the TAG fraction (0.39%), followed by CE (0.27%) and PL (0.22%). These data indicate that different plasma fractions have different *c*9,*t*11-CLA contents.

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CLA (*cis*-9,*trans*-11-CLA, 18:2), recently given the trivial name rumenic acid (RA; 1), is the main conjugated dienoic FA in human serum lipids (2). In general, the term CLA refers to a mixture of positional and geometric isomers of octadecadienoic acid (18:2) having a conjugated double bond. Animal studies have shown that CLA may inhibit carcinogenesis (3,4), lower body fat (5,6), increase lean body mass (7,8) decrease risk of atherogenesis (9,10), and exhibit antidiabetic characteristics (11). Conjugated linoleic isomers are present in human food and occur abundantly in dairy products (12) as well as in meat from ruminants (13), hydrogenated fats (14), and used frying oil (15). The distribution of CLA isomers has been examined using Ag^+-HPLC (16,17) in a number of natural products including milk, cheese, meat, adipose tissue, and tissues from animals fed commercial CLA mixtures. Biological activity has been attributed to *cis*-9,*trans*-11 dienoic acid (18) and *trans*-10,*cis*-12 dienoic acid (19), the two major CLA isomers.

Iversen *et al.* (20) and Harrison *et al.* (21) were the first to report that conjugated isomers of linoleic acid (LA) are associated with human plasma phospholipids (PL), and that a relatively uniform distribution of serum CLA exists among PL (36%), TAG (36%), and cholesteryl esters (CE) (28%). However, Fogerty *et al.* (22) found that only 16–34% of CLA was present in PL, whereas 58–78% of CLA was found in TAG and 2–8% in CE. Subsequent studies suggest that dietary CLA is incorporated into circulating TAG and PL (23,24), but investigations of the distribution of CLA into the other major lipid fractions are limited. Although the majority of human studies indicate that CLA accumulates predominantly in the TAG fraction, the discrepancy among study results remains unresolved.

CLA isomers are usually quantified by GLC analysis as their methyl esters, prepared by a variety of acid-/base-catalyzed reactions (25). Acid-catalyzed methylations using HCl/methanol and BF_3/methanol have been reported to change the isomer distribution of CLA and generate allylic methoxide from CLA (26), whereas base-catalyzed methods (i.e., those using sodium methoxide in methanol) are considered to be milder methods for methylating the TAG and PL fractions; however, they fail to methylate nonesterified FA (NEFA).

A growing literature shows that different isomers of CLA may have different physiologic effects. Therefore, it is very important to avoid modifying the original isomer when analyzing CLA in biologic fluids and tissues. The first objective (Experiment 1) of this set of studies was to develop methods for complete methylation of the *c*9,*t*11-CLA found in the major fractions of human plasma, followed by preliminary quantification of *c*9,*t*11-CLA in these fractions. Our second objective (Experiment 11) was to apply these methods to a small subset of human plasma samples to confirm the data collected in the initial methodological phase of the study. We hypothesized that different methodologies would be required

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Abbreviations: CE, cholesterol ester; MTBE, methyl *tert*-butyl ether; NEFA, nonesterified fatty acid; RA, rumenic acid; TMSDAM, trimethylsilyldiazomethane.

for different plasma fractions and that distribution of *c*9,*t*11-CLA among fractions would not be uniform.

MATERIALS AND METHODS

Materials. Phosphatidylcholine (PC), cholesteryl linoleate, and a mixture of CLA isomers (FFA) containing 80% *cis*-9,*trans*-11, 17% *cis*-9,*cis*-11, and 1% *trans*-9,*trans*-11 18:2 were purchased from Matreya (Pleasant Gap, PA). A FAME standard (C_8 to C_{24}), oxalic acid ($C_2H_2O_4$), sodium methoxide in methanol ($NaOCH_3$, 0.5 N), and trimethylsilyldiazomethane (TMSDAM) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Freshly opened reagents were redistributed to small amber glass bottles and sealed to prevent progressive deterioration during storage at 4°C. A vacuum manifold, silica Maxi-Clean™ cartridges (600 mg), and precoated silica gel plates (*ca.* 0.1 mm thick, 20 × 20 cm) were purchased from Alltech Associates, Inc. (Deerfield, IL). All chemicals and reagents used were of analytical grade.

Subjects and samples. For the initial methodological phase of the study, healthy women (25 to 38 yr; $n = 4$) donated blood that was collected in heparinized tubes in the morning (nonfasting sample). Blood was centrifuged at 1500 × *g* for 10 min at 4°C. Plasma was removed, pooled, and stored at −40°C. These samples were used to develop the various methylation methods. For the second phase of the study, nonfasting blood samples were collected from healthy women (19 to 38 yr; $n = 10$) and extracted as described above.

Lipid extraction. Plasma lipid was extracted using the modified method of Ingalls *et al*. (27). Briefly, plasma was thawed and vortexed for 15 s, then 700 µL was transferred into a microcentrifuge tube and centrifuged at 1500 × *g* for 2 min at 4°C to remove proteins; 200 µL duplicates were transferred into screw-capped glass tubes (15 mL) containing 300 µL of 0.005 N HCl and 3 mL chloroform/methanol (2:1, vol/vol). This mixture was vortexed until a complete emulsion had formed. Tubes were then centrifuged at 1500 × *g* for 15 min at 4°C. The chloroform/methanol layer was transferred to a Pasteur pipette column containing anhydrous Na_2SO_4 layered over a glass wool plug. The column was rinsed with an additional 1 mL of chloroform/methanol (2:1, vol/vol), and the samples were brought to dryness under a stream of nitrogen in a heated (45°C) water bath.

Column chromatography. Separation of lipid fractions was accomplished using silica Maxi-Clean™ cartridges (600 mg) and the method of Hamilton and Comai (28) modified to allow processing of several samples at a time using a vacuum pump. Briefly, the column was loaded with 1.5 mg of plasma lipid; CE and TAG fractions were eluted with a combination of hexane/methyl *tert*-butyl ether (MTBE) (200:3, 12 mL for CE followed by 12 mL for TAG). After removal of TAG, the column was acidified with 12 mL hexane/acetic acid (100:0.2, vol/vol). This fraction contained no lipid and was discarded after confirmation by TLC. NEFA were then eluted using 12 mL hexane/MTBE/acetic acid (100:2:0.2, by vol); MAG/DAG/cholesterol were eluted as a single fraction with 12 mL of hexane/acetic acid (100:0.2, vol/vol). Finally, the PL fraction was eluted with 20 mL MTBE/methanol/ammonium acetate (pH 8.6; 10:4:1, by vol). Each fraction was dried under nitrogen at 45°C for further analysis.

Thin-layer chromatography (TLC). TLC was performed on glass microscope plates coated with silica gel G (*ca.* 0.1 mm thick), and a mixture of *n*-hexane/diethyl ether/acetic acid (90:10:1, by vol) was used as the solvent. Lipid spots were visualized under UV light by spraying with a solution of 2′,7′-dichlorofluorescein in 95% ethanol to check the purity of each fraction; 50% H_2SO_4 in 95% ethanol was used to visualize spots after charring at 100°C.

Preparation of FAME by various methods (Experiment 1). For all the methylation procedures, 1 mL concentrated lipid solution (2 mg/mL hexane) was pipetted into a glass tube, and the following methylation procedures were performed.

(i) Method A: *$NaOCH_3$/methanol (26)*. A plasma lipid extract (2 mg) was transferred into a screw-capped glass tube (15 mL); 2 mL of 0.5 N sodium methoxide solution was added to the tube. The reaction mixture was heated for 2 h at 55°C. After the tube had been cooled to room temperature, 5 mL of 0.2 N HCl solution was added and extracted with MTBE/hexane (1:3, vol/vol; 3 × 2 mL). The reaction mixture was washed with water (2 × 2 mL) and passed through a Pasteur pipette column containing a glass wool plug, a 4-cm filter bed of silica gel, and 1 cm anhydrous $MgSO_4$. Sample was then concentrated under nitrogen to approximately 500 µL and transferred into a GLC vial.

(ii) Method B: *Acetyl chloride (CH_3COCl)/methanol-chloroform*. The reaction was carried out as previously described (29), except that benzene was substituted with chloroform. A plasma lipid extract (2 mg) was transferred into a screw-capped glass tube (15 mL) and dried under nitrogen. Anhydrous methanol (2 mL) and then 250 µL of CH_3COCl were added to the tube while the tube was gently shaken. The reaction mixture was heated at 100°C for 1 h. After the tube had been cooled in water at room temperature for 2 min, 5 mL 6% K_2CO_3 solution was slowly added to stop the reaction and neutralize the mixture before extraction. The reaction mixture was extracted with MTBE/hexane (1:3, vol/vol; 3 × 2 mL), and the solvent was passed through a Pasteur pipette column containing a glass wool plug, a 4-cm filter bed of silica gel, and 1 cm anhydrous $MgSO_4$. Sample was concentrated under nitrogen to approximately 500 µL and transferred into a GLC vial.

(iii) Method C: $NaOCH_3$/methanol followed by TMSDAM (30). Anhydrous methanol (0.5 mL) was added to plasma lipid extracts (2 mg), followed by the addition of 40 µL TMSDAM, and allowed to react overnight (12 h) at room temperature. Excess TMSDAM was decomposed with 2% acetic acid in hexane until colorless, followed by the addition of 1.5 mL 5% $NaHCO_3$ (wt/vol) and extraction with MTBE/hexane (1:3, vol/vol; 3 × 2 mL). The organic layer was removed and passed through a Pasteur pipette column containing a glass wool plug and a 4-cm silica gel overlaid with 1 cm anhydrous $MgSO_4$. Sample was concentrated under nitrogen to approximately 500 µL and transferred into a GLC vial.

(iv) Method D: Hydrolysis of plasma lipid/TMSDAM (31). Ethanolic KOH (1 mL) solution (0.5 M in 95% ethanol) was added to a plasma lipid extract (2 mg), and the reaction mixture was heated for 2 h at 55°C. After cooling to room temperature, water (5 mL) was added, the solution acidified with 6.0 N HCl and extracted with MTBE/hexane (1:3, vol/vol; 3 × 2 mL). Solvent was passed through a Pasteur pipette column containing a glass wool plug, a 4-cm filter bed of silica gel, and 1 cm anhydrous $MgSO_4$ and evaporated under nitrogen. The lipid sample was then treated with TMSDAM as described in Method C.

(v) Method E: *$NaOCH_3$/methyl acetate(CH_3COCH_3)/diethyl ether (32).* A plasma lipid extract (2 mg) was dried under nitrogen, and sodium-dried diethyl ether (1 mL) was added, followed by 20 µL methyl acetate and 40 µL of 0.5 N $NaOCH_3$. The reaction mixture was vortexed to ensure thorough mixing. After 1 h at room temperature, the reaction was stopped by adding 30 µL saturated oxalic acid in diethyl ether. The mixture was centrifuged at 1500 × *g* for 2 min and dried under a gentle stream of nitrogen. Hexane (1 mL) was added, and the mixture was passed through a Pasteur pipette column containing a glass wool plug and a 4-cm silica gel overlaid with 1 cm anhydrous $MgSO_4$. Sample was concentrated under nitrogen to approximately 500 µL and transferred into a GLC vial.

(vi) Method F: Analysis of CE in whole plasma (33). This method was used only for the methylation of the CE fraction of the whole plasma. Briefly, CE (1 mg) was dissolved in 2 mL petroleum ether; 1 mL of methanolic base ($KOCH_3$, 2 N) was added. The reactants were mixed by inversion for 1 min. An aliquot of the upper phase was used for TLC and GLC analyses.

Analysis of FAME. Samples were analyzed using GLC (model 6890; Agilent Technologies, Wilmington, DE) equipped with a capillary column (Quadrex 007-23-60-0.25F; 60 m, 0.25 mm i.d., with 0.25 µm film thickness; Cyanopropyl; Quadrex, New Haven, CT). Helium was used as carrier gas at a constant flow mode with linear velocity set at 22 cm/s. The FID was heated to 260°C, and detector gas flows were set at 40, 450, and 49 mL/min for hydrogen, compressed air, and nitrogen, respectively. Samples (1 µL) were injected in the splitless mode (injection temperature 260°C, purge at 0.75 min) with an initial oven temperature of 50°C with a 4-min hold time, increased at 10°C/min to 150°C and then 1°C/min to the final temperature of 191°C. Identities of selected CLA peaks and other FA peaks were established by comparing retention times to a 14-component C_8–C_{24} FAME standard mixture, a 9,11-CLA mixture containing 80% *cis*-9,*trans*-11; 17% *cis*-9,*cis*-11; and 1% *trans*-9,*trans*-11 18:2, respectively, and an anhydrous milk fat reference standard obtained from the Commission of the European Communities (CRM 164; European Community Bureau of Reference, Brussels, Belgium).

Preparation of FAME of major plasma lipid fractions of a small subset of samples (Experiment 11). To confirm the applicability of these data to a larger sample size, plasma lipids (nonfasting) from a subset of female subjects (19 to 38 yr; $n = 10$) were collected, fractionated, and then methylated according to the methods described above for the pool plasma samples. Because NEFA and MAG-DAG fractions were found not to contain much *c*9,*t*11-CLA, they were combined for analytical purposes.

RESULTS

Experiment 1. (i) Effect of the methylation procedure on FAME in whole plasma. Data in Table 1 can be used to compare the estimates of *c*9,*t*11-CLA concentration (% FAME) in human plasma lipid samples when analyzed by different methylating procedures. Compared to Method E ($NaOCH_3$/methyl acetate), use of Methods A, B, C, and D ($NaOCH_3$/methanol, CH_3COCl/methanol, $NaOCH_3$/TMSDAM, and KOH/TMSDAM) resulted in lower *c*9,*t*11-CLA content. The apparent loss of this CLA isomer was most pronounced in Methods A and C. Four other FA—16:0 (palmitic), 18:0 (stearic), 18:1 (oleic), and 18:2 (linoleic), which constitute 60 to 70% of the total plasma lipid—were also greatly influenced by these various esterification methods. For example, esterification of 16:0 and 18:2, the two plasma FA in greatest concentration, were considerably reduced in Method C as compared to Method D (Table 1). TLC analysis with hexane/diethyl ether/acetic acid (90:10:1, by vol) as the solvent system suggested incomplete FA methylation of the lipid samples obtained from Methods A, B, and C. To determine whether the CE and PL fractions were being completely methylated, known amounts (2 mg) of cholesteryl linoleate and PC were methylated as described in Methods A, B, C, and E and analyzed by TLC. Results indicated that PC was fully methylated with all methods, whereas cholesteryl linoleate was completely methylated only with Method E (Fig. 1). (Method D is not a transesterification method and therefore was excluded from this experiment.) However, methylation of the whole plasma with this method resulted in a chromatogram with many additional unidentified peaks after GLC analysis (Fig. 2A).

(ii) Effect of the methylation procedure on FAME in major lipid fractions. Results of column chromatographic separation of lipid fractions are as follows: CE, 1.2 mg, 37%; TAG, 0.8 mg, 25%; NEFA, 0.2 mg, 6.2%; MAG/DAG/cholesterol, 0.3 mg, 9.4%; and PL, 0.5 mg, 15.6%. Only 6.2% of the total lipid was not recovered following separation of fractions. The CE fraction was predominant, with TAG being the second major fraction. When the CE fraction (representing the major fraction of plasma) was treated with $NaOCH_3$ (0.5 N) at 55°C for 2 h, 4 h, or kept overnight at room temperature (Method A), the reaction was found to be incomplete by TLC; thus, Method A was unsuitable for methylation of the CE fraction. When the CE fraction was hydrolyzed with 0.5 M ethanolic KOH at 55°C for 2 h and then acidified with dilute 0.2 M HCl followed by overnight methylation with TMSDAM as described in Method D, many additional peaks (Fig. 2B) were detected by GLC analysis. No attempts were made to identify the unknown peaks. We then tested a methylation procedure

TABLE 1
FA (% identified FAME) Composition of Whole Human Plasma Using Various Methylation Methods

	Methylation method				
FA[a]	Method A $NaOCH_3$/methanol[b]	Method B CH_3COCl/methanol[c]	Method C $NaOCH_3$/TMSDAM[d]	Method D KOH/TMSDAM[e]	Method E $NaOCH_3$/methyl acetate[f]
14:0	0.87	1.15	0.59	0.70	1.02
14:1 (*c*9)	0.13	0.20	0.80	0.07	0.30
15:0	0.20	0.31	0.17	0.22	0.24
16:0	18.34	20.13	13.66	18.34	22.26
16:1 (*c*9)	1.26	1.30	1.20	1.34	0.30
18:0	6.40	7.39	5.36	7.52	5.90
18:1 (*c*9)	18.86	21.01	15.47	20.36	17.82
18:2 (*c*9,*c*12)	30.01	32.47	24.30	34.08	28.21
18:2 (*c*9,*t*11)[g]	0.19	0.21	0.19	0.20	0.36
18:3 (*c*9,*c*12,*c*15)	0.88	0.98	0.81	1.20	0.33
20:4 (*c*5,*c*8,*c*11,*c*14)	5.33	6.00	5.32	6.50	5.00

[a]These 11 FA (14:0, myristic acid; 14:1, myristoleic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:2, CLA, 18:3, linolenic acid; and 20:4, arachidonic acid) accounted for 82, 91, 67, 90, and 81% of the total human plasma FA composition after methylation with $NaOCH_3$/methanol, CH_3COCl/methanol, $NaOCH_3$/TMSDAM, KOH/TMSDAM, and $NaOCH_3$/methyl acetate, respectively.
[b]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C.
[c]Samples were treated with acetyl chloride in methanol/chloroform for 1 h at 100°C.
[d]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C followed by TMSDAM overnight (12 h) at room temperature. TMSDAM, trimethylsilyldiazomethane.
[e]Samples were saponified with 0.5 N KOH in 95% ethanol for 2 h at 55°C and then methylated with TMSDAM overnight (12 h) at room temperature.
[f]Samples were treated with methyl acetate and sodium methoxide in methanol (0.5 N) in diethyl ether for 1 h at room temperature.
[g]Rumenic acid.

utilizing alcoholysis in an essentially nonalcoholic solution (33) (Method F). TLC analysis again revealed incomplete methylation; therefore, FAME were not analyzed on GLC. Despite such efforts, none of these methods was able to methylate the CE fraction completely. Finally, treatment with $NaOCH_3$/methyl acetate in diethyl ether (Method E) was

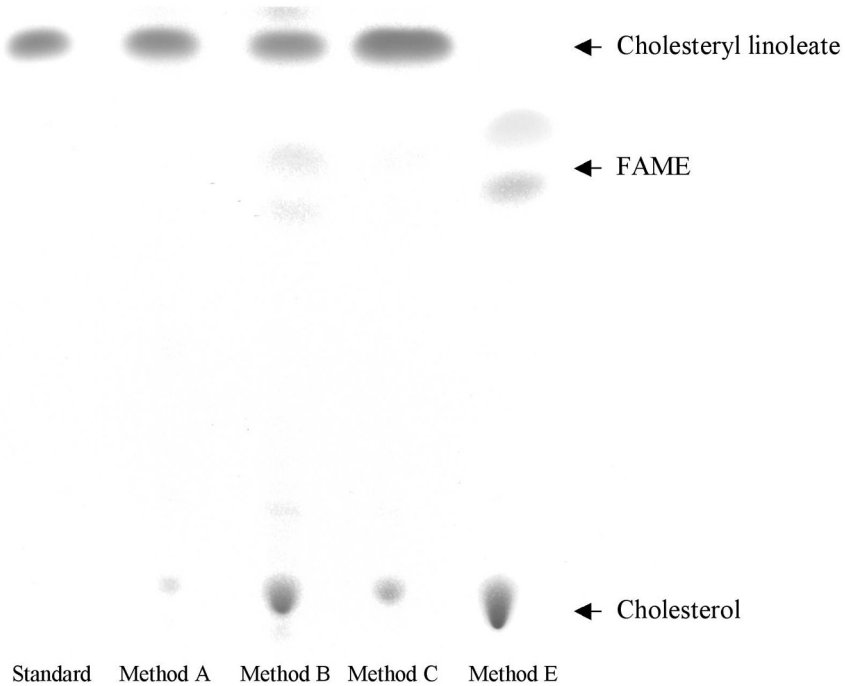

FIG. 1. Illustration of TLC plate with incomplete and complete methylation of cholesteryl linoleate (from left to right): cholesteryl linoleate standard; $NaOCH_3$ (0.5 N)/methanol (55°C, 2 h, Method A); CH_3COCl/methanol-chloroform (2:0.5 mL, vol/vol) (100°C, 1 h, Method B); $NaOCH_3$ (0.5 N)/methanol (55°C, 2 h)/TMSDAM (overnight at room temperature, Method C); $NaOCH_3$/methyl acetate/diethyl ether (room temperature, 1 h, Method E). The TLC plate was developed in hexane/diethyl ether/acetic acid (90:10:1, by vol), sprayed with 50% H_2SO_4 in 95% ethanol, and charred at 100°C. TMSDAM, trimethylsilyldiazomethane.

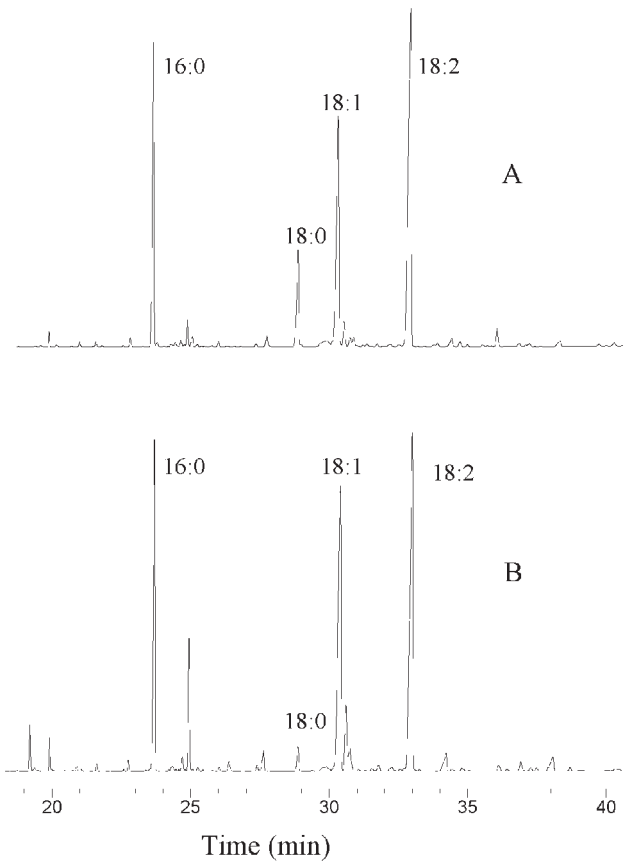

FIG. 2. Gas chromatograms of total plasma (A) and cholesterol ester fraction (B) after saponification with ethanolic KOH and methylation with TMSDAM (Method D); known peaks have been identified. For abbreviation see Figure 1.

found to fully methylate the CE fraction. Figure 1 shows the completeness of methylation of cholesteryl linoleate by this method. TAG, MAG/DAG/cholesterol, and PL were found to be completely methylated with Method A, whereas NEFA were methylated with Method C only. The FA composition of the plasma lipid classes are shown in Table 2. These data show that the greatest enrichment of *c*9,*t*11-CLA (% FAME) was in the TAG fraction (0.31%), followed by CE (0.14%) and PL (0.13%).

Experiment 11. Determination of c9,t*11-CLA in major lipid fractions of a small subset of plasma samples.* Results obtained from this experiment are shown in Table 3. These data indicate that the greatest enrichment of *c*9,*t*11-CLA is present in the TAG fraction (0.39%), followed by CE (0.27%) and PL (0.22%).

DISCUSSION

Results from this study confirm that, for the quantitative analysis of *c*9,*t*11-CLA in biological samples, care should be taken when choosing the methylation procedures. For example, methylation of the *c*9,*t*11-CLA isomer in CE is not achieved using sodium methoxide even under conditions of lengthy reaction times (2 h, 4 h, 55°C, or left overnight at room temperature) (Method A). Because a direct esterification method would be desirable when analyzing a large number of samples by GLC, such a method (Method B) was also investigated. Our data suggest that this direct esterification procedure is not suitable for *c*9,*t*11-CLA isomer quantification owing to incomplete methylation of the FA in whole plasma. Shantha *et al*. (34) have reported artifact formation

TABLE 2
FA (% identified FAME) Composition of Human Plasma Lipid Fractions in a Single Pooled Human Plasma Sample

	Lipid fraction				
FA[a]	CE[b]	TAG[c]	NEFA[d]	MAG/DAG/cholesterol[c]	PL[c]
14:0	0.50	1.42	2.36	1.00	0.70
14:1 (*c*9)	0.03	0.11	0.24	0.49	0.27
15:0	0.21	0.22	0.43	0.28	0.33
16:0	10.29	19.52	22.66	19.0	21.30
16:1 (*c*9)	1.79	1.87	0.60	2.58	0.22
18:0	9.60	4.96	35.47	33.31	11.21
18:1(*c*9)	17.18	32.32	7.93	4.56	6.95
18:2 (*c*9,*c*12)	51.93	21.28	4.47	3.03	17.91
18:2 (*c*9,*t*11)[e]	0.14	0.31	0.07	0.00	0.13
18:3 (*c*9,*c*12,*c*15)	0.54	1.82	0.14	1.68	0.20
20:4 (*c*5,*c*8,*c*11,*c*14)	6.10	0.82	0.37	0.21	6.25

[a]These 11 FA (14:0, myristic acid; 14:1, myristoleic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:2, CLA, 18:3, linolenic acid; and 20:4, arachidonic acid) accounted for 98, 85, 75, 66, and 65% of the FA composition in cholesteryl esters (CE), TAG, nonesterified FA (NEFA), MAG/DAG/cholesterol, and phospholipid (PL) FA fractions, respectively.
[b]Samples were treated with $NaOCH_3$ in methanol/methyl acetate (Method E).
[c]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C (Method A).
[d]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C followed by TMSDAM overnight (12 h) at room temperature (Method C).
[e]*c*9,*t*11-CLA. For other abbreviation see Table 1.

TABLE 3
FA (% identified FAME) Composition of Human Plasma Lipid Fractions[a]

FA	Lipid fraction			
	CE[b]	TAG[c]	NEFA/MAG/DAG/chol[d]	PL[c]
14:0	0.53 ± 0.07	1.35 ± 0.29	2.81 ± 0.49	0.24 ± 0.02
14:1 (*c*9)	0.03 ± 0.03	0.15 ± 0.02	0.18 ± 0.02	0.18 ± 0.02
15:0	0.16 ± 0.01	0.26 ± 0.04	0.40 ± 0.05	0.17 ± 0.01
16:0	11.92 ± 0.44	19.75 ± 1.32	19.35 ± 1.22	29.15 ± 1.56
16:1 (*c*9)	3.48 ± 0.71	3.33 ± 0.62	0.72 ± 0.12	0.67 ± 0.10
18:0	1.08 ± 0.04	4.11 ± 0.51	23.18 ± 1.54	14.28 ± 0.23
18:1(*c*9)	19.12 ± 0.73	28.58 ± 1.31	9.51 ± 0.7	11.13 ± 0.54
18:2 (*c*9,*c*12)	49.64 ± 1.9	20.67 ± 1.93	4.02 ± 0.51	23.28 ± 1.32
18:2 (*c*9,*t*11)[e]	0.26 ± 0.03	0.39 ± 0.03	0.06 ± 0.01	0.22 ± 0.03
18:3 (*c*9,*c*12,*c*15)	0.42 ± 0.01	1.07 ± 0.17	0.50 ± 0.01	0.16 ± 0.14
20:4 (*c*5,*c*8,*c*11,*c*14)	4.98 ± 0.37	1.20 ± 0.29	0.89 ± 0.15	6.33 ± 0.77

[a]Values represent means ± SEM (n = 10).
[b]Samples were treated with $NaOCH_3$ in methanol/methyl acetate for 1 h at room temperature (Method E).
[c]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C (Method A).
[d]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C followed by TMSDAM overnight (12 h) at room temperature (Method C); NEFA and MAG/DAG/cholesterol fractions were combined together since they were found not to contain much *c*9,*t*11-CLA. chol, cholesterol.
[e]*c*9,*t*11-CLA; for other abbreviations see Tables 1 and 2.

when using this method. However, the formation of artifacts in our human plasma sample was not detected, perhaps because other FA present in trace amounts also eluted at that region. Method D is not a transesterification method and therefore was not applied to confirm the methylation on standard CE and PL. However, methylation of the whole plasma with this method resulted in a chromatogram with many additional peaks after GLC analysis (Fig. 2). No attempts were made to identify the unknown peaks. One possible explanation for these additional peaks and a lower *c*9,*t*11-CLA content could be the structural changes in double bonds during saponification. Indeed, Chahine *et al*. (35) observed structural changes while using this method to saponify unsaturated FA.

The data obtained from our pooled plasma lipid fractions (Table 2) were confirmed by analyzing a small subset of plasma samples (n = 10) (Table 3). These data are consistent in that both data sets suggest that the highest percentage of *c*9,*t*11-CLA is present in the TAG fraction, followed by CE and PL. The NEFA and DAG/MAG fractions were found to contain very little *c*9,*t*11-CLA. Fogerty *et al.* (22) also reported the highest percentage of *c*9,*t*11-CLA in the TAG fraction, followed by PL and CE in human blood serum. The levels of other major FA (e.g., 16:0, 18:0, 18:1, 18:2) in the PL and TAG fractions were also found to be similar to those reported by others (36,37).

Sodium methoxide in methanol is considered to be a good reagent that requires mild reaction conditions for transesterification. Bannon *et al.* (38) and Craske *et al.* (39) have discussed the merits of the $NaOCH_3$/methanol reagent, including low cost, reagent stability, and mild reaction conditions. However, the $NaOCH_3$/methanol reagent does not convert FFA to methyl esters, and strict anhydrous conditions are required. The methylation procedures tested in the present study using the $NaOCH_3$/methanol reagent gave little agreement with the reported values of the total plasma *c*9,*t*11-CLA content. For example, total plasma *c*9,*t*11-CLA concentrations ranged from 0.12 to 0.54% of total FA in Australian (n = 15), Swedish (n = 123), Finnish (n = 403), and American (n = 10) volunteeers (22,40–42). In these studies, the differences in total *c*9,*t*11-CLA content might be due to differences in the subjects' population/diet or in the methylation procedures. In most of the preceding studies, $NaOCH_3$/methanol, methanolic HCl, or BF_3-methanol have been used for transesterification, thereby ignoring the contribution of *c*9,*t*11-CLA in CE, as it would likely not be methylated but may be isomerized. Recently, Yamasaki *et al.* (43) and Park *et al.* (31) reported the isomerization of *t*,*c* and *c*,*t* isomers to *t*,*t* isomers of CLA while using methanolic H_2SO_4 or methanolic HCl and BF_3-methanol for transesterification. A similar phenomenon was also reported for oleic acid (44), and artifacts were produced from some lipids during BF_3 methylation (45).

In the current study, one possible reason for the *c*9,*t*11-CLA not being methylated in CE using sodium methoxide in methanol (Method A) could be the partial solubility of CE in methanol; the reverse occurs in Method E, where diethyl ether is used to dissolve the CE along with methyl acetate, thereby methylating the *c*9,*t*11-CLA completely. Longer reaction times required for methylating of CE and the partial solubility of CE in methanol also have been reported by Christie (25) and Stoffel *et al*. (46). Another explanation as to why whole plasma *c*9,*t*11-CLA content was estimated to be lower when methylating with methylation procedures other than Method E could be the presence of trace amounts of water from the atmosphere or absorbed on glassware, resulting in irreversible hydrolysis; the use of methyl acetate in Method E might have preferentially hydrolyzed the lipid itself, thus giving higher estimates of *c*9,*t*11-CLA content. Further, a rapid hydrolysis of FAME has been reported while transesterifying a variety of polar lipids (32).

Of all methods tested, we found no single method able to methylate *c*9,*t*11-CLA satisfactorily in the major fractions or the whole plasma lipid extract. Therefore, methylation of whole plasma lipid extract by any single method may result in incorrect estimation of *c*9,*t*11-CLA concentration. When methylation of the major plasma fractions is necessary, these results suggest that the CE fraction (which represents the major plasma lipid fraction) should be methylated with sodium methoxide and methyl acetate in methanol. A combination of methylating methods can be used (Methods E and C) when methylating the whole plasma sample to ensure complete methylation of all FA in plasma.

In conclusion, our data show that the highest enrichment of *c*9,*t*11-CLA is present in the TAG fraction, followed by CE and PL, and that different methodologies would be required for different plasma fractions. Further research is needed to determine whether physiologic state (fed vs. fasting) influences CLA distribution among fractions.

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